Intrathecal gene transfer by adeno-associated virus for pain

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Current Opinion in Molecular Therapeutics 2005 **7**(5):431- 439
 ISSN 1464-8431

Chronic pain is among the most prevalent medical problems, affecting more than half of patients with advanced cancer and many with other common diseases. Current analgesics often fail to provide satisfactory symptom relief and frequently cause severe side effects. Intrathecal (IT) gene transfer is an attractive method for pain research in rodent models, because it allows targeting of a wide variety of secretable peptides and proteins to the spinal cord, an important neural center for the processing of nociceptive signals. The potential of IT gene transfer for improving opioid therapy and for validating new analgesic targets, such as cytokines involved in spinal glial activation, is discussed. The IT space has been notoriously resistant to efficient gene transfer, limiting therapeutic gene expression to less than 2 weeks with most vector systems. Recent progress with adeno-associated virus (AAV) technology allowed efficient long-term gene expression, facilitating studies reflective of the chronic nature of many pain states. AAV is one of the most advanced gene therapy vectors currently undergoing clinical trials for a variety of disorders. In patients, AAV vectors could be administered intrathecally by a lumbar puncture, a safe procedure routinely performed at the bedside. AAV vectors may therefore become an important tool for translational studies to validate newly identified therapeutic targets in clinical pain states.

Keywords β-endorphin, adeno-associated virus, chronic pain, gene therapy, glial activation, interleukin-10, intrathecal, spinal cord

Introduction

Chronic pain is among the most common problems in clinical medicine. Available analgesic treatments fail in many patients, particularly those with advanced cancer. In the search for new approaches, many studies focus on the spinal cord (SC), because it is an important center in the neural processing of pain information. Pain signals can be stopped or reinforced as they pass through the SC, making the underlying molecular mechanisms attractive drug targets. The lack of efficient systems for delivering traditional drugs to the SC complicates long-term studies in animals and impedes the translation of many new approaches to the clinic. Two pitfalls exist in targeting the SC if drugs are administered by the common systemic routes (ie, pills taken by mouth, patches applied to the skin or intravenous injections). Firstly, the drug has to cross the blood-brain barrier (BBB), which is only possible for small hydrophobic compounds. Secondly, once across the BBB, the drug must not cause untoward effects by acting indiscriminately on the supraspinal structures of the central nervous system (CNS, ie, the brain). Solving the pharmaceutical dilemma of appropriate drug delivery may serve as a new tool for animal studies and may make it possible to test new treatment paradigms for chronic pain in patients.

Intrathecal (IT) adeno-associated virus (AAV) gene transfer will be reviewed as a new means of drug delivery to the SC for studies on chronic pain. The rationale of this approach is based on the strategic location of the IT space (encompassing the SC and accessible by a bedside procedure) and on the potential of AAV to lead to a true long-term effect after a single administration, applicable to chronic syndromes. An overview of pain as a target for gene therapy and studies with non-AAV vectors will be provided, emphasizing two areas: spinal opioids, because of their established clinical efficacy, and 'glial activation', because this pain mechanism is supported by extensive preclinical studies and awaits validation in patients. The biology of AAV vectors and their recent use for gene transfer studies in the pain field will be reviewed and potential scientific hurdles on the road to clinical testing of IT AAV gene transfer for pain will be identified.

Pain as a target for IT gene therapy and approaches with vectors other than AAV

Clinical challenges and approaches for new therapies

Older classification systems still used by the Food and Drug Administration define pain as acute or chronic, and mild, moderate or severe. Acute pain, for example, from a ruptured stomach ulcer or a recent operation, is usually managed successfully by opioid analgesics, such as morphine, in conjunction with an appropriate intervention aimed at the underlying cause. However, chronic pain is a major medical challenge because it is often excruciating and refractory to available treatments. Chronic pain affects a large patient population, 50 million patients in the US alone, according to estimates by the Society for Neuroscience [1]. It can develop or persist without a clearly identifiable cause or may be brought about by a persistent or incurable medical illness. It is among the most frequent reasons for medical consultations and a leading cause of disability, for example, in patients with a 'failed back' who have endured multiple spine operations without experiencing relief. It has a significant socio-economic impact through medical cost and lost productivity.

