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Fat Grafting as a Vehicle for the Delivery of Recombinant Adeno-Associated Viral Vectors to Achieve Gene-Modification of Muscle Flaps*

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Abstract

Background—The combination of gene therapy and plastic surgery may have the potential to improve the specificity that is needed to achieve clinically applicable treatment regimens. Our goal was to develop a method for gene-modification that would yield sustainable production of gene products but would be less time consuming than existing protocols.

Methods—An adeno-associated virus (AAV) was used to deliver gene products to pectoralis muscle flaps. Gene-modification was accomplished via either direct injection or novel fat grafting techniques.

Results—Gene product production was observable by both *in vivo* imaging and immunohistochemical staining. Gene products were not detected in tissues that were not in contact with the fat grafts that were incubated with the viral vector, indicating that the transduction stayed local to the flap.

Conclusions—Using novel recombinant AAV vectors, we have developed a method for gene delivery that is highly efficient and applicable to muscle flaps.

Introduction

Traditionally, plastic surgical procedures aim to reconstruct function and appearance through the use of techniques that involve the transposition of tissues from nearby or distant areas on the patient's body. However, recent publications in the plastic surgical literature have introduced applications of plastic surgical principles that are therapeutic rather than reconstructive.¹⁻⁵ These studies have reported experiments in which free flaps were gene-modified through isolated perfusion with a viral vector. These gene-modified muscle tissues were shown to be a reproducible source of gene products which could be used to deliver proteins or enzymes to targeted regions of the body to produce anti-tumor effects, through immunomodulation, or to promote the healing of infected wounds, through the expression of antimicrobial proteins. While promising, these protocols utilized surgical methods that

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required free tissue transfer and *ex vivo* perfusion of tissues with viral vectors, making such procedures technically demanding and subjecting the transferred tissues to prolonged ischemia times.

The purpose of this study was to develop a technically simple and efficient method for gene-modification of flap tissues that could be used for the targeted delivery of gene products. The initial methods developed as part of this work utilized direct intramuscular injection of a viral vector; however, systemic spread and gene-modification of distant, non-target organs prompted the use of fat grafts as an adjunct vehicle for gene-modification of flap tissues.

Materials and Methods

Viral Vector Preparation

A recombinant adeno-associated virus (rAAV) vector was used for gene delivery and modification of flap tissues. This rAAV vector was derived using a PCR shuffling technique from human and novel nonhuman primate viral isolates.^{6,7} Specifically, a rAAV vector containing the gene for either green fluorescent protein (GFP) or the enzymatic biomarker luciferase was constructed. The cDNA was cloned using standard molecular biology techniques into the high expression pAM AAV cis plasmid containing the hybrid CBA promoter and WPRE 3' sequence. Once the pAAV-CBA-lacZWPRE was constructed, it was used to generate high titer rAAV vectors expressing the chosen tag using transfection techniques with helper plasmids as we have previously reported in the literature.⁸⁻¹²

Flap Surgery

Male C57BL/6 mice ranging in weight from 30 to 35 grams were anesthetized via intraperitoneal injection of ketamine/xylazine (87/13 mg/kg). The right side of the chest was shaved and prepped in a sterile fashion. Using a surgical microscope, a vertical skin incision was made to the right of the sternum approximately 5 to 10 mm in length. After dissection, the lateral edge of the right pectoralis muscle was identified and we proceeded to elevate the muscle off of the sternum medially. The muscle flap was dissected back to the point of origin and the vascular pedicle was identified. At this point, gene-modification was accomplished by either direct muscle injection or by fat grafting with virally incubated fat as described below. The flap was inset in its original location using 6-0 Prolene sutures. The skin was closed and covered with dermal adhesive to prevent the animals from reopening the wounds.

Gene-Modification by Direct Injection of rAAV

Using a 50 ul Hamilton syringe with a 30 gauge needle and a microinfusion pump to time the injection over five minutes, 15 ul of viral vector, either rAAV-GFP (2×10^{12} vg/ml) or rAAV-luciferase (5×10^{12} vg/ml), was injected intramuscularly into the flap. In the initial phases of our work, we injected volumes between 50-100 ul and noted that this lead to some leakage of injectate. When we lowered the volume of the injectate to 15 ul, no leakage was appreciated and the injectate appeared to remain within the fascia of the muscle. Each injection was performed after the muscle flap was elevated, using the surgical microscope for clear visualization of needle placement in the muscle belly.

