**Original Article**

**Effect of recombinant adenovirus coding for endomorphin-2 on neuropathic pain in rats**

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**Abstract:** Objective: To construct a transgene expressing human endomorphin-2 by linking the signal peptide of mouse nerve growth factor (PN) to a human endomorphin-2 DNA sequence containing a short linker recognized by the protease FURIN and test the analgesic effect of endomorphin-2 on neuropathic pain. Methods: The transgene was inserted into the cosmid pAxCAwt to generate PN-EM-2-pAxCAwt. The recombinant adenovirus Ad-PNEM2 was packaged and propagated in HEK293 cells. After the Ad-PNEM2-infected NIH3T3 cells had been cultured, protein expression was examined by immunofluorescence and ELISA. A CCI rat model was constructed and the Ad-PNEM2 was administered intrathecally. The rats’ pain thresholds (PWL) were measured and the presence of endomorphin-2 in the cerebrospinal fluid was confirmed through ELISA. Results: The Ad-PNEM2 expressed endomorphin-2 smoothly and abundantly in NIH3T3 cells at a significantly higher rate than the viral control (P<0.01) or blank control (P<0.01). The expressed endomorphin-2 was mainly observed in the cytoplasm. The concentration of endomorphin-2 in the cerebrospinal fluid increased 1 day after injection and peaked between 7 and 14 days after injection. After injection, PWL approached normal levels in the operated study group. No significant change was observed in the control groups. There was a significant correlation between PWL and endomorphin-2 level (r = 0.944, P<0.001). Conclusion: The constructed human endomorphin-2 transgene was expressed effectively, and endomorphin-2 expressed by the recombinant adenovirus altered the threshold to thermal stimulus and showed good analgesic effect.

**Keywords:** Endomorphin-2, nerve growth factor, adenovirus, pain, gene therapy

**Introduction**

Endorphins are one kind of opioid peptide in the nervous system. Opioid peptides are critical to endogenous analgesia. The activation of this system can prevent the conduction of peripheral and central pain signals and eliminate or alleviate this unpleasant sensation. Many studies have shown that endorphins are good endogenous analgesics. Endorphin-2 (EM-2) is a carboxy-amidated tetrapeptide that binds to the mu-opioid receptor with high affinity and selectivity [1]. It has one promising and exciting feature: it is effective against neuropathic pain, which even morphine cannot mitigate easily [2, 3]. Endorphins are easily degraded by various proteases [4]. This may be one reason why the analgesia produced by endorphins is shorter in duration than that produced by morphine [5]. This also hampers its direct application as a drug.

Many researchers have attempted to find a way to overcome the fact that some proteins cannot be administered directly in clinical therapy. Gene therapy is one promising means of overcoming this obstacle. The key to gene therapy is clear expression of the gene in question. Endorphin peptides have been isolated from bovine and human brains [6]. Great effort has been made toward finding the genes that encode endorphins, even to the point of a project utilizing human proteomes to search for the precursor proteins of endorphin-1 and endorphin-2 [7]. Despite these efforts, a breakthrough is still needed. We built several stringent DNA sequences for endorphin-2 using a genetic code table and selected one of them based on human codon usage bias. One article has shown that the engineered transgenes of endorphin-2 can be used for the gene therapy, which was found effective against neuropathic pain in rats [8]. Mizoguchi et al.
Effect of EM-2 on neuropathic pain

found that the antinociceptive effects of endomorphin-2 may involve the release of dynorphin A via mu-opioid receptors on both the spinal and the supraspinal level [9, 10]. This study was similar to another that administered engineered beta-endorphin to rats with inflammatory pain. Hao et al. [11] constructed a non-replicating herpes simplex virus (HSV)-based vector containing a synthetic endomorphin-2 gene. The HSV-based vector transduced DRG neurons and the constructed transgene expressed endomorphin-2 that reduced nociceptive behaviors in response to mechanical and thermal stimuli after injection of complete Freund's adjuvant into the paw. These findings suggest that transgene-mediated expression of endomorphin-2 at the spinal level may act in vivo both peripherally and centrally through mu-opioid receptors, reducing the perception of pain. A team led by Wolfe designed a tripartite synthetic gene to direct the production, cleavage, and amidation of EM-2. This transgene was inserted into a replication-defective herpes simplex virus (HSV) vector (vEM2). Subcutaneously inoculated vEM2 was delivered in retrograde fashion to the dorsal root ganglion and found to reduce the mechanical allodynia and thermal hyperalgesia in the spinal nerve ligation model of neuropathic pain [8].