Chronic pain affects 57 to 70% of patients with cancer [2-4], making it one of the most common complications in oncology. The treatment of chronic cancer pain typically follows the World Health Organization guidelines or similar principles [5]. Depending on individual pain intensity, non-opioid analgesics, weak opioids or strong opioids are prescribed. Opioids in the class of morphine are the cornerstone in the treatment of moderate-to-severe cancer pain [6-8]. While adoption of this treatment approach has improved results, it fails in a significant number of patients. Recent studies report failure rates of 12 to 66% for cancer pain therapy [9-12]. To elucidate causes of pain treatment failure, Weiss *et al* studied the attitude of patients towards their prescribed opioid doses [12]. Of 514 patients treated for pain, 73% experienced moderate-to-severe pain, yet 60% of this group (ie, those with unrelieved pain) did not wish to increase their opioid dose, and 10% even wished to reduce it. Major reasons given by patients for not wanting additional therapy with opioids were the mental side effects (ie, sedation, cognitive dysfunction and hallucinations) and the physical side effects (ie, constipation and nausea) at the prescribed dose level [12]. A 'Clinical Crossroads' discussion presented the dilemma in the words of a 44-year-old woman with lung cancer: "so the biggest trade-off is that I'm in pain in order to stay lucid. I want to be awake and know everything that's going on, for as long as humanly possible. I do not want to die in pain. I do not want to be in pain. But I want to know what is going on around me" [13]. In clinical practice, pain therapy is often empiric, several drugs are tried alone or in combination to control pain and minimize side effects, and relies heavily on the most effective analgesic drugs (ie, -opioid agonists). The first of the gene therapy approaches outlined in this review, IT pre-pro-β-endorphin (ppβ-EP) gene transfer, is based on the clinical observation that opioids are effective even when delivered only to the SC. It has the potential to be active in many different pain states and to improve upon current therapies due to a superior delivery technique, while it does not attempt to target any specific mechanism that might be responsible for a pain state.

Mechanism-specific pharmacological pain therapy is emerging. Woolf divided pain into two broad categories: adaptive pain, which protects from survival and promotes healing, and maladaptive pain, which is pain as a disease [14]. Four clinical pain principles, nociceptive, inflammatory, neuropathic and functional, were used and several physiological principles as underlying mechanisms, nociception, peripheral sensitization, phenotype switch, central sensitization, neuron glial interaction, increased facilitation, structural reorganization and decreased inhibition were outlined [14]. One of the most clinically important pain diagnoses is neuropathic pain, a chronic pain state that is caused by damage to a peripheral or central neural structure. It is typically associated with hyperalgesia and/or allodynia, the former being exaggerated pain sensitivity and the latter being a normally innocuous stimulus coded as painful. Neuropathic pain can occur as an isolated medical problem or as a complication of other diseases, for example, more than half of cancer patients have neuropathic pain [15]. The second of the gene therapy approaches outlined in this review, suppression of glial activation by IT interleukin (IL)-10 gene transfer, is based on a pain mechanism observed in rodents, glial activation, which will be discussed below. Suppression of glial activation has great potential because it targets a specific mechanism, while it faces the uncertainty of whether or not this mechanism is as important in patients as it appears to be in rodent models.

The SC as a 'gate' in pain processing and as a target for therapy

The SC is a main target of established pain treatments, such as opioids, and of many novel pain therapies. The SC fulfills a critical role in the neural processing of pain information because it is the lowest level of the CNS where sensory input can be integrated with other neural activity. The SC is an important site of plastic cellular changes in the response to chronic nociceptive stimulation, which can alter and potentially aggravate pain perception. Nociceptive information reaches the SC through peripheral sensory nerves, which convey nociceptive stimuli from any site in the body, for example, the sciatic nerve in the case of a hind limb injury. The peripheral sensory nerves synapse on neurons within the dorsal horn, a specialized region of the SC. From the dorsal horn, secondary pain projection neurons relay the information to the brain. Under conditions of acute stress, a 'fight or flight' situation, pain perception can be suppressed, for example, the severe pain from traumatic limb amputations is often not felt by the victim in the immediate aftermath of an accident. This phenomenon is at least partially mediated by dynamic SC mechanisms, which are under the control of higher centers. Thus, the dorsal horn of the SC acts as a 'gate' that can stop pain information.