Gene-Modification Using Fat Grafts as Vehicles for rAAV Delivery

A 1 mm³ globule of fat, harvested from the epididymal fat pad of a donor mouse, was injected with 15 ul of rAAV-luciferase (5×10^{12} vg/ml) using a 50 ul Hamilton syringe with a 30-gauge needle. This fat was incubated on ice for approximately 30 minutes before it was washed in saline solution. The fat was subsequently diced into 1 mm³ pieces and implanted

into the pectoralis muscle flap belly under direct visualization with the operative microscope.

Tissue Histology

Animals were sacrificed 5 weeks after surgery. The left and right pectoralis muscles were harvested along with several internal organs, including the heart, liver, and kidneys. Specimens were either frozen using 2-methylbutane and liquid nitrogen or placed in formalin for further analysis. The frozen blocks were sectioned at a thickness of 12 microns and viewed under direct fluorescence at both 100× and 400× magnification. The formalin fixed specimens were sectioned at a thickness of 14 microns and subsequently stained for the presence of the marker.

GFP staining was accomplished by fixing the slides and blocking them using serum-free protein block for 10 minutes. After washing, the primary antibody (Cell Signaling 4B10) was applied at a concentration of 1:165 in Dako antibody diluent for 30 minutes and washed again. The secondary antibody (biotinylated horse anti-mouse, Vector) was applied at 1:200 in protein block for 30 minutes and the final stain was visualized using DAB (Dako).

Luciferase staining was completed in a similar manner. Fixed slides were blocked for 10 minutes in serum-free protein blocker and subsequently incubated in the primary antibody (MBL Int. Corp. PM016) for 30 minutes at a concentration of 1:250 in Dako antibody diluent. After washing, the slides were incubated with the secondary antibody (biotinylated goat anti-rabbit, Vector) diluted to 1:200 in protein block for 30 minutes. Once again, the stain was visualized using DAB (Dako).

Tissue Analysis

For both the frozen and formalin fixed slides, 10 high-powered fields were obtained from each group using identical settings and analyzed using digital pixel densitometry on Adobe Photoshop CS3 (Adobe Systems Incorporated, USA). To achieve this, the Color Range tool was used to select the pixels on one image that were considered positive for gene expression at the setting of 100% range. This selection was then saved and applied to the subsequent images. For each high-powered field, the number of pixels selected as a result of the Color Range was recorded. The average of these values was calculated for each group of images and the standard deviation was also obtained. A student's T-test was used to compare the averages from the two groups and a p-value of less than 0.05 was used to demonstrate significant difference.

***In Vivo* Imaging of Luciferase Activity**

Mice were serially imaged using an In Vivo Imaging System Series 100 (Xenogen) to determine the localization of the rAAV-luciferase virus. Intraperitoneal injections of XenoLight RediJect D-Luciferin Ultra (Caliper Life Sciences) at 150 mg/kg were administered and mice were imaged according to the manufactures specifications. Combined images and luminescent measurements were generated using Living Image software (Version 2.50.1, Xenogen). These composite images were then normalized to remove any background luminescence. Zones, referred to as Regions of Interest (ROI), were identified and used to analyze specific areas where the luminescence was most evident.

Results

Recombinant AAV viral vectors demonstrate superior gene-modification of muscle flap tissues compared to traditional AAV serotypes

In order to select the ideal viral vector for gene-modification of muscle flap tissues, a human serotype (AAV1) which had previously been shown to be highly efficient for muscle tissue transduction was compared to a novel, recombinant AAV vector that was previously developed by our laboratory by PCR shuffling to combine components of human and nonhuman primate AAV vectors.⁹ Both vectors were loaded with the gene for GFP and injected into pectoralis muscle flaps at identical titers (2×10^{12} vg/ml). Direct fluorescence microscopy of muscle tissues at 5 weeks demonstrated significantly higher levels of GFP expression in flaps that were injected with the recombinant AAV vector (Figure 1).