To facilitate maturation of the constructed vector, the code sequence of human endomorphin-2 was ligated to the end of the signal peptide of mouse nerve growth factor with a linker recognized by a protease Furin as described by Finegold et al. [12]. After we obtained the translated transgene, we constructed an adenoviral vector to serve as the gene delivery carrier. In the rat CCI model of neuropathic pain, not only did the constructed transgene express human endomorphin-2 successfully, but this endomorphin-2 also changed the threshold of thermal hyperalgesia in CCI rats very effectively. Researchers have explored the applications of gene therapy in the treatment of chronic pain for years. The studies mentioned above, including our own, suggest that this transgene of endomorphin-2 may be a promising option.

Methods and materials

Strain propagation and cell culture

Escherichia coli strain DH5-α (Gibco-BRL, UK) and TG1 (Institute of Genetics, Fudan University, Shanghai China) was transformed with recombinant plasmid vectors in LB culture media. NIH3T3 cells, obtained from the American Type Culture Collection (Manassas, VA, U.S.), were transfected with recombinant adenoviral vectors and maintained as subconfluent stocks in Dulbecco's Modified Essential Medium (DMEM, Gibco-BRL, UK) containing 10% fetal bovine serum (PAA, Germany).

Transgene and recombinant adenovirus

Although the endogenous analgesic protein endomorphin-1/2 has been found in primates, its location within the genome is still unknown. It is known, however, that the amino acid sequence of human endomorphin-2 is Tyr-Pro-Phe-Phe-NH2. We translated this amino acid sequence into DNA sequences and selected one of them for use. Two primers were designed as follows: the forward primer 5′-ACG CGA ATT CAC CAT GTC CAT GTT GTT CTA-3′ and the reverse primer 5′-ATA TGG ATC CTT AGA AGA AGG GGT AGC GCT TGC TCT TGT GAG TCC TGT TGA AAG GGA TTG TAC CAT GGG CCT GGA AGT CTA GAT CCA GA-3′.

A polymerase chain reaction was performed. The vector plasmid pTCNE (from our previous work) was the substrate template of the reaction, which consisted of one cycle of 95°C for 3 minutes, then 30 cycles of 95°C for 0.5 minutes, 58°C for 0.5 minutes, and 72°C for 1 minute, followed by a single 5-minute extension cycle at 72°C. The purified, digested PCR products, 401 bp in size, were subcloned into the pUC19 by EcoRI and BamHI to generate pUC19-PN-EM-2. This was digested with EcoRI and BamHI to generate a fragment encoding human endomorphin-2. The inserted fragment was verified by automated DNA sequencing using a T7 Sequencing™ Kit (Pharmacia Biotech, Sweden). The fragment was blunt-ended and inserted into the SwaI site of the cosmid vector pAxCAwt (Takara, Kyoto, Japan) to generate the vector PN-EM-2-pAxCAwt for preparation of recombinant adenovirus Ad-PNEM2. This was digested with EcoRI and BamHI to generate a fragment encoding human endomorphin-2. The inserted fragment was verified by automated DNA sequencing using a T7 Sequencing™ Kit (Pharmacia Biotech, Sweden). The fragment was blunt-ended and inserted into the SwaI site of the cosmid vector pAxCAwt (Takara, Kyoto, Japan) to generate the vector PN-EM-2-pAxCAwt for preparation of recombinant adenovirus Ad-PNEM2. The recombinant adenovirus was prepared with an Adenovirus Expression Vector Kit (Takara) according to the manufacturer’s protocol. The recombinant virus was identified using PCR. The titer of the virus block was determined using the TCID50 method.
Effect of EM-2 on neuropathic pain

In vitro expression of transgene

NIH3T3 cells were cultured in six-well plates at 2×10⁵ cells per well. The cells were divided into a study group, a virus control group, and a blank control group. Recombinant adenovirus expressing EGFP was used as the control virus. The MOI (multiplicity of infection) of the experimental virus was 10. The cultured cells were infected with propagated recombinant virus one day after the NIH3T3 cells were seeded in six-well plate.