In nociceptive, inflammatory and neuropathic pain states, neurons in the dorsal root ganglion (DRG) and the posterior horn of the SC 'change their function, chemical profile or structure' and thereby reshape the processing of sensory signals [16]. Many changes observed under these circumstances constitute therapeutic targets, for example, cyclooxygenase-2, which is induced in neurons of the SC and other CNS sites in response to hind paw inflammation [17].

Only certain SC targets may be suitable for gene therapy strategies. Targets requiring widespread gene transfer into neurons are problematic, for example, a majority of neurons in all or several DRGs or in the dorsal horn at many spinal levels is technically unrealistic with the current vector technology. On the other hand, many SC targets can be manipulated with peptides or proteins that act in a paracrine manner. These targets are realistic objects for gene therapy, because it would be sufficient to achieve gene transfer into a small fraction of cells in or surrounding the SC, which in turn would continuously secrete a therapeutic gene product into the space surrounding the SC, ie, into the IT space.

The IT space and its accessibility in the clinical and laboratory setting

The IT space is contained by the thecal sac, surrounds the SC and is filled with cerebrospinal fluid (CSF). It is on the brain side of the BBB, which allows even large and hydrophilic substances such as proteins to reach cells in

the SC, eliminating concerns of crossing the BBB. A major attraction of the IT space is its relative accessibility in patients. While all other sites in the CNS can only be reached by a surgical procedure, the IT space can be accessed through a lumbar puncture (LP), sometimes referred to as a 'spinal tap'. LPs are often performed to obtain CSF for diagnostic purposes and under certain circumstances to deliver drugs. An LP is a safe bedside procedure, which causes only minor-to-moderate discomfort if performed by an experienced physician. An LP is a greater burden to a patient than, for example, an intravenous injection. It can be repeated, but in practice this is usually avoided or kept to a minimum.

In human and non-human primates, the thecal sac is several centimeters longer than the SC, creating a pocket below the conus medularis (the lower end of the SC), which contains only CSF and movable nerve roots (the cauda equina). This anatomic configuration makes an LP straightforward, because a long needle can be safely inserted between the lower spinal vertebrae and advanced until CSF is retrieved without the risk of damage to the SC. In lower mammals, on the other hand, the thecal sac follows the outline of the SC and the individual nerve roots very closely, making it challenging to access the IT space. In rats, we are currently using two alternative techniques, either a lumbar approach [18], as described by Storkson *et al* [19], or IT catheters inserted through the cisterna magna into the IT space and advanced to the caudal end of the SC, as originally developed by Yaksh *et al* [20,21].

IT opioid gene therapy

Opioids are presently the only class of drugs effective enough to control most severe pain states. Pure opioid agonists such as morphine have no ceiling effect (ie, worsening pain can always be suppressed by higher doses), and limitations of their use are based upon the appearance of side effects [22]. Thus, enhancing the selectivity of the drug response would be of major benefit in patient care. Selectivity of drug action can be achieved in several ways. For some drug classes, agents have been developed that interact with sub-populations of receptors, such as β_1- or β_2-adrenergic drugs for heart disease or asthma. No such drugs are available in the opioid field. Attempts to enhance the selectivity of the opioid drug response by modifying receptor selectivity have always failed because the -opioid receptor (OR) is the critical receptor for analgesia and side effects [23]. An alternative approach is to limit the site of action of a drug to regions that mediate the desired action and not those that are problematic. Spinal opioid targeting by IT delivery is such a strategy. It has been widely used to administer opioids, leading to excellent analgesia with limited side effects. Analgesia after a single administration lasts only a few hours, which led to the use of implanted devices. In a recent, randomized controlled trial in cancer pain patients, IT opioid delivery by implanted pumps lowered opioid side effects and improved pain control [24], thereby further supporting the concept of spinal opioids for clinical use. However, complication rates with this approach have traditionally been as high as 25% [25], mainly due to infections and CSF leakage. Devulder *et al* noted meningitis in 10% of individuals treated [26], while Nitescu *et al* reported 20% with bacterial catheter colonization and 0.5 to 25% with meningitis [27]. Though the trial by Smith *et al* suggested lower complication rates, it is uncertain whether these will be reproducible in the community setting without the dedicated multi-specialty teams that conducted the research [24]. Furthermore, since the time of publication three years ago, IT pump implantation has failed to be widely adopted by oncologists. Replacing pumps by a long-acting gene vector would have great clinical appeal for the treatment of cancer pain, because oncologists could deliver the gene therapy intrathecally by an LP as they routinely do with IT chemotherapy. In a clinical setting, continuous release of spinal opioids by gene therapy would be combined with a systemic opioid drug that is adjusted for worsening or breakthrough pain. Preclinical studies suggest that this may be a particularly effective combination, due to spinal-systemic synergy enhancing the analgesic efficacy [28].