Direct injection of viral vectors into muscle flaps leads to transduction of distant organs

In order to further investigate the specificity and localization of gene-modification, flap tissues and distant organs were analyzed histologically. While direct fluorescence microscopy of fresh frozen tissue sections indicated strong GFP production in the flap tissues and no GFP in distant organs, immunohistochemical staining of fixed specimens showed GFP production in flap tissues and liver tissues (data not shown). To clarify these conflicting data, a model for whole-body, real-time detection of a biological gene product (luciferase) was used. Recombinant AAV vector, loaded with the gene for luciferase, was directly injected into pectoralis muscle flaps as described above (Figure 2a-b). Luciferase expression and activity were assayed at one week using *in vivo* imaging after luciferin administration. Whole-body images demonstrated detectable levels of enzyme activity in the injected, gene-modified pectoralis muscle flaps, but also consistently showed equal or stronger amounts of activity throughout the mid-abdomen, a distribution suggestive of hepatic expression (Figure 2c). Hepatic transduction and luciferase expression was confirmed with immunohistochemical staining using anti-luciferin antibody.

Fat grafting as a vehicle for the delivery of rAAV-luciferase

Gene expression by non-target tissues seen following direct injection of vector into pedicled muscle flaps prompted us to explore the use of an intermediary vehicle to improve the targeting of gene delivery. To accomplish this, fat grafts were isolated and incubated in a solution containing viral vector. The fat grafts were subsequently processed into smaller pieces that were implanted into the bellies of muscle flaps (Figure 3a-b). Similar to the direct injection method, IVIS imaging of fat grafted mice indicated luciferase activity in the region of the targeted pectoralis muscle flap (Figure 3c). In contrast to the directly injected mice, IVIS imaging of the fat grafted animals showed little or no activity outside the targeted area.

To better understand the findings from the biological imaging, immunohistochemical analysis of tissue sections was performed. This demonstrated observable luciferase in the fat-grafted pectoralis muscles and an overall paucity of enzyme in non-target tissues such as the contralateral pectoralis and liver. Overall positivity of luciferase staining was compared between the directly injected mice and those that underwent gene-modification using fat graft vehicles for vector delivery using pixel densitometry of tissue sections. In both methods of vector delivery, the target pectoralis muscle showed highly positive staining for luciferase enzyme (Figure 4). This was not the case for liver sections, which showed significantly lower luciferase enzyme when fat grafting was used as a vehicle for viral vector delivery (Figure 5).

Discussion

Recent reports in the literature have shown methods for introducing genes into plastic surgical flap tissues and applications of these gene-modified flaps to deliver gene products to specific regions of the body to achieve healing of infected wounds and produce cytokine-mediated anti-tumor effects.^{1-5,13} Such gene-modification of tissues opens a new direction in the field of plastic surgery, one that deviates from the traditional goal of reconstruction to address potentially therapeutic applications.¹⁴ Our study introduces a novel method for using fat grafts to produce gene-modified flaps for targeted gene delivery.

The major innovation in this study was the finding that autologous fat grafts could serve as a vehicle to achieve highly targeted delivery of viral vectors into muscle tissues with sustained production of functional proteins. This is in sharp contrast to the results that were seen with direct injection of the vectors into muscle tissues, where even with the use of a micro-infusion pump, the injectate became systemic and lead to widespread transduction of non-targeted tissues. Interestingly, immunohistochemical examination of the gene-modified muscle sections showed gene-modification of both implanted fat cells and most of the myocytes in the entire muscle belly. We believe that the fat graft was able to absorb virus particles during its 30 minute incubation and then, after implantation, slowly release unbound particles into the surrounding tissue. This longer infusion time allowed the muscle surrounding the fat graft to bind the virus, while not bathing it in a bolus volume leading to systemic spread.

Another factor possibly contributing to the targeting of tissues seen in our studies is the use of a recombinant AAV vector for gene delivery.⁷ The specific serotype used in our studies was notable for its muscle tissue tropism and selected from a panel of recombinant vectors that were produced by using a PCR shuffling technique from human and novel nonhuman primate viral isolates. As demonstrated in our direct injection experiments, this heightened affinity for myocytes alone does not prevent transduction of non-targeted tissues. It does, however, result in a significant improvement in local gene expression and may have contributed to our success with the fat grafting protocol for gene-modification of flap tissues.

