RESEARCH ARTICLE

Gene-gun particle with pro-opiomelanocortin cDNA produces analgesia against formalin-induced pain in rats

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Endogenous opioid peptides play an essential role in the intrinsic modulation and control of inflammatory pain, and could be therapeutically useful. These opioid peptides are synthesized as parts of larger precursor molecules. One such precursor molecule is pro-opiomelanocortin (POMC). In this study, we developed a gene-gun method for the transfer of POMC cDNA *in vivo*, and investigated its therapeutic effect on inflammatory pain in a rat model of formalin-induced pain. Human POMC cDNA was cloned into a modified pCMV plasmid and delivered to the skin of rats by gene gun. Three days after gene-gun injection, 1% formalin was injected. Endorphin levels were measured in the serum and skin after the formalin test, and skin histology was used to detect endorphin after green fluorescent protein (GFP; control) or POMC cDNA transfer. There was no significant difference in the results of acute nociceptive tests between the experimental and control groups. There was also no dif-

ference in response between the groups to phase 1 of the formalin test. However, rats which received POMC cDNA via gene-gun injection showed a significantly reduced response in phase 2 of the formalin test. Endorphin immunoreactivity in the skin increased approximately three- to four-fold in experimental animals compared with GFP-treated controls at day 3 after injection. The phase 2 response in animals treated with formalin and naloxone did not differ significantly from the control, implying that the analgesic effects of POMC cDNA particle injection in phase 2 of the formalin test are reversed by naloxone. There are two major findings from this study. First, *in vivo* DNA delivery by gene gun to the skin is feasible. Second, the production of β -endorphin is insufficient to block phasic pain, but is effective against sensitization of the afferent neurons during phase 2 of the formalin test.

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Introduction

We are now in an era where the molecular mechanisms involved in the pathogenesis of clinical disorders are being unraveled and fully elucidated. As molecular-based knowledge and advanced technology continue to expand, there is increasing focus on gene therapy as a potential treatment for a number of disorders in various types of patients. Endogenous ligands, in particular the opioid peptides, are expressed by resident immune cells in inflamed peripheral tissue. Interleukin-1 β and corticotropin-releasing factor can stimulate the release of these opioid peptides, resulting in local analgesia and suppression of the immune system.¹ Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception during inflammation.² It is likely that these endogenous opioid peptides, when secreted from

immune cells peripherally at sites of inflammation, occupy the opioid receptors on sensory nerves, and cause analgesia by inhibiting the excitability of these nerves. This peripheral endogenous opioid-mediated analgesia could possibly be artificially introduced or induced by implantation of a therapeutic 'pain killer' gene to control inflammatory pain such as postoperative pain. An attractive candidate gene with potential for such therapeutic effects in the control of inflammatory pain is the pro-opiomelanocortin (POMC) gene. The expression of POMC produces opioid peptides including the β -endorphins, other shorter endorphins, adrenocorticotrophic hormone (ACTH), and melanocyte-stimulating hormones (MSH). β -endorphin can induce analgesia when transported to peripheral inflamed tissue by activating peripheral μ -opioid receptors on sensory neurons to inhibit pain within inflamed tissue.³ Up-regulation of P-selectin and PECAM-1 and the co-localization of L-selectin and β -endorphin in immunocytes imply an important role for these adhesion molecules in the recruitment of immunocytes containing β -endorphin to sites of painful inflammation.⁴

To date, viral vectors have been widely used in most gene therapy trials. This often requires the construction of complex recombinant viruses and expensive viral production facilities. Furthermore, the use of adenovirus, retrovirus, and herpes simplex virus as vectors has been hindered by viral cytotoxicity, host immune rejection, and poor efficiency.⁵ Adenoviral vectors can infect both mitotic and post-mitotic cells. However, their stability and the long-term expression of transgenes using first generation adenoviral vectors have been hampered by immune rejection.⁶ Recent deaths implicating gene therapy have further aroused concerns worldwide. Therefore, the relatively high cytotoxicity and immunogenicity of viral vectors hamper their potential use in the clinical context.⁷

Particle-mediated gene transfer has been used to deliver cytokine genes for cancer therapy. *In vivo* cytokine gene transfer by gene gun reduces tumor growth in mice.⁸ In this study, we set out to deliver POMC cDNA by gene gun into the skin of rats (Figure 1) to achieve a peripheral analgesic effect.

