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measurements made. Therefore, this method holds great promise for the use of single base-pair polymorphisms in a scan of the entire human genome for genetic association with a DNA pooling methodology, although the expenses involved are considerably greater than those for RFLPs.

CONCLUSION

Two methods of DNA pooling analysis of SNPs are presented. The RFLP method is less accurate than the Taqman method but should still be sensitive enough for the rapid screening of candidate polymorphisms. With the Taqman method, the allele estimates are extremely accurate but the set-up costs per polymorphism are large (approximately \$800) and the optimization of the assay may present difficulties. On the other hand, the cost of genotyping samples individually by any method far exceeds this cost. As the Taqman technology becomes more developed, it will probably become a more viable technology for large-scale screening of many polymorphisms.

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Use of Fluorescent Microspheres to Localize In Vivo Gene Transfer Injection Sites

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ABSTRACT

*The potential for using gene therapy to treat a variety of disease states is growing rapidly. Many vector types and delivery systems have been developed that allow the optimization of protein production levels and kinetics for a given therapeutic gene product. In cases in which a transient, localized delivery of gene product is desired, any determination of the locale of transfected tissue by non-marker genes is problematic. We describe a technique by which the use of fluorescent microspheres can help in identifying potentially transfected tissue. Adenovirus containing the gene for **b**-galactosidase (**b**-gal) was mixed with fluorescent microspheres and injected into rat skeletal muscle and porcine myocardium. The injection sites could be visualized under ultraviolet light and correlated with **b**-gal enzyme expression. This method is simple, inexpensive and generally useful for in vivo gene transfer experiments.*

INTRODUCTION

A variety of gene-therapy based treatments have demonstrated efficacy in animal models (1,2,5-8,10-12) and several have entered or approach clinical trial status (3,4,9). Among other things, the research preceding these studies requires the determination of gene product expression levels, expression kinetics and protein localization in transfected tissues. When the gene product cannot be detected visually, its localization can be difficult because a relatively small piece from a large tissue mass is often involved. One approach to this problem is to place sutures at the site of transgene administration, although this is impractical when the treated area is remotely located in the body (e.g., catheter-mediated administration). An alternative method would be the co-injection of a marker

dye with the transgene preparation; however, anecdotal reports have suggested that dyes such as methylene blue can inactivate adenoviral vectors (I. Kovesdi, personal communication).

Therefore, a method is required that will allow for the localization of gene transfer sites without inactivating the therapeutic vector. We have developed a technique in which fluorescent microspheres are mixed with an adenoviral transfection vector and injected into muscle tissue. After harvesting the treated tissue, the site of injection can be localized using a UV lamp and the degree of therapeutic protein expression determined without undue dilution by untreated tissue. This method could be applied to any tissue system that would allow for the trapping of fluorescent microspheres upon treatment. It is also quite flexible in that these microspheres—normally used for flow cytometric analyses—are commercially available and can be obtained in a variety of sizes and colors.

MATERIALS AND METHODS

The replication deficient adenovirus containing an expression cassette encoding nuclear-targeted β -gal (AdLacZ) was kindly provided by Dr. Imi Kovesdi of GenVec, Inc. (Rockville, MD, USA). A549 cells were obtained from ATCC (Rockville, MD, USA). Polystyrene fluorescent microspheres (2.0% solids suspended in water) labeled with a proprietary FITC-like dye were purchased in 0.1, 0.2, 0.5, 0.75, 1.0, 1.7, 1.9, 2.8, 4.2 and 5.0 μ m sizes from Polysciences (Warrington, PA, USA). Skeletal muscle injections were done in Sprague-Dawley rats and cardiac injections were performed in Hanford mini-pigs (Charles River Laboratories, Wilmington, MA, USA). All surgeries were carried out with the approval of the Animal Care and Use Committees of Parke-Davis or the Washington Hospital Center.

