Fine Structure of Astroglial Integration into Host Brain Following Xenografting

MARC R. DEL BIGIO, M.D., PH.D., FRCP, CATHERINE COLIN, AND CLAUDE M. JACQUE, PH.D.

Abstract. Previous investigations showed that fragments of fetal rabbit brain transplanted into striatum of neonatal shiverer mouse give rise to cells that migrate through host tissue and differentiate into astroglia and oligodendrocytes within 2 weeks. We studied the integration of transplanted astroglia at the ultrastructural level using pre-embedding labeling with a monoclonal antibody which recognizes an epitope associated with rabbit but not mouse glial fibrillary acidic protein. The morphology of early migrating donor cells does not distinguish them from cells arising in host germinial matrix. Once the cells complete their migration they integrate into host brain in a structurally normal manner. Transplanted astroglia form perivascular foot plates with host capillaries. They also send extensive processes into the neuropil where intimate contacts with neurons and synaptic structures are formed. Oligodendrocytes send processes to nearby axons where they form normal-appearing myelin. During the rejection process, which may begin at 4 weeks, donor astroglia show evidence of reaction with increased intermediate filament content. Donor cells are attacked by leukocytes, including eosinophils, and subsequently degenerate. We conclude that cross-species transplantation of glial cells can result in entirely normal structural integration into host brain.

Key Words: Astrocyte; Rabbit; Rejection; Shiverer mouse; Transplantation; Ultrastructure.

INTRODUCTION

Glial cell transplantation has been proposed as a method for introducing lost or missing cellular products into damaged brains and as a potential method for enhancing neural regeneration (1–3). The study of such experimental models requires a knowledge of how well the transplanted cells are capable of integrating into host brain. At the light microscopic level a variety of models have been used (4–8). Few papers, however, address the integration of transplanted glial cells at the ultrastructural level. Among these, some have focused on myelin production by transplanted oligodendrocytes (9–12) or integration of transplanted Schwann cells into brain (13, 14). Transplanted astroglia (15–17) and C6 glioma cells (18) have also been shown at the ultrastructural level to assume a perivascular position. None of the methods of identifying grafted cells (i.e., by markers in lymphosomal or nuclear compartments) allow a detailed assessment of integration by astroglia into host brain.

Migration of transplanted fetal rabbit brain cells in the neonatal shiverer mouse brain has been well characterized at the light microscopic level using a monoclonal antibody designated Tp-GFAP1 which under certain fixation conditions recognizes an epitope associated with rabbit but not mouse glial fibrillary acidic protein (GFAP) (19–21). In this circumstance the entire cytoplasmic compartment of donor astroglial cells is labeled. The shiverer mouse, which is a dysmyelinating mutant lacking myelin basic protein (MBP), was originally chosen as a recipient in this laboratory because it allowed the identification of transplanted oligodendrocytes, which form normal myelin, as well as transplanted astrocytes. Although not necessary to study the integration of xenografted astrocytes into mouse brain, we continue to use the shiverer mutant in this experiment because we have detailed knowledge of glial cell integration (21) and subsequent cell rejection in this experimental model (22–24). Furthermore, it was demonstrated that rabbit astroglial migration is similar after xenografting into mice with normal myelination (25). Using conventional and immunoelectron microscopy we report on the fine structure of rabbit astroglial cell migration, integration, and rejection which follows transplantation into mouse brain.

MATERIALS AND METHODS

All animals were treated in accordance with the French Committee on Animal Ethics and the experimental protocols were approved within the institution.

The Tp-GFAP1 mouse monoclonal antibody recognizes rabbit but not mouse astroglia under certain fixation conditions (19, 20). To assure that it would be useful for the proposed ultrastructural study, we compared recognition of rabbit astrogial by polyclonal anti-bovine GFAP (Dako) and Tp-GFAP1 (gift of V. P. Collins) in a conventional immunochemical study using whole rabbit brains at ages E21, E26, and P2 and in coronal brain sections through the frontal lobes at ages P10, P20, and adult. All rabbits were perfused through the heart with ice cold 3% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brains were removed, sectioned coronally, and frozen in O.C.T. medium (Miles Laboratories) by immersion in methyl butane cooled with liquid nitrogen. Cryostat sections (10 μm) were single labeled with Tp-GFAP1 (1/50) diluted in PBS and revealed by sheep anti-mouse Ig conjugated to FITC (Stilent). Simultaneous double labeling with Tp-GFAP1 and anti-GFAP (1/200) additionally used sheep anti-rabbit Ig conjugated to rhodamine (Dako) as a secondary antibody. We also
studied the Tp-GFAP1 recognition of mouse and rabbit astroglia under various fixation conditions. In mouse brain fixed with paraformaldehyde alone or in combination with glutaraldehyde (0.1–2.0%), Tp-GFAP1 did not recognize astroglia that were labeled by anti-GFAP. However, Tp-GFAP1 did label astrocytes in adult mouse brain which had been frozen without prior fixation (data not shown). On sections of adult and fetal rabbit brain the sensitivity of Tp-GFAP1 diminished at glutaraldehyde concentrations ≥0.25%.