Results

The relationship between gene-gun therapy and antinociception

The results of the paw withdrawal latency test for each group are shown in Figure 2. There was no significant difference detected between the groups. Neither treatment with POMC nor treatment with GFP effectively prolonged the latency in the plantar test. In attempting to produce the robust nociceptive responses associated with a strong inflammatory reaction, we have shown that 1% formalin produced moderate, yet reliable, nociceptive responses in the ipsilateral hind paw. The contralateral paws did not significantly alter their flinching behavior during phase 1 or 2.

Effect of POMC gene-gun injection on the formalin-induced phase 2 response

POMC cDNA (group B) injection decreased the magnitude of the formalin-evoked phase 2 flinching response ($P < 0.05$) when compared with control groups (group A). However, there was no significant difference among control groups (group A), POMC plus naloxone (group C), POMC plus naloxone methiodide (group D) and con-

Figure 1 The gene gun uses a 400- pounds per square inch helium pulse to accelerate plasmid-coated gold particles through cell membranes of skin cells. (a) Gene-gun therapy in rat treated with POMC gene particle; (b) Gold particle in plantar.

Figure 2 The pain responses for the ipsilateral and contralateral hind paws using paw withdrawal latency test in GFP and POMC treated rats. Data are represented as mean $\pm$ s.e.m. Statistical comparisons between groups were made by two-tailed unpaired *t* test. No significant difference was noted between the ipsilateral and contralateral paws in GFP and POMC treated rats.

tralateral formalin injection groups (group E) in phase 1 and 2 ($P > 0.05$) (Figures 3 and 4).

Effect of POMC gene-gun injection on blood levels of endorphin

The levels of β -endorphin measured in serum are shown in Figure 5. Levels of β -endorphin increased significantly in both POMC-treated and POMC plus naloxone-treated animals when compared with the GFP-expressing group ($P < 0.05$), but there were no significant difference between POMC-treated and POMC plus naloxone-treated animals (Figure 5).

Figure 3 Time course of formalin-induced flinching behavior in control (group A), POMC gene-gun injection (group B), POMC gene-gun injection with naloxone (group C), POMC gene-gun injection with naloxone methiodide-treated (group D) and POMC gene-gun injection with contralateral paw formalin injection (group E). Each point represented as mean $\pm$ s.e.m. number of flinches per min.

Figure 4 Flinch responses during the formalin test phase 1 and 2 in control (group A), POMC gene-gun injection (group B), POMC gene-gun injection with naloxone (group C), POMC gene-gun injection with naloxone methiodide-treated (group D), and POMC gene-gun injection with contralateral paw formalin injection (group E) rats. POMC injection suppressed the phase-2 pain, whereas naloxone as well as naloxone methiodide reversed the analgesic effects of POMC. Data are presented as the mean $\pm$ s.e.m. Statistical comparisons between groups were made by ANOVA with individual comparisons identified using the post hoc test. * $P < 0.05$, showing a significant difference between GFP (group A) and POMC-only (group B) rats in phase 2.

Figure 5 Effect of POMC gene-gun injection on the blood levels of endorphin at day 3 after gene-gun injection. Data are presented as the mean $\pm$ s.e.m. Statistical comparisons between groups were made by ANOVA with individual comparisons identified using the post hoc test. * $P < 0.05$, showing a significant difference when comparing GFP (group A) with POMC-only (group B) and POMC plus naloxone-treated (group C) rats, but there were no significant difference between POMC-only (group B) and POMC plus naloxone-treated (group C) rats.

Figure 6 Effect of POMC gene-gun injection on skin levels of endorphin at day 3 after gene-gun injection. Data are presented as the mean $\pm$ s.e.m. Statistical comparisons between groups were made by ANOVA with individual comparisons identified using the post hoc test. * $P < 0.05$, showing a significant difference when comparing GFP (group A) with POMC-only (group B) and POMC plus naloxone-treated (group C) rats.

Effect of POMC gene-gun injection on skin levels of endorphin

The mean endorphin level in the skins of animals expressing exogenous POMC (group B) was 48.8 ± 9.4 (mean $\pm$ s.e.m.) pg/g per tissue site, the highest value of all the tested groups (Figure 6). The endorphin levels in the skin of all test rats were within the sensitivity limit of our radioimmunoassay and were therefore detectable.

Time course of endorphin and ACTH serum levels in blood

Endorphin and ACTH serum levels were measured on day 3, 7 and 14 after POMC gene-gun injection (Figure 7a and b). Endorphin and ACTH serum levels in POMC injection group were above the control groups in their respective time period ($P < 0.05$).