In Vitro Adenoviral Transfection

A549 cells were grown to approximately 90% confluency in DMEM (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA) before trans-

fection with adenovirus as described in Table 1. The cells were maintained in an environment of 5% CO₂/95% air, 90% relative humidity and 37°C until assayed for enzyme activity by means of a commercially available β -galactosidase kit (Galacto-Star Assay System; Tropix, Bedford, MA, USA).

Skeletal Muscle Injections

Sprague-Dawley rats (400 g) were anesthetized with 30 mg/kg Telezol (Tiletamine/Zolazepam; Fort Dodge Animal Health, Fort Dodge, IA, USA) delivered intramuscularly (i.m.). A small incision was made in the skin of the hindlimb to visualize the greater femoral muscle and 2×10^{10} particles (10^9 FFU per injection) of AdLacZ in PBS were injected i.m. The total injection volume was 100 μ L and contained 10% fluorescent microspheres (vol/vol). In initial acute studies, the hindlimb was harvested 15 min after injection, sectioned into 0.5 cm slices and examined under UV light (300 nm UV lamp generating 7 W/cm² intensity; Ultra-Lum, Carson, CA, USA) to determine the location of the injection site. AdLacZ transfection/bead co-localization studies were performed identically except that the muscle was harvested at 1, 3, 7, 14, 21 or 28 days after injection. The tissue sections were then lightly fixed (30 min) in 2% formaldehyde/ 0.2% glutaraldehyde in PBS and viewed for focal bead sites under UV light. These same sections were subsequently stained for the presence of enzyme activity by using an in situ β -galactosidase staining kit (Stratagene, La Jolla, CA, USA).

Histology

Skeletal muscle tissue sections used to demonstrate microsphere fluorescence and/or β -gal activity (time course described above) were embedded in paraffin, sectioned at 5 μ m, placed on slides and stained with hemotoxylin and eosin to determine the inflammatory response to the adenovirus and microspheres.

Endocardial Injections

Pigs (40 kg) were anesthetized with isoflurane gas. The animals were placed

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on a respirator and monitored for heart rate, blood pressure and EKG throughout the procedure. The right femoral artery was isolated and an 8F catheter sheath indwelled. An injection catheter (7F, Cordis Webster, Baldwin Park, CA, USA) was fed into the sheath and through the thoracic aorta and coronary artery into the left ventricular chamber. Using fluoroscopy to guide the injections, six injections of 10^{10} particles of AdLacZ in PBS containing a 1:10 dilution (vol/vol) of fluorescent beads were injected into the wall of the left ventricle. The catheter was removed, the incisions were closed and the animal was returned to its cage. Twenty-four hours after injection, the animal was killed, and the heart was harvested and sectioned horizontally into 0.5 cm slices from the apex to the base of the organ. Injection sites were visualized under UV light and the tissue was lightly fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS before staining for β -gal activity using the staining kit.

RESULTS AND DISCUSSION

Fluorescent Bead Optimization

A series of Polysciences yellow-green fluorescent microsphere sizes were analyzed for localization after acute injection into skeletal muscle. Test beads ranging from 0.1–5.5 μ m were prepared to a 1:10 dilution (vol/vol) in PBS before injection. None of the bead preparations clogged the 31-gauge needle used for injections. The smaller beads resulted in a more diffuse center of injection, and the largest beads were very concentrated at the injection site (data not shown). We chose the 1.7 μ m beads that provided a reproducible and readily apparent injection site marker for all subsequent experiments. (This choice was appropriate for our tissue system and may differ depending on the application.) Fluorescent microspheres were diluted to between 1:5 and 1:100 (vol/vol) in PBS and an additional series of acute injection studies were performed in skeletal muscle. The bead center was visible in all diluted injections, but the more concentrated bead preparations gave much brighter foci under UV light. Figure 1

illustrates the difference between a 1:10 and a 1:100 dilution injection of microspheres in the hindlimb muscle.