Cytological immunofluorescence

Sterilized 24 mm cover slips were put into each well of every six-well plate (Corning) and NIH3T3 cells were seeded. The cells were grown as subconfluent polarized monolayers. The cover slips with cells were collected carefully 24 hours after the recombinant virus and the controls were added to their respective groups of cells. The cover slips with cells growing on them were rinsed three times with PBS (5 minutes each time), fixed with 4% formaldehyde for 15 minutes, and again rinsed three times with PBS. The fixed cells were stained by incubation with primary antibodies (rabbit anti-endomorphin-1 and endomorphin-2 affinity purified polyclonal antibody (Chemicon, Temecula, CA, U.S., No. AB5106) and diluted in 1:200 in PBS with 5% BSA blocking buffer). The cover slips were incubated in blocking buffer and then rinsed three times (5 minutes each) in PBS. They were then exposed to Cy3-labeled secondary antibodies (Cy3-labeled goat anti-rabbit IgG, 1:1000, Sigma) for 1 hour at room temperature in the dark. Cells were stained with Hoechst 33342 (1:1000, Sigma) for 1 minute and rinsed as before. The rinsed cover slips with PBS were mounted with a drop of 50% glycerin onto slides and imaged using an Olympus 10×20 IX70 inverted fluorescence microscope.

Enzyme immunoassay

The culture medium was sampled 1 day, 3 days, and 7 days after viral infection. The concentration of endomorphin-2 was measured using a sandwich ELISA kit (E074SHu, Uscln Life Science Inc. Wuhan China) according to the manufacturer’s protocol as follows: 100 µL of standard, control, and sample were added to flat-bottom 96-well microtiter plates and incubated for 2 hours at 37°C. The liquid was then removed from each well. Detection reagent A (100 µL) was added and incubated for 1 hour at 37°C. Each well was aspirated and washed three times with wash buffer. Detection reagent B (100 µL) was added to each well and incubated for 1 hour at 37°C. Each well was aspirated and washed three times. Substrate solution (90 µL) was added to each well and incubated for 30 minutes at room temperature in the dark. The reaction was terminated with stop solution. The optical density was read within 30 minutes at 450 nm on a microplate reader (Bio-Rad Laboratories). Endomorphin-2 concentration was determined according to the standard curve from standard data.

Animals

The adult male Sprague-Dawley rats (250−300 g at the time of arrival) used in experiments was provided by Sino-British SIPPR/BK Laboratory Animal Ltd. (Shanghai, China). Rats were housed in temperature- (23 ± 3°C) and light-controlled rooms (12:12 light:dark; lights on at 07:00) with standard rodent chow and water available ad libitum. Animals were divided into six groups by treatment: 1) Sham, normal saline injection; 2) sham, control-virus injection; 3) sham, virus injection; 4) operated upon, normal saline injection; 5) operated upon, control virus injection; and 6) operated upon, virus injection. All procedures were approved by the Institutional Ethics Committee of Eastern Hepatobiliary Surgery Hospital of Shanghai, China and were in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. NIH (publication No. 96-01).

Surgery

Animal subjects were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the common sciatric nerve was exposed. Proximal to the sciatric trifurcation, the nerve was freed of adhering tissue, and four ligatures (4-0 chromic gut) were tied loosely around it with a 1.0-mm interval between each. The incision was closed in layers. An identical dissection was performed on sham subjects, except no ligatures were tied.

A polyethylene (PE10) catheter (length, 15 cm; OD, 0.61 mm) was prepared and sterilized with...
Effect of EM-2 on neuropathic pain

Behavioral testing (paw-withdrawal method)

The threshold of thermal pain was determined by paw withdrawal latency (PWL), visual observation of the rats' paw withdrawal reflex induced by a radiant heat instrument. The investigator performing the behavioral tests was blind to the treatment assignments. The data for each group were collected on the day the viruses were injected and 1 day, 3 days, 7 days, 14 days, and 28 days after that. A movable radiant heat source, placed underneath a glass table, was aimed at the plantar surface of one hindpaw. Care was taken to stimulate the hindpaw only when it was in contact with the glass floor of the test apparatus. Stimulus onset activated a timer, which was stopped manually upon paw withdrawal. Latencies of the reflex were measured from the onset of radiant heat until paw withdrawal to the nearest 0.1 second. The latencies for both paws were monitored.