The method of transplantation has been described in detail elsewhere (21). Briefly, periventricular tissue was harvested from fetal rabbits on gestational day 25, diced into fragments (maximum dimension 0.3 mm) in DMEM culture medium, incubated with bismuthenidine 10 mg/ml at 37°C for 1 hour, coated with sterile charcoal powder, and implanted into the right side dorsal striatum of 13 shiverer mice (age 1 or 5 days) which had been cold anesthetized. The mice were returned to their mothers, allowed to survive for 3, 6, 12, 20, or 28 days after the grafting procedure, then overdosed with pentobarbital and perfused through the left ventricle with ice cold 4% paraformaldehyde and 0.1% glutaraldehyde in 0.13 M phosphate buffer with 0.2 mM CaCl₂. The brains were removed intact and left in the same fixative overnight at 4°C.

Previous experience with the xenograft model has shown that transplanted glial cells do not express the Tp-GFAP1 epitope until 1–2 weeks after transplantation (26). Therefore at 3 and 6 day survival periods (4 mice), the brains were trimmed to expose the graft site which was identifiable by charcoal particles and cells with blue fluorescent chromatin. Brain tissue surrounding the graft and contralateral tissue for comparison were post-fixed with 2% osmium tetroxide for 30 minutes, stained en bloc with a saturated aqueous solution of uranyl acetate for 30 minutes, dehydrated in graded ethanol solutions, and embedded in Epon 812. Semithin sections (1 μm) were stained with toluidine blue and ultrathin sections were contrast-stained with lead citrate. At post-graft survival periods of greater than 6 days (9 mice) the brains were bisected and trimmed in the parasagittal plane using a Vibratome until the graft site, identifiable by charcoal, was approached. Then 100 μm thick sections were floated on 0.125 M PBS and examined by fluorescence microscopy to identify the blue fluorescence of bismuthenidine-labeled chromatin in transplanted cells. Sections containing transplanted cells were selected for pre-embedding labeling with antibodies to mouse monoclonal IgG1 Tp-GFAP1 (1/50) and rabbit polyclonal anti-bovine GFAP (1/100; Dako). Sections were cryoprotected in 30% sucrose in PBS for 2 hours, cryofractured by dipping into liquid nitrogen for 30 seconds, thawed in PBS, incubated in PBS 0.1 M with 4% bovine serum albumin, incubated overnight at 4°C with the primary antibody diluted in PBS, and washed in PBS. Sections for Tp-GFAP1 were incubated with biotin-conjugated sheep anti-mouse immunoglobulin (1/50; Amersham) for 4 hours at room temperature, washed in PBS, incubated with streptavidin-conjugated peroxidase (1/100; Boehringer-Mannheim) for 2 hours at room temperature, washed, and reacted with 3,3′-diaminobenzidine (DAB; Dako) for 20 minutes. Sections for GFAP were incu-
bated with pig anti-rabbit immunoglobulin conjugated to pero-
oxidase (1/100; Dako) for 4 hours prior to coloration with DAB.
The tissue sections were examined by light microscopy for la-
beled cells prior to post-fixation with osmium tetroxide, en bloc
staining with uranyl acetate, and flat embedding in Epon. La-
beled cells were all photographed by light microscopy using
water immersion objectives and then the tissue was trimmed to
allow mounting on an Epon pellet. Ultrathin sections were ex-
amined by electron microscopy without lead citrate contrast-
ing. Tissue controls consisted of age-matched, intact or sham-graft-
ed (i.e. stab wounded) animals. Samples of the rabbit brain
fragments following bisbenzimide incubation were also immor-
sion fixed and processed for conventional electron microscopy.
Staining controls consisted of sections processed without pri-
mary antibody incubation.