Microsphere/Adenovirus Interaction

To use the fluorescent beads as an injection site marker, we first demonstrated that bead/adenovirus interactions would not negatively affect the viability of the virus or its ability to transfect tissue and express the target protein. Initially, we examined this in vitro by mixing beads and virus at various concentrations and measuring the ability of the mixture to transfect A549 cells (an adenovirus host cell line). Table 1 demonstrates that the beads do not prevent the virus from producing active β -gal under these conditions, but rather may have enhanced the uptake or expression of the viral vector by an unknown mechanism. We then used our skeletal muscle model and compared injections containing 2×10^{10} viral particles without beads to those containing the same viral titer, but with a 1:5, 1:10 or 1:50 dilution (vol/vol) of fluorescent microspheres. Tissues were harvested 24 h after injection and sliced serially, perpendicular to the muscle fibers. Figure 2 shows a representative set of muscle slices stained for the presence of β -gal; here, the no-bead control is compared to the same injection containing the 1:10 dilution of beads. While quantitative analysis was not done, we found no grossly detectable difference in stained area, stain intensity or distance travelled from the injection site for the two injection types. The staining appeared slightly lighter at the center of the bead injection sites because the yellow beads mix with the dark blue stain

Table 1. Effects of Fluoresbrite Fluorescent Microparticles on Adenoviral Transfection/Expression in A549 Cells

Dilution	β -gal Activity (% of Control)	
	Mean	SEM
1:0	135.8	
1:5	365.0	25.4
1:10	227.3	15.7
1:20	214.0	1.0

A549 cells were transfected with adenovirus encoding the *LacZ* gene at an MOI of 50 per cell. Fluorescent microspheres (1.7 μ m) were either excluded (control) or added at the indicated dilutions in PBS. Enzyme activity 24 h after transfection is presented for each bead dilution as a percentage of the control activity. Each value represents the mean of four separate trials except for the 1:20 dilution that represents duplicate measurements.

to give a lighter, green color. There was no evidence of increased β -gal expression in the skeletal muscle as was seen in vitro in A549 cells.

Endocardial Injection

After finding no negative interaction between beads and the LacZ adenovirus, we attempted to co-localize the fluorescent microspheres with β -gal activity in the left ventricular wall of pigs in which adenovirus was administered by catheter-mediated endocardial injection. After harvesting 24 h after injection, sectioning, fixation and staining of the cardiac tissue, discrete bead centers and β -gal staining foci were found to be

Figure 1. Detection of injection site in skeletal muscle using fluorescent microspheres. Rat skeletal muscle injected with a 1:10 (A) or 1:100 (B) dilution (vol/vol) of fluorescent beads in PBS. Note the drop off in signal with dilution. The approximate site of injection is also marked with a suture in Figure 1A.

co-localized in the heart. In any section demonstrating bead presence, there was corresponding staining for the marker enzyme. However, there were tissues in which β -gal staining was seen but no beads were found. Of the 24 injection sites analyzed in pigs for this study, we found three samples (13%) that were sectioned so that no beads were found to be associated with β -gal staining. This finding suggests that the injection fluid containing adenovirus can travel further from the injection site than the beads and, depending on how the tissue section is cut, it is possible to miss the bead center but still cut through the sphere of transfection, that is, the volume of tissue demonstrating β -gal expression. Figure 3 demonstrates both of these scenarios in a section of pig left

ventricle. The lack of beads in certain sites that display protein expression indicates the importance of matching bead size and dilution to the test system being used. Optimizing these parameters in a given tissue system will maximize the collection of transfected tissue.