Previously published methods by Gurtner and colleagues relied on methods that utilized technically demanding free tissue transfers that relied on perfusion of the isolated flap tissues with a viral vector, a step that inevitably prolongs ischemia time.¹⁻⁵ In our studies, we sought to not only shorten tissue ischemia times, but also technically simplify gene-modification procedures by developing a method that could be applied to pedicled flaps.¹⁵ Our initial attempts with direct injection of pedicled flaps with viral vector particles in solution resulted in impressive gene-modification of target muscle tissue, but also lead to systemic spread of the vectors and transduction of distant, non-targeted liver. The subsequent use of fat grafts as a vehicle for delivering the viral vectors into the target tissues involves an additional step. While this invariably made the overall process more time consuming than direct vector injection, its ability to result in highly targeted gene-modification of muscle tissue in a non-ischemic, pedicled flap model makes the use of fat grafting an appealing alternative to free tissue perfusion protocols.

Despite the positive results of our current experiments, gene therapy using fat grafts is in its early stages of study and future analysis is still required. Our protocol has only been applied to the mouse model, and it is possible that at least minor changes would have to be made to translate the methods to larger animals. Larger scale transductions would likely require implantations of virus-incubated fat to multiple areas to result in clinically detectable gene transfer. Such large-scale fat transfers might prove more difficult than using cubes of fat, as

described in our methods. For this reason, future studies should be aimed at using minced or liposuctioned fat that could be injected, rather than implanted, which would actually allow for more customization by allowing the surgeon to infect only parts of the flap in contact with areas of concern. In addition, future studies could be done to determine methods other than muscle flaps that could be used to place transduced fat grafts in contact with a desired anatomical location. Directly implanting the fat in a subcutaneous plane or in a peritumoral environment should also be considered.

Conclusions

While the use of fat grafts for gene-modification involves additional steps to the gene delivery protocol, there are considerable advantages in this approach. Fat grafting makes it possible to specifically target the delivery of viral vectors to precise anatomical locations, while limiting operative times, due to the ability to incubate autologous fat grafts preoperatively, *ex vivo*. Furthermore, future studies could analyze fat grafting into tissues other than muscle flaps, such as implantation directly into or around tumors or wounds, thus eliminating the need to subject patients to additional surgeries and improving the efficiency of gene transfer.

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Fig. 1.

a. Frozen sections of pectoralis muscle were analyzed under direct fluorescence at 100x magnification. Muscle injected with AAV1 (left) demonstrated fewer areas positive for GFP than that injected with the recombinant AAV (right). b. Pixel densitometry was performed to quantify the GFP present in each high-powered field. Data are mean $\pm$ SD. * $p < 0.05$ demonstrated a significant result.

Fig. 2.

a. Incision was made on the right side of the chest and the lateral edge of the pectoralis muscle was identified. The right pectoralis muscle flap was dissected off of the sternum and up to the point of origin. b. The viral vector was injected intramuscularly and the flap was inset in its original location using 6-0 Prolene sutures. c. Mice were imaged using the In Vivo Imaging System Series 100 for the presence of the active luciferase enzyme. Even one week after surgery, widespread luminescence was visible over both chest and the abdomen.

Fig. 3.

a. Incision was made on the right side of the chest and the lateral edge of the pectoralis muscle was identified. The right pectoralis muscle flap was dissected off of the sternum and up to the point of origin. b. A fat sample, infected with the viral vector, was implanted into the muscle flap. The flap was inset and the skin was closed using a 6-0 Prolene suture. c. Mice were imaged using the In Vivo Imaging System Series 100 for the presence of the active luciferase enzyme. At one week after surgery, luminescence was isolated over only the right pectoralis muscle.

A.

B.

Fig. 4.

a. Formalin fixed sections of control pectoralis muscle, directly injected pectoralis muscle, and fat grafted pectoralis muscle were analyzed at 100× magnification. Both the directly injected muscle (middle) and the fat grafted right muscle (right) demonstrated more areas positive for luciferase than the control left muscle (left). b. Pixel densitometry was performed to quantify the luciferase present in each high-powered field. No significant difference was calculated between the directly injected and fat grafted muscles. Data are mean ± SD; n = 3 for each group.

A.

B.

Fig. 5.

a. Formalin fixed sections of liver from a control mouse, liver from a directly injected mouse, and liver from a fat grafted mouse were analyzed at 100x magnification. The liver from the directly injected mouse (middle) demonstrated more areas positive for luciferase compared to the liver from both the control mouse (left) and liver from the fat grafted mouse (right). b. Pixel densitometry was performed to quantify the luciferase present in each high-powered field. * $p < 0.05$ demonstrated a significant result. Data are mean $\pm$ SD; $n = 3$ for each group.