RESULTS

In the immunohistochemical study of the rabbit brain
during development, the monoclonal antibody Tp-GFAP1
recognized only very rare subpial astrocytes on the ven-
tral brain surface of the E21 rabbit. Labeling of this cell
population progressively increased in intensity and dis-
tribution at each successive age studied. At age P2 some
stellate astroglia were labeled in the fimbria and very
rarely the internal capsule. By P10 many stellate cells
were labeled in the corpus callosum. Their staining in-
tensity increased into adulthood. In the striatum, perivas-
cular astroglia were labeled at P20. Not until adulthood
were astroglia in all regions labeled by the Tp-GFAP1
antibody. Long, straight astroglial processes were found
only in adults, primarily in the white matter bundles of
the striatum (Fig. 1). The polyclonal anti-GFAP antibody
labeled astroglial cells, radial glia, and some ependymal
cells in immature animals. Double labeling showed that
expression of the Tp-GFAP1 epitope was more restricted
than that recognized by the polyclonal anti-GFAP at all
ages except in the adult rabbit brain where labeling pat-
terns were coincident (not shown). This was not a func-
tion of antibody dilution as a tenfold increase in the Tp-
GFAP1 concentration caused minimal change in labeling
pattern.

Electron microscopic examination showed that the E25
tissue fragments destined for grafting consisted of small
cells with round nuclei, clumped chromatin, and small
quantities of cytoplasm which contained abundant polyp-
ribosomes (Fig. 2). Although occasionally closely adher-
et to neighboring cells, only rarely were well-formed
junctions identified. In addition there were grossly swol-
len cell processes and short axon segments representing
damaged and edematous cells.
In all four mice with grafts of 3 or 6 days duration, transplanted tissue was readily identified in the dorsal striatum or periventricular region as a mass of small cells which resembled the subependymal germinal matrix of the host brain (Fig. 3). The graft sites were associated with charcoal deposition, some of which was engulfed by macrophages or glial cells, hemorrhage, and small cavities which represent focal infarction resulting from the grafting procedure. Graft centers were loosely organized and contained viable cells with small quantities of cytoplasm, dying cells, and cell debris (Fig. 4). At the periphery of the graft, however, the cells were clearly viable. By 3 days many elongated unipolar cells were present in the corpus callosum and striatum at the graft edge (Fig. 5). These cells have large nucleoli and contain abundant mitochondria, polyribosomes, and rough endoplasmic reticulum in what appears to be their leading process (Fig. 6). Their appearance is consistent with that of an immature migrating cell. Similar cells could occasionally be identified in the non-grafted hemisphere at the edge of the germinal matrix. Subjectively they were not as abundant in this location. By 6 days some reactive host astrocytes were present in the vicinity of the graft.

At graft sites of 12, 20, and 28 days duration a variable number of small germinal matrix-like cells with Hoescht-labeled nuclei persisted along with scattered hemosiderin- or charcoal-containing macrophages. Medium-sized blood vessels had also developed at the graft sites. Astroglial cells labeled with the Tp-GFAP1 antibody were identified in the striatum up to 4 mm from the graft site in 6/9 mice. Labeling occurred at the surface of the tissue sections which had been incubated. In five animals there were scattered cells (range 2–10) which were well labeled throughout. In one animal dozens of cells were labeled. Cell shape varied considerably. When located in striatal white matter bundles, donor astroglia were bipolar with delicate parallel straight processes, particularly in the animal with many cells (Fig. 7). When located in striatal gray matter, donor astroglia were stellate and usually in contact with one or sometimes two small blood vessels as well as neurons (Fig. 8). Either phenotype could be identified at both 12 and 28 days.

A total of 10 Tp-GFAP1-labeled cells were studied in detail by electron microscopy on serial sections. Ultrastructural examination of cells with a moderate degree of labeling showed that the DAB reaction product decorated filamentous material in the cytoplasm (not shown). Unfortunately, details of the cytoskeleton were obscured by the reaction product. Examination of the cells with long processes in the white matter revealed narrow processes among unmyelinated and thinly myelinated axons. No specific contacts could be identified nor could we identify with confidence the "end" of any given process. Other processes of these cells were in contact with capillary
basal lamina and at their termination they simply tapered off, generally not forming significant contacts with adjacent astroglial cells (Fig. 9). Stellate cells labeled by Tp-GFAP1 wrapped around capillaries and were in direct contact with the basal lamina of endothelial cells (Figs. 10, 11). The cell "bodies" tended to be rather small with an ovoid nucleus generally located near a blood vessel and many branching processes. Their cytoplasm contained a normal complement of organelles (Fig. 12). Golgi apparatus were found only near the nucleus while mitochondria and rough endoplasmic reticulum were pres-

**Fig. 7.** Light micrograph of an unstained full thickness plastic section showing a Tp-GFAP1-labeled donor astrocyte lying among axons of the host striatal white matter, 28 days after transplantation. Long delicate processes (arrows) run in parallel with axons and surround the nucleus (n). Another process of (perhaps) a different cell is wrapped around a capillary (c). Bar = 20 μm.