Time Course and Histology

After demonstrating the usefulness of fluorescent microspheres for the acute detection of injection sites, we wanted to know the longer term fate of the beads. Figure 4 depicts a time course of β -gal expression and microsphere fluorescence in rat skeletal muscle through 28 days. Macroscopic detection of β -gal enzyme activity ends at day 7, and discrete fluorescent foci are

Figure 2. Adenovirus transfection efficiency in skeletal muscle with or without the inclusion of fluorescent microspheres. Adenoviral preparations containing 2×10^{10} particles without fluorescent beads (A) or with the inclusion of a 1:10 dilution (vol/vol) of beads (B) were injected into rat skeletal muscle. (A) and (B) are serial slices of the same muscle from two different animals. Muscle transfection was determined by staining for β -gal 24 h after injection.

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readily visible out to 28 days. Although we cannot predict the ultimate length of time the beads will remain intact, it is apparent that the fluorescence outlasts the ability of the adenovirus to produce visibly detectable protein.

Sections of the tissues used in this time course were then stained with hematoxylin and eosin so we could better understand the interaction of the beads with the muscle tissue. During the harvest of our *in vivo* muscle tissues, we have seen transient edema between days 3 and 7, but no other gross signs of an inflammatory response such as redness, etc. Figure 5 shows the microscopic situation with muscle fibers surrounded by what appear to be macrophages loaded with beads at days 3 and 28 after injection. There is a mild, general inflammatory response at day 3 that is typical of adenoviral injections we have seen previously in muscle tissue, which present large numbers of monocytes and macrophages, as well as low numbers of PMNs, lymphocytes, etc. This generalized response has receded by day 28 leaving clusters of bead-laden macrophages in what ap-

pears to be an inert granuloma with no significant fibrotic response. These engulfed microspheres are still localized *in vivo* and visible under UV light at that time (Figure 4). Of note, the beads are no longer fluorescent on the slides after the tissue embedding and staining protocol. It is presumed that the chemical treatments involved in processing and staining the tissues has stripped the beads of their marker dye.

Therefore, this method allows for the localization of target proteins or oligonucleotides, etc. that do not generate a visible product, making it difficult to otherwise mark a treatment site. In the case of the heart or skeletal muscle, this could include antisense treatment, treatment with growth factors or growth factor inhibitors, anti-inflammatory agents or any other system for which a delivery and propagation scenario can

Figure 3. β -gal activity and fluorescent bead co-localization in left ventricular tissue injected from the endocardial surface using a catheter. An adenovirus preparation (1×10^{10} particles) containing a 1:10 dilution (vol/vol) of fluorescent microspheres was injected by catheter into the left ventricular wall of a pig. Arrowheads indicate sites of β -gal expression. Note that in some cases staining can be present without beads being visible. Muscle transfection was determined by staining for β -gal 24 h after injection.

Figure 4. Time course of macroscopic β -gal activity and fluorescent bead localization in rat skeletal muscle. Adenoviral preparations containing 2×10^{10} particles with the inclusion of a 1:10 dilution (vol/vol) of beads were injected into rat skeletal muscle. Tissues were harvested at days 3, 7, 14, 21 and 28 and stained for β -gal activity and viewed under UV light for bead presence.

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Figure 5. Microsphere clusters in rat skeletal muscle tissue 3 or 28 days after co-injection with *LacZ*-encoding adenovirus. Adenoviral preparations containing 2×10^{10} particles and a 1:10 dilution (vol/vol) of fluorescent microspheres were injected into rat skeletal muscle. Tissues were harvested at days 3, 7, 14, 21 and 28 and fixed, embedded and stained with hematoxylin and eosin. The micrographs (400 $\times$) show representative bead clusters from tissues 3 and 28 days after injection.

be envisioned. Obviously, the technique is not constrained to the heart but could be used in any tissue in which the beads are entrapped at the treatment site. This localization of the marker confers an advantage on the microsphere system that is lacking in the currently available dye systems. The flexibility of the protocol is also enhanced by: (a) the variety of bead sizes available and (b) the fact that beads of different colors can be used (yellow-green, red and blue) allowing for multiple treatment types or time points to be differentiated within a single experiment.

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