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Biotinylated Dextran Amine as a Marker for Fetal Hypothalamic Homografts and Their Efferents

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Abstract

We have explored the use of biotinylated dextran amine (BDA) as a marker for labeling fetal brain grafts and their connections with the host. As a model system we used transplantation of the hamster suprachiasmatic nucleus, the site of an endogenous biological clock governing circadian rhythms. Similar transplants into arrhythmic hosts have been shown to restore behavioral function with a period specific to the donor. For locomotor rhythms, efferent connections are not necessary. For other responses, including endocrine rhythms, efferent connections may be necessary. In order to visualize homografts and their efferents, injections of BDA, an anterograde tracer, were made into the anterior hypothalamic (AH) region containing the SCN or into the dorsal cortex (CTX) of fetal hamster brains. The fetal AH or CTX was microdissected out and stereotactically implanted into the third ventricle of intact, adult hamsters. After 2, 4, 8, or 12 weeks, hosts were sacrificed and their brains were processed for detection of BDA by either histochemistry or immunofluorescence. BDA intensely labeled graft neurons, their dendrites, and axons with minimal or no spread to the adjacent host brain. Labeled graft axons could be followed for long distances (>1 mm) into the host brain and graft-derived varicosities formed close contacts with host neurons. BDA-labeled graft neurons, located at the perimeter of the graft, also extended dendrite-like processes into the host parenchyma. We conclude that BDA is a useful marker for fetal homografts and their efferents for survival times of less than 2 months.

Keywords

neural transplantation; suprachiasmatic nucleus; circadian; anterograde tract tracing; prelabeling

INTRODUCTION

Neural transplantation has been a valuable approach to explore the potential for functional restoration after brain injury and damage (4, 11) as well as to understand the normal functional organization of brain systems that mediate behavior and physiology (16). An example of the latter is in the field of circadian rhythms research, where transplantation has been used to investigate the signals and pathways by which the circadian clock communicates with the rest of the brain. The endogenous biological clock that controls mammalian circadian rhythms is located in the suprachiasmatic nuclei of the anterior hypothalamus and regulates a wide variety of circadian rhythms in behavior, autonomic function, and neuroendocrine physiology (27). Fetal grafts of the SCN restore robust behavioral rhythms to SCN-lesioned rats and hamsters (9, 12, 16), and cross-genotype grafting between clock mutant and wild-type animals has shown that restored rhythms are due to the presence of the donor pacemaker (21).

The precise mechanisms by which SCN grafts restore function are still under investigation, but there is evidence for both neural efferents (18) and diffusible factors (24) as mediators of circadian clock function. In order to assess graft outgrowth and map the pattern of host innervation, markers must be used to identify the grafted tissue and its efferents. For SCN graft studies, delineation of the graft and its borders is most often accomplished by the use of gross morphological features and specific neurochemical markers that characterize the SCN (1, 14, 16). Limitations to these techniques include the fact that graft and host tissue often appear to merge, that neurochemical markers are only expressed in a subpopulation of SCN cells and fibers, and that these markers are found in the host brain as well as the graft making it difficult to unambiguously identify their origin. In order to circumvent these limitations it is useful to take advantage of species-specific markers (3, 26) or genetically modified cell lines and transgenic species (20, 23). Again, there are disadvantages in that the species-specific marker may not be expressed in all regions of the donor brain including the SCN (24), and transgenic donors are not available for all animal models, such as the hamster and the rat. In addition, with the use of cross-species transplantation there is considerable risk for graft rejection requiring the use of immunosuppressive agents, such as Cyclosporin A, following grafting (6).