**Fig. 8.** Light micrograph of a semithin plastic section (stained with toluidine blue) showing a Tp-GFAP1-labeled astrocyte, 12 days after transplantation. The cell body and nucleus (arrow) lie near a capillary and fine processes extend outward among neurons in three directions. Bar = 20 μm.

**Fig. 9.** Transmission electron micrograph showing the advanced process (arrows) of a Tp-GFAP1-labeled astrocyte whose cytoplasm is filled with electron-dense reaction product. The process surrounds and lies on the basal lamina of a capillary endothelial cell (e) which has been cut in longitudinal section (proved by serial sectioning). Bar = 1 μm.

**Fig. 10.** Light micrograph showing a Tp-GFAP1-labeled astrocyte in a 100 μm thick unstained plastic section (prior to sectioning). The cell processes radiate in all directions from the nucleus (arrow). The major process surrounds a capillary (C). Bar = 10 μm.
Fig. 11. Transmission electron micrograph showing the same cell illustrated in Figure 10. A broad process extends from the nucleus (n) toward the capillary (C) and numerous heavily labeled processes (arrow) leave the capillary and extend into the neuropil. Bar = 2 μm.

Fig. 12. Higher magnification micrograph of the main cell process (illustrated in Fig. 11) lying between the nucleus (n) and the thin endothelial cell (e) basal lamina (small arrow). Although the reaction product somewhat obscures details, the Golgi apparatus (g) and mitochondria (m) appear normal. Some membranous inclusion debris (large arrow) is evident in the cytoplasm. Bar = 1 μm.

ent in the most distal processes. Delicate branching processes radiated into the surrounding neuropil, either directly from the cell body or distal to capillary contacts. Examination at high magnification revealed intimate contact between donor cells and host dendrites and synapses (Fig. 13). Capillaries contacted by donor astrocytes showed no abnormal features. In some cases donor astroglial cells contacted pericytes rather than endothelial cells. In addition to astrocytes, transplanted oligodendrocytes could be identified by the production of normal myelin (in contrast to shiverer mouse myelin which lacks a major dense line). These are not illustrated as they have been the subject of previous publications (10, 12).

In this experimental model it was previously shown that microglia are activated in the vicinity of the graft within 2 days of transplantation and that the activation persists for at least 4 weeks. Although T cell invasion has rarely been identified as early as 18 days after grafting, by 4–5 weeks the majority of animals exhibit an intense T cell response at the implantation site and at sites of distant migration. All grafted cells are rejected by 9–10 weeks (22–24). In the present study, signs of cell-mediated rejection by the host, manifest at the light microscopic level by mononuclear cell accumulation around blood vessels ("cuffing"), were evident in two animals, both with grafts of 28 days duration. One of these animals had many Tp-GFAP1-labeled cells. While some donor astrocytes appeared normal, others were contacted by microglia and mononuclear cells (Fig. 14). The processes of such cells had an abnormally dense accumulation of filamentous material indicative of a reaction by the donor astrocyte (Fig. 15). Despite the increased intermediate filament content, labeling was relatively weak, perhaps indicating loss of the Tp-GFAP1 epitope early in the degenerative process. Unlabeled cells associated with normal-appearing myelin, and therefore probably donor oligodendrocytes, exhibited organelle swelling and accumulation of membrane-bound autophagic debris (Fig. 16). Small mononuclear cells, likely lymphocytes, were often identified in the brain tissue, sometimes in proximity to donor cell myelin with normal structure. Some unlabeled cells were surrounded by mononuclear cells and eosinophils. These cells, presumed to be rabbit glial cells under direct cellular attack by the host immune
system, exhibited vacuolization of cytoplasmic invaginations in the nuclei (Fig. 17).