75% alcohol before insertion. Rats were anesthetized as above. The lumbar region was shaved, prepared with 10% povidone-iodine, rendered kyphotic, and incised 1.5–2.0 cm longitudinally in the midline at the level of the iliac crests. The paraspinal musculature was dissected bluntly from the vertebra. The sixth spinous process was removed to facilitate epidural catheter insertion. After making a small hole into the L5 and L6 intervertebral space with a blunted needle, the epidural catheter was inserted approximately 2 cm into the rat's body. The catheter was settled in the fascia, tunneled under the skin to the cervical region, flushed with saline, and sealed with electrocautery. The incision was closed in layers.

Enzyme immunoassay

The cerebrospinal fluid was collected through the catheterized tubes at different points in time after rats were anesthetized. The concentration of endomorphin-2 in the cerebrospinal fluid was determined by ELISA as described above.

Figure 1. Construction of recombinant virus. A: The code fragment of the transgene was detected by PCR in pUC19-PN-EM-2. 1: Detected vector. 2: Positive control; B: Selection of forward insertion positive clone. 1, 3–6: Insertion positive clone. 2, 7: Reverse insertion positive clone; C: Screening of positive virus. 1, 2: Positive single virus. 3, 4: Negative virus. 5: Negative control. 6: Positive control; D: Identification of RCA adenovirus. 1: Propagated virus stock. 2: Positive control (880 bp). 3: Negative control.
Effect of EM-2 on neuropathic pain

Statistical analysis

Data are expressed as mean ± SD. Data were analyzed using an unpaired student’s t-test. The linear correlation coefficient was used to determine the correlation between quantitative variables. A P value < 0.05 was considered significant.

Results

Construction and amplification of viral vectors

The DNA sequence of endomorphin-2 was obtained using genetic biochemistry. The transgene was driven by the human cytomegalovirus (CMV) promoter. To facilitate the expression of the transgene, the code DNA of endomorphin-2 was led by the signal peptide of mouse nerve growth factor just as the expression gene in pTCNE. A plasmid clone vector, pUC19-PN-EM-2, was constructed by ligating the PCR product into pUC19 (Figure 1A). The PCR segment was sequenced. Next, a recombinant adenoviral vector, PN-EM-2-pAxCAwt, was generated by inserting the segment containing the code sequence of endorphin-2 from pUC19-PN-EM-2 into the SwaI site in pAxCAwt. The forward insertion positive clone (508 bp) was selected based on the results of a PCR analysis (Figure 1B).

A recombinant adenovirus, Ad-PNEM2, was packaged with the help of HEK293 cells. Single-clone viruses were screened out by PCR and propagated (Figure 1C). Although propagation of E1 region replacement adenovirus vectors in HEK293 cells resulted in the rare appearance of replication-competent adenoviruses (RCA), it also indicated that there was no RCA virus in the virus stock (Figure 1D).

Expression of transgene in cells

No endomorphin-2 expression is naturally present in NIH3T3 cells, which are suitable for transgene research because of their the negligible expression background. The expression of EM-2 in NIH3T3 cells was detected on the first day after infection by recombinant EM-2-expressing virus, and the concentration of EM-2 in cell culture media had significantly increased by the third and seventh days after infection relative to animals injected with the of EGFP-control virus (P<0.01) or the blank control (P<0.01). A clear, gradual increase in EM-2 expression was observed (Figure 2).

The control virus Ad-EGFP was able to efficiently infect the cultured NIH3T3 cells at MOI=10. The constructed recombinant virus served as a control and was found capable of infecting the cells. The expressed EM-2 was abundant in the cytoplasm but not in the nucleus. The expressed product was clearly observed in cells infected with the viral control Ad-EGFP or in cells exposed to normal saline (Figure 3).