For these reasons, it is desirable to explore other means of identifying the graft cells and efferents. One such option is to label the graft prior to implantation, and a number of tracers have been explored for this purpose. These include fluorescent latex microspheres (7, 19), gold-filled viruses (2), horseradish peroxidase (13), 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) (10, 19), fluorescent tracers and Phaseolus vulgaris leucoagglutinin (PHA-L) (19), as well as the use of retroviral mediated gene transfer of a reporter gene (19). The application of these techniques varies depending on the goal of the experiment because some only label graft neurons (fluorescent latex microspheres, fluorescent tracers, and gold-filled viruses) other label both cells and neurites (HRP, DiI, PHA-L, and retroviral mediated gene transfer). Since we were interested in visualizing the integration of the graft with the host brain we needed a prelabeling technique that would label both the cells and the efferents of the graft. Given the large investment of time required for both retroviral mediated gene transfer (17) and DiI labeling (22), the fact that HRP is retained in neurons only for a short time (30) and that the efficacy of PHA-L has been reported to be unreliable (29), we explored the use of another prelabeling technique. In the present study, we report on the use of the anterograde tracer, biotinylated dextran amine (BDA), as a marker for prelabeling anterior hypothalamic and cortical grafts and their efferents. In addition, we directly compared the use of this marker with that of a species-specific neurofilament marker using rat-to-hamster anterior hypothalamic xenografts (17).

MATERIALS AND METHODS

Animals

Adult male Syrian hamsters, *Mesocricetus auratus*, 2–3 months of age, 100–120 g, Charles River, Wilmington, MA) were used as hosts for this experiment. Animals were housed in a 14:10 LD cycle under constant temperature with food and water available *ad libitum*. All treatments and protocols were reviewed and approved by the University of Cincinnati College of Medicine Institutional Animal Care and Use Committee.

Prelabeling and Preparation of the Donor Tissue

Pregnant female hamsters were sacrificed at day E15 (E0 = day of mating) by an overdose of sodium pentobarbital (200 mg/kg) by intraperitoneal injection and the fetuses were collected and stored in a sterile petri dish on ice. Donor tissue for xenografts was obtained from pregnant female Sprague–Dawley rats sacrificed by an overdose of sodium

pentobarbital (200 mg/kg, ip) at day E18–20. The fetuses were quickly removed by caesarean section and kept in a sterile petri dish on ice. After decapitation of the fetus, pressure injections of 2–3 μ L of 10% biotinylated dextran-amine conjugated to lysine (BDA; molecular weight 10,000, lysine fixable; Molecular Probe, Eugene, OR, dissolved in 0.9% sterile saline) were made into the fetal anterior hypothalamus (AH; $n = 23$) or dorsal cortex (CTX; $n = 9$) under direct visualization using a 10- μ L lure tip Hamilton syringe (Fischer Scientific, Pittsburgh, PA) and a 30-gauge disposable needle (Becton Dickinson and Company, Franklin Lakes, NJ). Control animals received either 0.9% saline injections ($n = 3$) or no injections ($n = 4$). Thirty minutes after receiving BDA injections, tissue blocks (0.5–1 mm²) containing the AH or CTX were microdissected out. The CTX tissue was taken from the frontal lobe just caudal to the olfactory bulbs on the dorsal surface. The AH was dissected out from the ventral surface of the hypothalamus by making coronal cuts 0.5 mm rostral and caudal to the optic chiasm, lateral cuts 0.5 mm on either side of the midline, and a horizontal cut 0.5 mm from the ventral surface. The volumes of the tissue grafts were equivalent in both dissections. The whole tissue grafts for each group were pooled in sterile Hank's Balanced Salt Solution (HBSS, Bio Whittaker, Walkersville, MD). The tissue was washed five times for 5 min in HBSS to wash off any extra tracer that might be present and to cut down on spread of the tracer to the host brain during transplantation. All dissections and subsequent transplantations took place within 2 h of harvesting the embryonic tissue.

Surgeries and Perfusions

For transplantation surgery hamsters were anesthetized with 75 mg/kg sodium pentobarbital by an intraperitoneal injection and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The skull was exposed and the nose bar was adjusted until lambda and bregma were leveled. A small hole was drilled at bregma just above the superior sagittal sinus. Special care was taken not to break the dura on the sinus. Two donor AH or two donor CTX tissue chunks were drawn up into a 5- μ L implant device (Wiretrol, Drummond, PA) and stereotaxically positioned over the drilled hole. The wiretrol was lowered at the level of bregma 7.6 mm ventral to dura. The tissue in the wiretrol was slowly expelled over a 1-min period through depression of the plunger. After all the tissue was expelled, the wiretrol and plunger were removed, the hole was filled with gelfoam (Henry Schein, Co.), and the skin was closed with wound clips.