**DISCUSSION**

The present study shows that xenografted rabbit astroglial cells migrate, survive, and become fully integrated into host mouse brain. While the first two behaviors were well established from previous light microscopic studies, we could only speculate on the degree of integration. Transplanted glial cells can be identified at the ultrastructural level by prelabeling with the carbocyanine fluorochrome Dil which, after photoreduction, appears as an electron-dense reaction product in lysosomes (11) or with polystyrene microspheres (15). The coexistence of the label and abundant intermediate filaments indicates that the cells are astrocytes. These studies have demonstrated that transplanted astroglia can settle in perivascular and perineuronal positions but the fine details of integration are not evident because the full extent of the cell is not labeled by those methods. In the present experiment we have taken advantage of the fact that the monoclonal antibody Tp-GFAP1 labels the entire cytoskeleton of donor astroglia unambiguously. The precise epitope labeled by the antibody is not known, although it could be a portion of the GFAP molecule which in mouse is masked by fixation or an intermediate filament-associated protein expressed differently in rabbit (V. P. Collins, personal communication). Donor cell morphology at the ultrastructural level suggests that grafted astroglia which have migrated into the striatum are in a position to function in a completely normal manner. Capillary contact is not unexpected because, in small rodents,

**Fig. 15.** The cytoplasmic filaments (arrow) in the vicinity of the nucleus (n) of the labeled cell are increased in density. A host mononuclear cell (M) of uncertain identity (perhaps a lymphocyte) contacts the grafted cell. E = endothelial cell. Bar = 1 μm.
brain capillaries are not surrounded by astroglia until approximately 10 days of age (27). In this experiment transplantation was performed well before this time, and donor cells are able to compete successfully with host cells for pericapillary positions. Although a structurally intact blood–brain barrier is not necessarily the result of the grafted astrocytes (28), it does indicate that there is no direct adverse effect of foreign astrocytes on brain capillaries. Data from other experiments show that astrocytes transplanted into adult brains can take up a pericapillary position (15). Direct contacts with neuronal structures including synapses, dendrites, and neuron cell bodies suggests that donor astrocytes could serve the normal metabolic functions expected of astrocytes, which include scavenging of potassium ions and neurotransmitters released by active neurons (29).

Unfortunately, labeling by Tp-GFAP is dependent on a certain degree of differentiation. Immature migratory cells cannot be identified by this method, perhaps because they do not express GFAP to the same extent (30, 31).

Fig. 16. Transmission electron micrograph showing a degenerating cell with many swollen organelles in a mouse with evidence of rejection, 28 days after transplantation. Contact with apparently normal myelin (arrows) suggests that the cell is a donor oligodendrocyte. Bar = 1 μm.

Fig. 17. Transmission electron micrograph showing a round cell in a mouse with widespread evidence of rejection, 28 days after transplantation. The clearing in the nucleus appears to represent swelling of a cytoplasmic invagination. The cell is surrounded by mononuclear cells including an eosinophil (arrow). The other surrounding processes (arrowheads) may be those of microglia. Bar = 10 μm.

Early grafts, as would be expected, closely resemble the host germinal matrix/subependymal cell layer which at later stages gives rise to migratory glial precursor cells (32–34). There is an ongoing debate concerning the stage at which germinal matrix cells become committed to the astroglial or oligodendroglial lineage (35). Once migration is underway, immature astroglia appear to extend vacuolated, growth cone-like processes in the direction of growth (36). In our model of glial cell migration it was postulated that bipolar astrocytes expressing the Tp-GFAP epitope are actively engaged in migration (37).

For several reasons we are unable to substantiate this postulate. First, because the DAB reaction product is deposited on the intermediate filaments, we are unable to discern structural differences between the cytoskeletons of stellate and bipolar cells. Second, bipolar cells could be observed both at early (12 day) and late (28 day) post-transplantation intervals, often coexisting with stellate cells. The major difference was their location; bipolar cells lay among axons of the striatal white matter bundles. Third, in rabbit brain, Tp-GFAP-labeled cells with long straight processes were identified only in the adult rabbit striatal white matter and not at earlier developmental stages. The latter two points suggest that the bipolar phenotype is one of a mature astrocyte lying among parallel axons and assuming a shape determined by its physical environment.

Rejection of xenografted glial cells in this experimental model has been shown to be a cell-mediated process. Microglia are activated within 2 days of the transplantation.
Entry of T lymphocytes into host brain begins at 4 weeks and the grafted cells are rejected by 9–10 weeks (22–24). The rejection process appears to involve direct cellular attack by host microglial cells, including microglia, and the initial response to rejection by donor astroglia is an increase in intermediate filament content. They then undergo degenerative changes which include loss of specialization and antigenicity, vacuolization of cytoplasmic organelles, and ultimately cell death. Similarly, the process of rejection of allografts from adult rat brain has been studied in detail at the ultrastructural level (38). In this model the initial response to foreign cells is encirclement by host microglia followed by cytotoxic lymphocyte-mediated killing. As has been demonstrated in other tissues, we show the potential importance of eosinophils in the rejection of transplanted brain cells (39).

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