EM-2 in cerebrospinal fluid

The fusion gene must be correctly expressed in the target cells before it can exert any biological effects. Some articles have shown that recombinant adenoviruses can infect cells in the spinal pia mater, causing those cells to express transgenes. The concentration of EM-2 was determined by gene expression. One day after the injection of recombinant virus Ad-PNEM2, the concentration of EM-2 in the cerebrospinal fluid was found to be significantly changed (P<0.05). After that, the expression level of the transgene increased over time. The concentration of EM-2 reached a peak between the seventh and fourteenth days. After that, expression descended and the level of EM-2 returned to baseline (Figure 4). Infection with control virus Ad-EGFP did not significantly change the level of EM-2 in the CSF relative to the NS group (P>0.05).

Effects of expressed EM-2 on neuropathic pain

EM-2 selectively acts at mu-opioid receptors, which belong to the G protein-coupled receptor
Effect of EM-2 on neuropathic pain

Family. Activation of the mu-receptor by EM-2 causes analgesia. After the rats' sciatic nerves were ligated, the PWL, representing the threshold of thermal pain, decreased significantly (P<0.05). This indicated that thermal hyperalgesia was induced by the ligation. The injection of recombinant adenovirus expressing human EM-2 caused the PWL in OR rats to increase gradually and significantly (P<0.05), but the two controls did not (P>0.05). In the group given control virus Ad-EGFP, the PWL did not show any significant change (P>0.05) (Figure 5). PWL showed a strong and significant linear correlation with the concentration of EM-2 in the cerebrospinal fluid (r=0.944, P<0.001) (Figure 6). In sham rats, no difference in PWL was observed among Ad-PNEM2, Ad-EGFP, or NS (P>0.05).

Discussion

Gene therapy is the application of genetic engineering to medicine. The greatest advantage of gene therapy over traditional treatments is that transgene-expressed peptides occur at high enough concentrations to maintain therapeutic effects over long periods, even when they are administered only once [13]. This minimizes systemic side effects [14, 15]. However, for some small molecules such as oligopeptides, problems remain. The most obvious of these problems is that the half-life of these molecules in the body is too short for them to remain at any concentration sufficient for therapeutic effect. This requires not only higher doses but also more frequent administration.

Because endomorphin-2 is a tetrapeptide with only four amino acid residues, it is suitable for use in gene therapy. The present study investigated the effects of a constructed recombinant adenovirus expressing an engineered transgene of human endomorphin-2. The effects of this endomorphin-2 on the threshold of thermal pain were measured using paw withdrawal latency in a rat CCI model. To ensure high-level endomorphin-2 expression, we employed a unique and efficient adenoviral vector (Ad-PNEM-2) encoding an engineered endomorphin-2 expression cassette controlled by a strong promoter (CMV promoter). After the recombinant
Effect of EM-2 on neuropathic pain

adenovirus infected the cultured NIH3T3 cells, large amounts of expressed endomorphin-2 were observed in those cultured NIH3T3 cells. A large quantity of endomorphin-2 was found to have been transferred into the cell culture medium. This indicated that the constructed transgene was normally expressed and that the mature protein had been produced.

The inoculation of recombinant adenovirus was carried out by injecting the virus into the cerebrospinal liquid in the lumbar subarachnoid space through an inserted PE-10 catheter. The virus infected the epithelial cells of spinal pia mater and the endomorphin-2 produced there was transferred to the outside. The concentration of endomorphin-2 in the cerebrospinal liquid became significantly higher than pre-inoculation levels \((P<0.05)\). Some of the expressed endomorphin-2 bound to mu-opioid receptors and changed the threshold of neuropathic pain in CCI rats. All of these findings suggest that our engineered transgene of human endomorphin-2 was successfully expressed and exerted analgesic activity.