Creating a gene vector with opioid activity requires the identification of an appropriate therapeutic gene. In principle, endogenous opioid peptides can exert the same activities as alkaloid opioids such as morphine. We chose the opioid peptide β-endorphin (β-EP) as the active drug for our gene therapy studies because of its potential to synergize with systemic opioids and because IT β-EP has evoked powerful analgesia in rats, cats, non-human primates [29,30] and humans [31-37]. The clinical studies showed that IT β-EP was safe over a wide dose range (30 g to 3 mg) and that its analgesic effect was short-lived, with pain control usually fading within a day of administration [31-37]. Endogenous β-EP is derived from the opioid peptide precursor pre-pro-opiomelanocortin (POMC). POMC is not suitable for pain gene therapy because it requires post-translational proteolysis by peptidases that are not expressed in most cells reached by IT gene transfer. Therefore, we developed an artificial opioid peptide precursor, ppβ-EP, which induces production of biologically active β-EP in primary cell cultures and leads to profound analgesia in rats if delivered intrathecally by retrovirally transduced fibroblasts or adenovirus vectors [38-40]. However, as typical of retrovirally transduced transplanted fibroblasts and of adenoviral gene transfer in immunocompetent animals, the duration of ppβ-EP gene-induced analgesia was less than 2 weeks. Therefore, we became interested in the use of a vector system with a potential for long-term gene expression, AAV.

IT gene therapy to suppress glial activation

Glial cells in the SC are an important new target of analgesic drug development, reflecting a recent paradigm shift [41,42 ,43]. Traditionally, research on SC pain mechanisms focused solely on neurons, because they were believed to be the active regulators of sensory signals and the plastic component when functional changes occur in chronic pain. The non-neuronal cells, on the other hand, include a family of cells collectively referred to as glia and were thought of as rather passive insulators. A number of observations questioned the concept and raised the possibility that glia are in dynamic communication with neurons: SC glial cells synthesize and/or secrete certain neurotransmitters, such as glutamate, or express neurotransmitter receptors [44-46]; participate actively in

neurotransmitter uptake from the extracellular space and in their directional transport [47]; and release several neuroexcitatory substances implicated in pain facilitation, including glutamate, prostaglandins and nitric oxide [45,48].

Glia consist of three major cell types, astrocytes, oligodendrocytes and microglia. Oligodendrocytes myelinate axons and hence make up a large proportion of white matter. Astrocytes and microglia are functionally active at the synapse. Astrocytes are derived from neural tissue during development, while microglia are derived from monocytes or other hematopoietic cell lines. Glial activation was consistently observed in the SC of rats with neuropathic pain [49-57]. Most readily notable was increased expression of glial fibrillary acidic protein (GFAP), observable in tissue sections [54]. Detailed studies led to three sets of observations: (i) the process of glial activation is driven by several pro-inflammatory cytokines, most prominently IL-1β, IL-6 and tumor necrosis factor (TNF)α. Although cytokines may be synthesized from multiple sources [57,58], astrocytes and microglia in the SC also release these cytokines [59]. Cytokines lead to facilitation of pain conduction and thereby induce and/or enhance the chronic pain state. (ii) Cytokines act in a paracrine manner, transcending the strict anatomical connections characteristic of neurons. Glial activation on one side of the SC can lead to glial activation and pain facilitation in the contralateral side leading to 'mirror image pain', a phenomenon that was well known but poorly understood previously [52]. (iii) Interventions capable of suppressing glial activation and cytokine induction reverse pain facilitation [60,61]. One such intervention that we are currently developing is IT gene therapy with IL-10.