Hamsters bearing AH grafts were sacrificed at 2 ($n = 12$), 4 ($n = 7$), 8 ($n = 4$), or 12 ($n = 4$) weeks after grafting. Hamsters with CTX grafts ($n = 9$) were sacrificed at 2 weeks after grafting. Animals were sacrificed with an overdose of pentobarbital (150 mg/kg by intraperitoneal injection) and perfused intracardially with 75 mL of 0.9% saline containing 0.1% sodium nitrite as a vasodilator followed by 150 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3). Brains were removed and postfixed for 4 h in the same fixative, and then transferred to a solution of 20% sucrose in PB until they were infiltrated and sank. Coronal sections (50 μ m) through the forebrain were cut on a freezing microtome, collected as a series of every fourth section, and stored in a cryopreservative solution (31) at -20°C until histochemical or immunocytochemical processing.

BDA Histochemistry

Every fourth section was washed in 0.1 M phosphate buffer containing 0.1% Triton X-100 (PB w/TX; Sigma, St. Louis, MO) then incubated in 0.1 M glycine to quench excess aldehyde reactivity. After washes in PB w/TX they were incubated in 0.5% H₂O₂ for 10 min to block endogenous peroxidase activity. Detection of BDA was performed using a modified avidin–biotin solution (Vector Elite kit, Vector Laboratories, Burlingame, CA) conjugated with horseradish peroxidase (HRP) for 1 h (1:250). The HRP was demonstrated using 2.5 mL of a 0.5% 3,3'-diaminobenzidine (DAB; Sigma) made up in 23 mL of PB plus 8.5 μ L of

30% H₂O₂ for single label detection or with 1.5 mL of DAB made up in 48 mL of Tris–HCL, 0.05 M, pH 8.0, plus 200 mg Ni (NH₄)₂–SO₄ and 17 µL of 30% H₂O₂ for double-label procedures (see below). Sections were washed thoroughly in 0.1 M phosphate buffer to stop the reaction. The sections were mounted and counter-stained with cresyl violet, dehydrated, cleared in Hemo-DE (Fischer Scientific), and coverslipped.

Combined BDA Histochemistry and Immunohistochemistry

Following BDA visualization with nickel-DAB sections were washed in 1% H₂O₂ solution then incubated for 1 h in 1% normal goat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) blocking serum in PB w/TX. Sections were then incubated for 24 h in primary antibody solution containing 1:10,000 rabbit anti-neurophysin (Incstar, Stillwater, MN) in PB w/TX with 5% normal goat serum plus 0.1% sodium azide. Neurophysin was detected using an unlabeled antibody peroxidase–antiperoxidase (PAP) technique. Briefly, the sections were washed in 0.05 M Tris, pH 7.6, plus 1.5% sodium chloride (1.5 T) and incubated for 1 h in goat anti-rabbit IgG (1:200; Sternberger Monoclonals Incorporated, Baltimore, MD) made up in 1.5 T. Next the sections were washed in 1.5 T then incubated for 1 h in ACTIVITY-SELECT rabbit PAP (1:200; Sternberger Monoclonals Incorporated, Baltimore, MD) made up in 1.5 T. After washes in 1.5 T, the peroxidase was detected using unenhanced DAB as described above.

Detection of Species-Specific Neurofilament Marker

Hamsters that received rat xenografts were sacrificed at 2 weeks postgrafting and brain sections were cut as described above. Rat specific neurofilament was detected by immunocytochemistry using a monoclonal antibody against rat NF protein (RMO 108), which was a generous gift of Dr V. Lee (15). This antibody recognizes a phosphorylated epitope of middle-molecular-weight NF protein (NF-M). Briefly, sections were washed, blocked in 1% normal donkey serum for 1 h and incubated overnight at 4°C in primary antibody at 1:1000. The antibody was detected by incubation in biotinylated donkey-anti mouse IgG (1:200 in PB w/TX, Jackson ImmunoResearch) and subsequent detection with a modified avidin–biotin–horseradish peroxidase procedure (Vector Elite kit, Vector Laboratories, Burlingame, CA). The HRP was chromogenically visualized with unenhanced DAB. Sections were washed, mounted, and coverslipped.