Immunohistochemistry of endorphin in the POMC gene-gun injection skin

Endorphin production in gene-gun-treated skin tissue was confirmed by immunohistochemical analysis, wherein sections of skin transfected with POMC cDNA par-

ticles were positive for endorphin immunoreactivity. In contrast, sections taken from the GFP-expressing controls or control animals from which the primary antibody was omitted, displayed no immunoreactivity (Figure 8a–d).

Discussion

The μ -opioid receptor is the principal physiological target for most clinically important opioid analgesics. However, chronic administration of opioids has been associated with drug tolerance and dependence. Exogenous opiate drugs have a strong tendency to induce tolerance and addiction, whereas native ligands do not. For instance, morphine is deficient in its ability to induce the desensitization and endocytosis of receptors.⁹ The rationale for using genes such as POMC is based on the role of endorphin as a potent native ligand. Our goal in this study was to use gene-gun delivery of POMC cDNA to induce analgesia to counteract inflammatory pain. Gene-gun delivery of POMC cDNA produced higher levels of endorphin than did injection of GFP-expressing plasmid, and correlated well with recovery. This supports a posi-

Figure 7 Serum levels of beta-endorphin (a) and ACTH (b) were measured at day 3, 7 and 14 after GFP and POMC gene-gun injection. Data are presented as the mean $\pm$ s.e.m. Statistical comparisons between groups were made by two-tailed unpaired *t* test. **P* < 0.05, showing a significant difference between the control groups and the POMC gene-gun injection groups.

tive role for the administration of POMC cDNA in the treatment of inflammatory pain.

In our study, analgesimetric tests showed no significant difference between the five groups except during phase 2 of the formalin test. Thus, local production of β -endorphin is not sufficient to alter phasic pain, but is effective against sensitization of the afferent neurons at phase 2 of the formalin test. This could reflect the limited effectiveness of POMC transfection and processing to attenuate the tonic, but not phasic pain of formalin tests.

Whether melanocyte-stimulating hormones or adrenocorticotrophic hormone are released to attenuate the phase 2 flinching response is not clear. However, the effect of naloxone in reversing the flinching response to formalin implies that most analgesic effects are derived from endorphin. Moreover, it has been shown that nociception induced by formalin is sufficient to activate the pituitary-adrenocortical system in rats, but the resulting release of corticosterone does not feed back nor reduce nociceptive processing.¹⁰ Although metabolic changes were not studied here, it is well known that POMC plays an important role in adjusting lipid metabolism because MSH produced in the CNS diffuses to the periphery and alters lipid metabolism thus encouraging its catabolism.¹¹

A variety of viral vectors, including adenovirus and vaccinia virus, as well as physical methods such as liposomes and the gene gun, have been used for DNA delivery during vaccine development. Finegold *et al*¹² delivered β -endorphin cDNA into the spinal space using an adenoviral vector, and successfully transfected cells on the pia mater to secrete β -endorphin, hence setting a paradigm for paracrine therapy for chronic pain. How-

Figure 8 Effect of POMC gene-gun injection on endorphin expression in skin. (a) GFP-treated detection of endorphin in the negative control; (b) immunohistochemical detection of endorphin (arrows) in the POMC-treated group; (c) immunohistochemical detection of endorphin in the negative control that omitted the primary antibody; (d) no significant inflammatory cell infiltration on H&E stain. Original magnification $\times 200$. Bar: 100 μ m.

ever, the use of adenovirus, retrovirus, and herpes simplex virus has been hindered by viral cytotoxicity, host immune-rejection, and poor efficiency. An alternative method of gene transfection that avoids many of these problems is particle-mediated gene transfer using the gene gun. To avoid the possible side-effects of viral vectors, we delivered the pCMV-hPOMC plasmid with a gene-gun system. The gene gun approach has the advantages of safety and simplicity, producing high-level gene transfection without the risks associated with viral manipulation.

POMC was the target gene for our study because previous data have shown that it is the precursor of beta-endorphin gene that plays an important role in the effective increase of beta-endorphin peptide secretion and producing its antinociceptive response. Beutler *et al*¹³ demonstrated that addition of pre-pro-sequence of mouse nerve growth factor to beta-endorphin in primary fibroblast was necessary for the secretion of beta-endorphin. They showed that the pre-pro-sequence is cleaved from pre-pro-sequence/beta-endorphin construct before secretion, resulting in bona fide beta-endorphin. Wilson *et al*¹⁴ also studied the antinociceptive effects of preproenkephalin cDNA delivery and showed that preproenkephalin selectively blocked hyperalgesia without disrupting baseline sensory neurotransmission and this blockade of sensitization was reversed by administration of the opioid antagonist naloxone.