IL-10 is an anti-inflammatory cytokine that can suppress IL-1β, IL-6 and TNFα in inflammatory processes outside the CNS, such as in inflammatory bowel disease or rheumatoid arthritis [62]. Therefore, we hypothesized that given the cytokine suppressive activities of IL-10, it would have similar activity in the CNS for pain. Using various models of neuropathic pain, we and others demonstrated that recombinant IL-10 suppressed nociceptive behavior in rats if administered intrathecally [63,64]. The brief half-life of IL-10, approximately 2 h in the SC, limits pain reversal to no more than several hours. Therefore, we sought to achieve chronic delivery of IL-10 via IT gene delivery. In the first approach, IT adenoviral vectors led to efficient expression of IL-10 in rats for up to 2 weeks and concomitant control of nociceptive behavior in two different models of neuropathic pain [65]. As expected for adenoviral vectors [66], IL-10 expression was lost at time points beyond 2 weeks, resulting in the recurrence of nociceptive behavior in all animals. In our second approach, we explored non-viral IT gene transfer using naked plasmid DNA [67]. Pilot studies support that a single vector application has a definitive but short-lived effect, while prolonged therapeutic benefit can be observed with repeated applications. Specific *cis*-acting sequences in the plasmid and injection in hypertonic diluent may lead to improved gene transfer [67]. In our third approach, we used recombinant AAV (rAAV) vectors, as discussed in further detail below.

IT marker gene transfer studies with vectors other than AAV

Achieving effective gene transfer in the IT space has been notoriously difficult. This led a number of investigators to explore the problem using marker genes. Studies showed that adenovirus transduces the meningeal linings, but that the expression declines rapidly after 1 week, due to an immune response [40,68]. IT gene transfer by electroporation of naked DNA leads to expression in SC neurons, DRG and the meninges, but expression disappears similarly after 1 week [69,70]. A large number of non-viral gene transfer methods failed to yield efficient expression, including polyethylenimine of different molecular weights, complexes with transferrin, psoralen-inactivated viral capsids or both, a variety of cationic lipids commonly used for transfection in tissue culture, and naked plasmid DNA [AS Beutler, MS Banck, unpublished data]. Meuli-Simmen *et al* reported successful intracerebroventricular and IT gene transfer in mice using plasmid DNA alone or complexed with cationic liposomes with significant expression in meningeal fibroblasts for up to 2 weeks [71]. Naked plasmid DNA was used in rats for IT gene transfer as mentioned in the previous section in the context of IL-10 [67].

AAV vectors and their application in experimental pain therapy

Recombinant AAV vector properties

AAV is a single-stranded DNA parvovirus. Its genome is simple in organization, with the left side coding for four replication proteins (Reps), the right side coding for three capsid proteins (Caps) and with palindromic inverted terminal repeat (ITR) elements in flanking positions on both sides [72]. There are at least ten AAV serotypes: AAV3 and AAV5 are human viruses, AAV1 and AAV2 may also be human viruses, as indicated by serological evidence, but were isolated from non-human primates, and AAV6 is > 99% identical to AAV1 and seems not to be discernibly different. For AAV7 and AAV8, only the Cap sequences are known, which were cloned from non-human primates by a PCR-based strategy [73].