Fluorescent Detection of BDA

Alternate sections were prepared as described above for BDA histochemistry but instead of chromogenic detection, either avidin–Texas Red or a tyramide amplification procedure from a TSA-Indirect kit (NEN Life Science Products, Boston, MA) was used. For avidin–Texas Red detection alone, sections were incubated in 1:100 solution made up in PB for 1 h. For tyramide amplification followed by avidin–Texas Red labeling, sections were first washed in 0.1 M Tris–HCl, pH 7.5, plus 0.15 M NaCl and 0.05% Tween 20 (TNT wash buffer). Sections were then incubated in 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent (supplied in kit) for 30 min. Next they were incubated in streptavidin–HRP (1:100) made up in TNT for 30 min, washed in TNT, and then incubated in biotinyl tyramide (1:50) for 10 min. These steps detect the biotin, amplify the signal, and then detect the amplified signal. The signal was then visualized with avidin–Texas Red (1:500) after a 30-min incubation.

Analysis

Viable grafts were defined by the presence of a Nissl-stained graft in the third ventricle and the absence of any evidence of gliosis or necrosis within the graft. Two independent observers examined all sections, and qualitative observations were recorded without

knowledge of the nature of the grafted tissue or survival time of the host. Photographs were taken under both brightfield and darkfield microscopy using a Nikon Microphot FX equipped with a 35-mm camera. Fluorescence sections were examined and images captured using a Bio-Rad MRC 600 confocal microscope with a krypton–argon laser.

RESULTS

All animals in this study had viable grafts, and in all animals sacrificed at 2, 4, or 8 weeks, prelabeled AH and CTX grafts contained histochemically detectable BDA. At 2 weeks after grafting, histochemically detected BDA completely filled fetal AH grafts. In each case, BDA labeled almost all neurons within these grafts, with no detectable spread of the tracer to the host brain except for labeling of a few ependymal cells lining the adjacent third ventricle (Figs. 1A and 1B). Darkfield illumination (Fig. 1B) of grafts at this survival time revealed numerous graft efferents exiting AH grafts at sites of attachment of the graft within the third ventricle where the ependymal cell layer is absent, and graft and host tissue appeared to merge (Fig. 1A). In addition to BDA-labeled graft fibers, we occasionally observed BDA-labeled cells in the host hypothalamus, adjacent to the sites of integration between the graft and host tissue (Fig. 1A).

Both the extent of outgrowth and the trajectory of BDA-labeled fibers as they entered the host hypothalamus were similar to that seen at the same postgraft survival time in AH xenografts using a species-specific neurofilament marker (Figs. 1C and 1D). For example, homografts and xenografts that possessed sites of attachment at the dorsal edge of the third ventricle both had labeled efferents that exited dorsally and coursed ventrolaterally to innervate the adjacent host hypothalamus. Similar host regions were innervated by both homografts and xenografts and included areas normally innervated by the host SCN, such as the dorsomedial and paraventricular nuclei of the hypothalamus. However, patterns of outgrowth were variable for both xenografts and BDA-labeled homografts, and single grafts rarely projected to the same set of targets.

In many cases, BDA-labeled fibers exiting AH homografts could be followed for distances greater than 1 mm into the host brain (Fig. 2A). BDA-labeled fibers and varicosities derived from the graft were often seen in close apposition to Nissl-stained host neurons in the medial hypothalamus (Fig. 2B). Analysis of the pattern of graft efferents could be readily combined with immunocytochemical identification of potential target neurons in the host brain. For example, BDA-labeled varicosities from AH homografts were seen to contact neurophysin-positive cells in the host paraventricular nucleus of the hypothalamus (Fig. 2C). Finally, in addition to labeling graft-derived axons, BDA also labeled the dendrites of donor neurons, which in some cases extended into the host brain at the graft–host interface (Fig. 2D).

At 2 weeks BDA also labeled neurons and fibers in fetal CTX grafts, as seen under both brightfield and darkfield illumination (Figs. 2E and 2F). However, unlike the case of AH grafts, BDA did not entirely fill CTX grafts and labeling was limited to cells within a subportion of the graft.