It has been demonstrated that gene-gun-mediated transfection of the skin allows the successful delivery of multiple genes. The levels of endorphin expression in serum and skin increased uniformly using this approach. We believe that part of the reason for the higher levels of endorphin expression was the effective transfection of POMC cDNA and the secretion of endorphins expressed from this cDNA. Processing of POMC peptides in skin is up-regulated through the expression of enzymes prohormone convertases 1 and 2 in human dermal fibroblasts.¹⁵ The high local concentration of secretion implies efficient synthesis and release of endorphin, which has been postulated to relieve phase 2 pain.

In the formalin test, phase 2 is thought to be associated with a facilitated state of the dorsal horn neurons in the spinal cord and various chemical mediators contribute to altering the functions of the peripheral afferent fibers.^{16,17} Recent evidence supports the presence of excitatory amino acid receptors on peripheral cutaneous axons, and subcutaneous formalin has been shown to induce the peripheral release of NO through activation of an NMDA-NO cascade in the peripheral nervous system.^{18,19} In addition, opioids have been reported to act peripherally, and their importance is being increasingly appreciated.²⁰ Of note, corticotropin releasing factor and interleukin-1 has been shown to activate their receptors on immune cells of inflamed tissues to release opioids that subsequently occupy multiple opioid receptors on sensory nerves and result in antinociception.¹ Interestingly, it has been shown that footpad inoculation with herpes simplex virus (HSV) vector encoding human proenkephalin gene produced a significant antinociceptive effect in the delayed ('tonic') phase 20–70 min after the administration of formalin which was blocked completely by intrathecal naltrexone.²¹ These results suggest that opioid receptors can feed back and reduce peripheral nociceptive processing of the formalin stimulus.

In conclusion, increasing focus is now being placed on gene therapy as a potential treatment for pain. POMC gene particle injection may provide analgesia for inflammatory pain as assessed by the formalin test. Further investigation of the clinical use of POMC for enhancing long-term relief of chronic pain is planned.

Materials and methods

Animal models

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee before the study began. Male Sprague-Dawley rats (300–350 g; NSC, Taiwan) were used in this study. Guidelines for pain experiments on conscious animals were adhered to throughout the experiments.²² The animals were housed two per cage, in (beta)-chip-lined metal cages in a central animal care facility with a 12-h light, 12-h dark cycle. They were fed rat chow and water *ad libitum*.

Human pro-opiomelanocortin (POMC) cDNA

Total RNA from a human pituitary gland tumor was isolated using Trizol Reagent (Clontech, Palo Alto, CA, USA). Complementary DNA was synthesized with reverse transcriptase using an oligo-dT primer (BcaBEST RNA PCR kit, TaKaRa Biomedicals, Otsu, Shiga, Japan). POMC cDNA was then amplified by PCR with specific upstream (5'-CAGGGTACCTGGAAGATGCCGAGATC-3') and downstream (5'-CCTGGGTACCGCTGTGCCCTCACTCG-3') oligonucleotide primers. The PCR product was 854 bp long, as expected, and contained the sequence coding the full-length POMC polypeptide. The amplified fragment was cloned into the pUC18 vector and white colonies (*LacZ* disruption) were selected. The plasmid DNA was purified using the Mini Plasmid DNA Preparation Kit (Qiagen, Valencia, CA, USA), digested with restriction enzymes, and separated electrophoretically on agarose gel. Plasmids with the correct insert were verified by DNA sequencing.

DNA-gold particle preparation

Plasmid DNA was precipitated onto 1.5–2.0 μ m gold particles. In this experiment, 28 mg gold particles and 105 μ g plasmid DNA were used.

Gene gun injection

We used the versatile Helios Gene Gun System (BioRad, Hercules, CA, USA), which allows rapid and direct transfer of DNA-coated gold microcarriers into a range of targets *in vivo*. Each pulse of helium expels the DNA-coated gold beads from a single 0.5-inch (12.7 mm) segment of gold bead-coated tubing, and results in the delivery of 0.5 mg gold and 1.875 μ g plasmid DNA per pulse (Figure 1).