Currently available rAAV vectors are derived from AAV2 (ie, they contain the ITRs of AAV2). The ITRs are the minimal *cis*-acting elements necessary for replication [74], allowing the construction of rAAV vectors in which the Rep and Cap coding regions are replaced by a choice of up to 4.3 kb of sequences containing a transgene and its regulatory elements. For production of rAAV vectors, *rep* and *cap* genes need to be provided *in trans*. Wild-type AAV is a 'dependovirus', which indicates that it requires a helper virus for replication during its natural life cycle, usually adenovirus. Similar helper functions are also required for making rAAV. Production of rAAV is most commonly carried out by co-transfection of three plasmids carrying the sequences for the recombinant vector genome, *rep*, *cap* and the adenoviral helper functions. AAV2-derived recombinant vector genomes may be packaged in Cap proteins of any of the other serotypes by exchanging the *cap* sequence of AAV2 in the appropriate plasmid with a *cap* sequence of any of the other serotypes. The resulting rAAV vectors are 'pseudotyped' and designated rAAV2/1, rAAV2/2, rAAV2/3 and so forth. rAAV pseudotyping can lead to marked improvements in gene transfer efficiency, as

reported for gene transfer into mouse muscle [75] and intraparenchymal gene transfer in the brain [76].

A direct consequence of the cell culture-based production method for rAAV is the requirement to isolate the recombinant virus. The starting material consists typically of 293 cells harvested 48 h after transfection and subjected to several freeze-thaw cycles. After removal of cell debris, the rAAV is concentrated by two iterations of ultracentrifugation in a CsCl gradient (in the case of the rAAV2/2 serotype, purification may also be performed chromatographically using a heparin column). While the purification/concentration of rAAV vectors is a highly perfected procedure in many AAV expert laboratories, it needs to be kept in mind that, even under the best circumstances, no rAAV preparation is pure. The following components may be found and need to be considered in the interpretation of any data: intact, fully infectious rAAV particles; defective rAAV particles that have no or decreased infectivity; empty viral particles lacking the rAAV genome; wild-type AAV virus; plasmid; and recombinant proteins encoded by the plasmid and produced during the 2 days between transfection and cell harvest.

Among the viral gene transfer vectors, rAAV is favored by many researchers interested in achieving stable long-term gene expression and in translating their studies into clinical trials, because, compared with adenovirus, rAAV causes less of an inflammatory response. In addition, rAAV elicits humoral immunity, but usually does not lead to any detectable cellular immunity, at least in rodent models. Unlike Moloney type retroviral vectors, rAAV has the ability to infect non-dividing cells and to maintain stable expression over time. Compared with lentiviral vectors, rAAV may be a safer choice, because it is derived from a non-pathogenic virus. A number of other features of rAAV vectors are important; they can accommodate a gene expression cassette of up to approximately 4.3 kb in size, which excludes the use of very large therapeutic genes, but appears adequate for any of the proteins or peptides currently considered for pain therapy. The kinetics of rAAV gene expression are unlike most other vector systems. There is usually a slow rise in expression during the first week following *in vivo* application; rAAV expression then reaches a plateau after approximately 1 month. From there on, rAAV expression appears to persist for the lifetime of a laboratory animal, at least in studies with rodents or dogs. For therapy of chronic pain studies, the potential for long-term efficacy is an important reason to use rAAV. Yet, when conducting studies in preclinical pain models, which use nociceptive behavior as a primary outcome, the slow onset of gene expression may require longer duration of experiments and larger group sizes.

The reason for the slow rise in transgene expression appears to be the requirement for conversion of the single-stranded rAAV vector genome to double-stranded DNA. Second-strand DNA synthesis occurs at different rates depending on the cell type and may not occur at all if certain cellular factors are missing. The identity and number of cellular proteins required for second-strand rAAV synthesis is not known, but it appears that they are related to DNA repair, are found at lower levels in metabolically inactive or quiescent cell types, and can be induced by agents that interfere with DNA synthesis or repair, such as hydroxyurea. To overcome these constraints, McCarty *et al* constructed rAAV plasmids containing a mutated ITR, resulting in the production of double-stranded or 'self-complementary' rAAV vectors (sc-rAAV), which can mediate gene expression without requiring second-strand synthesis [77]. In our experience with IT gene transfer, the use of sc-rAAV vectors constituted a significant step forward compared with traditional single-stranded rAAV (see below).

Intraparenchymal injection of rAAV into the SC or DRG for pain

rAAV vectors have been used in several pain studies to achieve gene transfer into the SC by direct intraparenchymal vector injection. This approach will probably never be applicable to humans because of the morbidity that would be associated with the procedure. Intraparenchymal vector injection yields only localized expression (limited to a few millimeters of the needle trajectory), which would necessitate applications at multiple spinal levels for most pain syndromes, requiring an extensive spine procedure. Regardless of this limitation, intraparenchymal rAAV is a useful tool for rodent studies as described below.