Figure 5. Change in threshold to thermal stimulus in rats. A: Operated, virus injection. B: Operated, control virus injection. C: Operated, normal saline injection. D: Sham, virus injection. E: Sham, control-virus injection. F: Sham, normal saline injection. G: Changes in PWL in rats operated in left hindpaw relative to un-operated rats, combined results as shown in A, B and C.
Neuropathic pain is chronic pain initiated or caused by a primary lesion or dysfunction in the nervous system. The mechanisms involved in neuropathic pain are complicated, including both peripheral and central pathophysiologic mechanisms. They are not fully understood. This article only deals with peripheral situations. Many studies have shown that neuropathic pain is likely to be only partially responsive to opioid therapy, if at all [16]. However, other findings have shown that neuropathic pain responds to high doses of opioids [17–19]. Gene therapy is a promising means of treating diseases on the genetic level, and it is expected that it will be applied to the treatment of chronic pain. It has been shown that beta-endorphin expressed in vivo from an engineered transgene encoded by a recombinant adenovirus can change the threshold of inflammatory pain induced by carrageenan [12]. Endomorphin-2 expressed by a constructed transgene in rats has been found to mitigate neuropathic pain [20]. Endomorphin peptides, including endomorphin-1 and endomorphin-2, have been isolated from bovine and human brains [21]. They are endogenous agonists for mu-opioid receptors. They have both high affinity and high selectivity [22]. Endomorphins have been found to be effective against neuropathic pain, even when morphine is not expected to have any analgesic effect. Endomorphins have shown obvious analgesic effects on hyperalgesia in acute and chronic stages of neuropathic pain induced by ligating the sciatic nerve [23].

The precise locations of endomorphins 1 and 2 on the human genome are unknown, so it is impossible to use standard gene therapy with endomorphin-2. We bypassed this problem by using the genetic principle to construct endomorphin-2 transgenes, which are distinct from the tripartite synthetic gene [20]. There is only one copy of the DNA sequence encoding human endomorphin-2. In the present study, endomorphin-2 was found to be abundantly expressed and present in the cytoplasm through immunofluorescence cell staining. A large quantity of endomorphin-2 was detected in cell culture media by ELISA. These outcomes indicate that our constructed transgene can be expressed in primate cells and that making an engineering transgene is a viable avenue to gene therapy.

The functional site of endomorphin-2, in this situation, is in the spinal cord [24]. This is far from the infection target of the viral vector. The transgene-expressed proteins must be transported outside of the cells and then transferred to mu-opioid receptors. The recombinant adenovirus Ad-PN-EM-2 was injected into the lumbar cerebrospinal fluid of rats, where it infected the epithelial cells of the spinal pia mater [25]. The engineered transgene expressed the peptide at a high level. The product not only entered the cerebrospinal fluid through the paracrine mechanism but was also transferred into the superficial cells of the spinal cord. The expressed human protein endomorphin-2 bound to mu-opioid receptors [24]. Once there, it triggered a reaction cascade that finally blocked the transduction of the pain signal through the nerve fibers. Horvath et al. first reported the antinociceptive function of intrathecally injected endomorphin-2 [21]. Ohsawa et al. showed that the antinociception induced by spinally injected endomorphin-2 contains an additional component, which is mediated by the spinal release of dynorphin A (1–17) acting on kappa-opioid receptors in the spinal cord [26]. These findings indicated that there may be a connection between kappa-opioid-receptor-containing neurons and mu-opioid-receptor-containing neurons, but most of the analgesic activity was generated through the binding of endomorphin to mu-opioid receptors.

**Conclusion**

This study shows that the designed transgene was successfully expressed at a high level in
vitro and in vivo and that the expressed endorphin-2 exerted analgesic activity against neuropathic pain. These results further support the idea that gene therapy is a viable means of treating chronic pain. Several problems must be addressed before human endorphin-2 can be used for gene therapy against chronic pain. The first is cloning of the original gene for human endorphin-2. If it is not found and cloned successfully, a more effective engineered transgene expressing human endorphin-2 must be designed. If both can be accomplished, then the more effective gene must be selected. The second is the gene-delivering vector, which must leave normal cellular biological order and the intrinsic expression of the original gene untouched. Interference with normal operation of the cell would be the worst possible side effect. Third, the method by which the gene-delivering vector is administered must be improved. A suitable method will be easy to perform and will not cause any injury or feelings of discomfort to the patients. There is still a long way to go before these problems are solved.

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Effect of EM-2 on neuropathic pain