In addition to histochemical detection, we found that BDA can also be effectively detected with fluorescence (Fig. 3). However, fluorescent labeling was possible only after using a tyramide-based amplification procedure (Fig. 3B); the use of fluorescent avidin conjugates alone was sufficient for detection of BDA in the graft but not for labeling of efferent fibers (Fig. 3A).

The percentage of BDA-labeled grafts that exhibited labeled efferents decreased with increasing time after implantation (Fig. 4). After a survival times of 2 and 4 weeks, 92 and 71% of BDA-labeled AH grafts, respectively, also possessed BDA-labeled efferents. In

contrast, after 8 weeks only 25% of labeled AH grafts also had BDA-detectable efferents. In addition, by 8 weeks after grafting the intensity of BDA reaction product within the graft itself had diminished markedly (data not shown). By 12 weeks BDA reaction product was no longer detectable in the graft or in any efferents (data not shown). Examination of Nissl-stained sections from these animals showed no obvious differences related to survival time in the degree of integration of the graft with the third ventricular wall.

DISCUSSION

We have found that BDA is a sensitive and viable marker for fetal AH and CTX homografts and their efferents for at least 4 weeks postgrafting. The BDA technique as used here has an advantage over other preimplantation labeling techniques in that it exhibits minimal spread after grafting into the host brain. This is in contrast to fluorescent dyes and fluorescent-labeled microspheres where there is the potential for false-positive labeling of the host cells due to diffusion of the tracer into the host brain and uptake by macrophages (19). The minimal diffusion we saw with BDA may also be related to the method of application of the tracer; i.e., that we prelabeled the graft by injecting the tracer into the fetal tissue prior to microdissection, rather than by incubating dissected fetal tissue chunks in a tracer solution.

The pattern of outgrowth observed from BDA-labeled AH homografts was very similar to that seen using xenografts and a donor-specific marker (17). For example, with both markers, AH graft efferents projected to targets normally innervated by the host SCN, such as the dorsomedial and paraventricular nuclei of the hypothalamus. However, as expected, there was variability among the patterns of outgrowth from AH grafts, regardless of whether they were xenografts or BDA-labeled homografts, and single grafts rarely innervated the same set of targets (17).

As noted in the Introduction, a limitation of the use of xenografts is the problem of immune rejection. The use of BDA as a method for prelabeled homografts circumvents this. Moreover, histochemical detection of BDA is relatively simple and fast compared to immunocytochemistry for other labels (e.g., PHA-L), and it can easily be combined with subsequent immunocyto-chemical visualization of other host or donor cells (Fig. 2C). In addition, dual fluorescent detection of BDA with neurotransmitter/neuropeptides should allow one to identify the neurochemical content of the BDA-labeled graft efferents. Although we did not analyze our material at an electron microscopic level, BDA is reported to endure EM fixation and processing and therefore the technique described here should be useful for ultrastructural studies as well (29, 32). Finally, while we have found BDA to reliably label whole tissue grafts, the question of whether BDA may label dissociated cells remains to be explored.

An alternative to prelabeled techniques is the use of mouse models and transgenic strains as sources of donor tissue. For example, transgenic mice expressing a tau-tagged green fluorescent protein can be used in grafting experiments where the transgene can serve as a histological marker of the graft and its outgrowth into a wild-type strain (20). On the other hand, BDA may be a useful alternative in transplantation studies using animal models where transgenic technology is limited, such as the hamster (16).

In addition to the labeling of graft fibers with BDA, we also occasionally observed BDA-labeled cells in the host hypothalamus, located adjacent to sites of integration between the graft and host ventricular wall. Since they were isolated at some distance from the graft, and not in proximity to BDA-labeled fibers, it seems unlikely that they were labeled by diffusion or release of tracer into the host. Instead, these may be cells of donor origin that have migrated from the whole tissue graft into the host brain. Based on their size and

morphology, these BDA-labeled cells appeared to be neurons, but we did not confirm their identity by double labeling with neuron-specific markers.