Eaton *et al* reported the use of intraspinal rAAV2/2 expressing brain-derived neurotrophic factor (BDNF) in a rat model of neuropathic pain [78]. The rationale for the study was derived from the previous observation that chronic delivery of BDNF to the SC from subdurally grafted cells attenuated the altered sensory behavior in neuropathic pain [79]. BDNF was expressed under the control of the cytomegalovirus promoter. rAAV2/2 vectors were quantified with an infectious unit (IU) center assay [80]. Access to the SC was gained through a laminectomy at the T12 level, a surgical procedure that removes the bony arch of a vertebral body, thereby exposing the dura overlying the SC. The dura was punctured with a needle and a glass pipette was inserted into the SC and advanced to a depth of 1 mm. 5×10^5 IU of rAAV2 was injected into the SC. BDNF expression was detected in the ventral and dorsal horn gray matter at the T12 level and spread 'several segments along the rostral caudal axis', suggesting that the rAAV2/2 virus diffused perhaps up to 2 to 3 mm from the injection site. BDNF expression was detectable at 1 week and at higher levels after 8 weeks. Chronic constriction injury was used as a neuropathic pain model. Pain intensity was assessed by recording nociceptive behavior in response to tactile and thermal stimuli. Nociceptive behavior was significantly ameliorated starting 1 week after vector injection and for the remainder of the study, which ended after 8 weeks.

Xu *et al* used rAAV2/2 to deliver the OR gene to the DRGs of the L4 and L5 nerve root in the rat [81]. They reasoned that increased levels of OR in the L4 and L5 DRGs would increase the analgesic effect of systemic opioids for nociceptive stimuli arising in the areas innervated by the L4 and L5 nerve roots. The OR was expressed under the control of the neuron-specific enolase promoter. rAAV vector quantification was not reported in this publication but can be inferred from a concurrent report by the same researchers, which describes that 'transducing units per

milliliter (TU/ml) was determined by transduction of cultured DRG neurons with serially diluted viral solutions' [82]. The titer of rAAV-enhanced green fluorescent protein viral stocks (used as control) was 4.2 x 10^8 TU/ml and the titer of rAAV- OR was 2.6 x 10^8 TU/ml. The left L4 and L5 DRGs were surgically exposed and 2 l of viral stock was injected into each DRG with a Hamilton syringe. The DRGs and the dorsal horn of the SC express the OR under normal conditions. The level of OR overexpression mediated by rAAV2/2 was quantified relative to basal expression levels. OR expression in the rAAV2/2-injected DRG was elevated to 1.2-fold of the basal level after 1 week, 1.4-fold after 10 days and 1.8-fold after 2 weeks. Expression remained elevated at approximately 2-fold until the end of the study at 3 to 6 months. OR overexpression led to increased analgesic efficacy of systemic morphine in chronic inflammatory arthritis rats when nociceptive stimuli were applied to the dermatomes corresponding to the gene therapy-treated DRGs.

IT rAAV gene transfer

The IT space has been chronically resistant to gene transfer with conventional rAAV2 vectors, for example, South *et al* failed to achieve IT gene transfer and had to perform intraparenchymal injections instead ("when the viral vector (was) administered into the CSF via an IT injection, very little if any uptake…occurred") [83]. Xu *et al* reported application of rAAV2 "into the subarachnoid space on the lateral side of the lumbar enlargement of the SC of 25- to 30-day-old rats" by puncturing the dura with a glass micropipette after a partial laminectomy [82]. The technique may result in an intraparenchymal rather than IT vector application, because the micropipette tip can touch or enter the SC, which may explain why expression in this study was anatomically limited to the SC parenchyma 2 mm along the rostral-caudal axis. Marker gene expression "started to appear in the dorsal horn at 1 week…and nearly doubled at 3 weeks" [82]. In the scope of studies on IT IL-10, rAAV2 was injected through a lumbar IT catheter (as described above) and there was evidence of analgesic efficacy in a rodent model of neuropathic pain. Therapeutic gene expression commenced shortly after vector administration, as indicated by reversal of nociceptive behavior within 1 to 3 days. Nociceptive behavior recurred after approximately 2 weeks, suggesting a loss of rAAV2 gene expression [84]. The latter may be related to early promoter shutdown or a variety of direct or indirect effects of IL-10, which are currently under investigation [ED Milligan, unpublished data].