Animal procedures

The paw withdrawal latency and formalin tests were performed on the 3rd day after plasmid injection. Group A served as the control group and received an injection of plasmid encoding green fluorescent protein (GFP). Group B was injected with the pCMV-hPOMC plasmid prepared in our laboratory. Group C was treated as group B except that 30 min before the formalin test, naloxone (1 mg/kg) was administered intraperitoneally to deter-

mine if any analgesic effect was mediated through the μ -opioid receptors. Group D was treated as group B, except that 30 min before the formalin test, naloxone methiodide (1 mg/kg) was administered intraperitoneally to determine if any analgesic effect was mediated through the peripheral or central μ -opioid receptors. Group E was injected with the pCMV-hPOMC plasmid in the ipsilateral paw, but administered formalin injection in the contralateral paw to determine if any analgesic effect was mediated through the peripheral or central μ -opioid receptors.

Paw withdrawal latency and formalin tests

Two nociceptive tests were conducted: the paw withdrawal latency test and the formalin test. An observer who was blinded to the treatment regimen performed all tests. The tests for latency in paw withdrawal from radiant heat were performed on the plantar area of hind paw. Briefly, the animal was placed on a glass plate (maintained at 30°C) for 5–10 min for adaptation to the thermal nociceptive threshold. The latency period between the application of a focused light beam and the hind-paw withdrawal response was measured to the nearest 0.1 s. The cut-off time in the absence of a response was 20 s. This value was then designated the response latency. Analysis of all outcomes was performed by observers blinded to the treatment regimen.

For the formalin test, 50 μ l 1% formalin was injected into the dorsal side of the right hind paw of each rat held in a Plexiglas observation chamber. The number of flinches was counted during the first minute, and for 1 min in every 5 min thereafter, until 60 min. The phase 1 response was defined as occurring in the first 10 min, and the phase 2 response as occurring over 10–60 min.

After the formalin test, the rats were anesthetized with 3% isoflurane plus pentothal (100 mg/kg), and skin and blood samples were taken.

Blood sampling and radioimmune assay for endorphin and ACTH

Three days after gene-gun injection, rats were deeply anesthetized with an overdose of anesthetic, and blood (2.0 ml) was sampled from the heart. ACTH and endorphin concentrations were assayed using a radioimmunoassay. This commercially available kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) has a linear range of measurement between 5 and 1000 pg/ml of ACTH and endorphin with a detection threshold of 5 pg/ml.

Extraction of opioid peptides from paw tissue

Plantar subcutaneous tissues (~0.5 g) were removed from injected paws at 3 days after POMC gene-gun treatment. Paw tissue samples were frozen on dry ice and stored at -70°C. The tissue was defrosted, weighed and then homogenized using a polytron tissue homogenizer (1 min) in a solution of PBS and 34 mg/l Bacitracin. Samples were centrifuged (3000 g, 15 min at 4°C), an aliquot (1 ml) was removed, lyophilized and stored at -20°C until further processing.

Immunohistochemistry

Skin samples were cryostatally cut into 10- μ m sections, fixed in acetone, rinsed in 0.1 M phosphate-buffered saline (PBS) for 20 min, and blocked in 2% normal goat

serum for 2 h at room temperature. Samples were then incubated with primary antibodies against β -endorphin (1:500; Chemicon, Temecula, CA, USA) in 10% normal goat serum and 0.3% Triton-X 100 octylphenoxypolyethoxyethanol (Sigma, St Louis, MO, USA) for 20 h at 4°C. Endogenous peroxidase activity was quenched by exposing the slides to 0.3% H₂O₂ and 10% methanol for 20 min, and the slides were then washed in PBS. The samples were then incubated for 1 h with biotinylated anti-rabbit immunoglobulin G and for 30 min with biotinylated anti-goat immunoglobulin G (Dako LSAB 2 kit, Dako, Carpinteria, CA, USA). They were then incubated with streptavidin-biotin-horseradish peroxidase complex (Dako LSAB 2 kit). The sections were stained with a diaminobenzidine/H₂O₂ solution, and the cytoplasm was counterstained with hematoxylin. A set of sections was stained in a similar way without the primary antibody to confirm the specific binding of the antibody to the protein.

Statistics

The data from the paw withdrawal latency, formalin test flinches and endorphin serum levels are expressed as the mean $\pm$ s.e.m. (standard error of mean). One-way analysis of variance with *post hoc* analysis was used to detect differences among groups. Differences are deemed significant at $P < 0.05$.

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