The major limitation we found in the use of BDA, compared to that reported for other pre-labeling tracers, was its persistence. Thus, although BDA reliably labeled efferents for up to 4 weeks following grafting, by 12 weeks BDA-labeled efferents were not detectable even though BDA was still evident within the graft itself. This was likely due to an overall reduction in our ability to detect BDA since the graft at 8 or 12 weeks also had a diminished intensity of BDA reaction product. Conceivably, this could be due to BDA diffusing out of the tissue after prolonged periods of storage in cryopreservative, or a change in the reactivity of the biotin substrate for histochemical detection. It is possible that tyramide amplification, which results in a more sensitive detection of BDA, would reveal BDA-labeled efferents at these longer postgraft survival times.

Interestingly, while BDA appeared to completely fill AH grafts, we found that in fetal CTX grafts, only subportions of the graft and its efferents were labeled. In most cases, these efferents were restricted and ramified into other portions of the CTX graft. We speculate that this is due to differences in the histogenic stage of the E15 hamster hypothalamus compared to cortex, in that neurogenesis in the hypothalamus is largely complete at this time while it continues postnatally in the dorsal cortex (8). Hence, fetal cortical grafts are much larger than hypothalamic grafts from the same age donor (17) and tend to completely fill the third ventricle (Fig. 2E). Because cortical grafts exhibit continued growth, it is possible that a restricted BDA injection would eventually end up occupying a smaller relative area of the graft.

In summary, BDA provides a simple and reliable anatomical marker for fetal homografts and their efferents. A limitation to this technique is that BDA labeling of graft efferents decreases in intensity by 8 weeks following transplantation. However, for grafting studies in which recovery of function occurs within 1–2 months, BDA provides a marker for the graft and its efferents that can be easily detected by histochemistry and readily combined with immunocytochemical detection of other host or donor markers.

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FIG. 1.

(A, B) Brightfield (A) and darkfield (B) photomicrographs of a BDA-labeled AH homograft implanted in the third ventricle. This section has been Nissl counterstained and shows BDA reaction product filling the graft (A) as well as numerous BDA-labeled fibers exiting the graft (B) at sites of attachment along the lateral walls of the third ventricle. In addition to labeled fibers, a few BDA-labeled cells (e.g., arrow) are seen in the host hypothalamus adjacent to the sites of integration. (C, D) Darkfield photomicrographs showing graft efferents of an AH xenograft visualized using a donor-specific neurofilament marker (C) and those from another BDA-labeled AH homograft (D). Grafts in C and D are both from animals sacrificed 2 weeks after implantation. The pattern and trajectory of labeled efferents is similar in both instances. Section shown in D was Nissl counterstained, whereas that in C was not, accounting for the difference in background color. Scale bar in A, C = 200 μ m.

FIG. 2.

(A–D) BDA-labeling of graft axons and dendrites. (A) Darkfield photomicrograph showing extensive outgrowth from a BDA-labeled graft (g) located in the rostral third ventricle. Scale bar = 200 μm . (B) High-power brightfield view of BDA-labeled fibers and varicosities (e.g., arrow) in close association with Nissl-stained neurons in the host hypothalamus. Scale bar = 50 μm . (C) Double-labeled section showing BDA-labeled fibers from an AH homograft forming close contacts with a neurophysin-positive neuron (arrow) in the host hypothalamic paraventricular nucleus. Scale bar = 20 μm . (D) BDA-labeled dendrite (arrow) from a cell within an AH graft cell extending into the host parenchyma at the site of attachment on the lateral wall of the third ventricle. Section is Nissl counterstained. Scale bar = 80 μm . (E, F) Brightfield (E) and darkfield (F) photomicrographs of a BDA-labeled fetal CTX graft that fills the third ventricle at this level. Note that the BDA-labeling is restricted to a subportion of the graft. Scale bar = 160 μm .

FIG. 3.

Fluorescent detection of BDA-labeling in AH grafts and their efferents. The section shown in A was processed using a fluorescent avidin conjugate alone, while that in B was processed using a tyramide-amplification procedure prior to labeling with the fluorescent avidin conjugate. Tyramide amplification allowed for sensitive visualization of BDA comparable to that seen after histochemical detection (Figs. 1A and 1B). Scale bar = 200 μm .

FIG. 4.

Percentage of AH grafts that exhibited BDA-labeled efferents at different times after grafting. The percentage of grafts with BDA efferents diminished between 4 and 8 weeks postgrafting; in addition, the intensity of labeling within the graft also decreased (not shown).