In an ongoing study, the two principal reasons why AAV gene transfer might be so difficult to achieve in the IT space are being addressed [AS Beutler, MS Banck, CE Walsh, unpublished data]. Firstly, there might be insufficient levels of Cap receptors on the target cells in the IT space to mediate efficient rAAV uptake, therefore, we tested several alternatives to rAAV2/2 and found that serotype 1 was superior. Secondly, the target cells might lack the factors required for converting the single-stranded viral genome to double-stranded DNA, a requirement for gene expression. To address this issue, we compared the double-stranded sc-rAAV vectors with conventional single-stranded rAAV and found that sc-vectors facilitated higher levels of expression. Combining both modifications (ie, an sc-rAAV2/1 vector) led to robust, stable IT gene expression for up to 4 months, the longest time-point tested thus far [85].

Conclusions

rAAV vectors are becoming an efficient tool for IT gene transfer in rodents. The technology may be particularly useful for studies on chronic pain requiring long-term delivery of peptides or proteins to the SC. Extrapolating from the experience with rAAV delivery to various other body sites (ie, muscle, liver and brain), it can be expected that intrathecally delivered rAAV will evade cellular immunity in rodents and will have minimal or no direct effects due to the vector itself, thereby permitting a clean evaluation of the transgene of interest. For possible future clinical developments, rAAV appears to be among the safest gene transfer options and a vector type for which clinical safety and efficacy data are currently accumulating from several ongoing clinical trials for various diseases, such as Parkinson's disease, retinal degeneration and hemophilia [86].

Chronic pain treatment is the objective of a gene therapy approach that is presently moving from the laboratory into phase I/II clinical trials. For example, Wilson *et al* [87], Goss *et al* [88], Hao *et al* [89] and Mata *et al* [90] used herpes simplex virus (HSV) to express pre-pro-enkephalin in the DRG. HSV vectors are injected subcutaneously and, based on their natural tropism, infect peripheral nerve endings resulting in central gene expression [87-90]. Clinical trials are planned in cancer patients with isolated painful vertebral metastases. This approach will likely be most relevant for localized pain syndromes, because vectors have to be delivered to the skin corresponding to all affected dermatomes. On the other hand, IT rAAV delivery may be practical in multifocal pain syndromes, for example, in patients with widely metastatic cancer, because a single injection can deliver the vector to many SC levels.

In order to evaluate the potential of rAAV as an IT gene transfer vector for clinical use, a number of hypotheses, which are related to the vector technology, need to be tested. Establishing in large animals whether a serological and/or cellular immune response occurs to IT rAAV will be critical [91]. Equally important will be to determine the biodistribution of IT rAAV, the target cell types and the frequency of integration events. Efficacy of the gene transfer needs to be assessed independently of a clinical response by determining transgene product levels and persistence in the CSF. Furthermore, questions specific to various therapeutic genes are important. For example, for spinal opioids, the distribution of the transgene product in the CNS and the consequences of irreversible persistence require a rigorous safety assessment. For IL-10, special issues would include the consequences of suppressing or modulating CNS immune responses chronically, thereby posing a potential risk to opportunistic CNS infections. The most important long-term benefit from the technology may be that it can serve as a platform for target validation in patients (ie, for determining whether the manipulation of a specific molecular mechanism is as effective in patients as in an

animal model). Thereby, an important role of IT rAAV may be to serve as a tool for translational research studies on chronic pain.

Acknowledgements

We would like to acknowledge research support from the American Society for Clinical Oncology, The G&P Foundation for Cancer Research, the TJ Martell Foundation and the National Institute for Neurological Disorders and Stroke (1K08NS046012-01) to ASB and from the National Institute for Drug Abuse (DA018156) to EDM.

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