Neural transplants in patients with Huntington’s disease undergo disease-like neuronal degeneration

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The clinical evaluation of neural transplantation as a potential treatment for Huntington’s disease (HD) was initiated in an attempt to replace lost neurons and improve patient outcomes. Two of 3 patients with HD reported here, who underwent neural transplantation containing striatal anlagen in the striatum during a decade earlier, have demonstrated marginal and transient clinical benefits. Their brains were evaluated immunohistochemically and with electron microscopy for markers of projection neurons and interneurons, inflammatory cells, abnormal huntingtin protein, and host-derived connectivity. Surviving grafts were identified bilaterally in 2 of the subjects and displayed classic striatal projection neurons and interneurons. Genetic markers of HD were not expressed within the graft. Here we report in patients with HD that (i) graft survival is attenuated long-term; (ii) grafts undergo disease-like neuronal degeneration with a preferential loss of projection neurons in comparison to interneurons; (iii) immunologically unrelated cells degenerate more rapidly than the patient’s neurons, particularly the projection neuron subtype; (iv) graft survival is attenuated in the caudate in comparison to the putamen in HD; (v) glutamatergic cortical neurons project to transplanted striatal neurons; and (vi) microglial inflammatory changes in the grafts specifically target the neuronal components of the grafts. These results, when combined, raise uncertainty about this potential therapeutic approach for the treatment of HD. However, these observations provide new opportunities to investigate the underlying mechanisms involved in HD, as well as to explore additional therapeutic paradigms.

Huntington’s disease (HD) is a progressive, untreatable, and fatal neurodegenerative disorder caused by increased CAG repeats in the huntingtin gene (1). The clinical evaluation of neural transplantation as a potential treatment for HD was initiated in an attempt to replace lost neurons and improve patient outcomes (see refs. 2–4). Preclinical rodent and primate experiments demonstrated the feasibility of using embryonic striatal grafts for the treatment of HD. Fetal striatal grafts survive (5), induce behavioral recovery (6, 7), and establish connectivity with the rodent brain (8, 9). Similar results have been reported with xenografts of porcine and human striatal tissue into rodents (2, 9–12). The human striatum is a small enough target to be approached surgically in HD with a realistic goal of achieving confluent graft integration into the host brain (2, 13, 14). Grafts can be functionally integrated (15–18), although such connectivity may not be necessary (19, 20). Similar behavioral results were reported in primate models of HD (21, 22).

The optimal donor age for human striatal graft survival was established in studies using animal models (4, 12, 23, 24). We chose to use embryonic striatal tissue derived from the far lateral portion of the lateral ventricular eminence to optimize striatal-like (P-zone) volume within grafts to greater than 50% (2, 3, 23, 25, 26), which was postulated to correlate with optimal behavioral improvement in rodent models of HD (4, 27–29). Others have used alternative methods of dissection, transplanting the entire ganglionic eminence (4, 30, 31), which includes at least 13 different cell types or nuclei that are not medium spiny neurons of the striatum (23).

Several programs initiated neural transplant trials to evaluate the safety, tolerability, and potential efficacy of human striatal transplantation for the treatment of HD (26, 30–32). Preliminary, open-label studies of neural transplants in patients with HD have demonstrated approximately 2 to 4 years of modest clinical benefits, followed by progressive clinical deterioration similar to the natural history of the disease (31, 32), although there is 1 anecdotal case report of more meaningful clinical benefit (33). The safety profile for neural transplantation in HD may differ significantly from neural transplantation in Parkinson’s disease (PD) (32, 34). Neuritic outgrowth of transplanted medium spiny-projection neurons to appropriate host target areas was postulated to mediate graft-derived behavioral benefits (35), although this afferent connectivity of the graft to the host has never been observed at human autopsy (25, 36). Additionally, recent reports of PD-like Lewy-body inclusions within grafts surviving long-term in patients with PD have raised concerns that genetically and immunologically unrelated grafts are susceptible to the disease processes as well (37–39).

The brains of 3 patients with HD who received neuronal transplants a decade earlier were evaluated at autopsy. Patients were clinically monitored, as described previously (31, 32). Here we report in patients with HD that (i) graft survival is attenuated long-term; (ii) immunologically unrelated grafts degenerate more rapidly than the patient’s brain, particularly the projection neuron subtype; (iii) cortical neurons project to transplanted striatal neurons; and (iv) microglial inflammatory changes in the grafts specifically target the neuronal components of the grafts. We also provide evidence that excitotoxicity and inflammatory mechanisms play a significant role in transplant degeneration and HD as well.

Results

Clinical Outcomes. Autopsy results from patient 2 (W.G.) of our series (32) have been reported previously (25). The evaluations...
reported here correspond to patients 1 (B.L.), 3 (M.C.), and 5 (M.S.) from the same series. Baseline data (age, CAG repeats, symptom duration, time from diagnosis, number of donors, and location of transplants), surgical and immunosuppression methods, clinical outcomes, and complications are described in Tables S1 and S2 and have also been previously described (32). More detailed clinical descriptions are contained in the SI Text.

**Graft and Striatal Gross Morphology.** Macroscopic examination of each patient’s brain showed prominent ventricular enlargement and highly atrophied striatal structures. Disease severity (40) was Grade 3 for patients 1 and 3, and Grade 2 for patient 5. Multiple grafts survived in 2 of the 3 patients (1 and 5). In patient 1, 3 of 16 transplants survived (Fig. S1). In patients 1 and 5, grafts were easily identifiable macroscopically and histologically in the host putamen. Complete loss of all caudate grafts was noted in all 3 patients, corresponding to the region where striatal atrophy was most severe. All histological analyses presented here therefore only pertain to patients 1 and 5. Volumetric analysis revealed that grafts replaced 2.9% and 3.8% of the corpus striatum volume of the right and left hemispheres, respectively, of patient 1, and 4.3% of the corpus striatum volume of the left hemisphere of patient 5 (see Fig. S2). The largest graft was identified in the left putamen of patient 1 and measured 39.7 mm³, similar in size to what was observed in an autopsy performed at 18 months after transplantation (3). Despite preservation of graft volume with time, brain atrophy was notable in all patients. Patient 1 exhibited a 56.5% putamenal shrinkage in comparison to control values and patient 3 demonstrated a 45% putamenal shrinkage, which is consistent with previous autopsy studies reporting a 5% yearly rate of striatal atrophy in HD (41) and a 43 to 47% putamenal atrophy in HD patients in comparison to age-matched controls (42).

**Graft Survival and Cellular Composition.** Immunohistochemical evaluation of surviving putaminal grafts demonstrated regions with striatal markers (P-zones) interspersed with areas of nonstriatal markers (non P-zones), similar to what was observed in a previous case evaluated 18 months posttransplantation (25). The P-zones of grafts in patient 1 were selectively immunoreactive for various markers of interneuron populations, such as acetylcholinesterase (AChE) (Fig. 1A), NADPH-d (Fig. 2 G and H), parvalbumin (PV) (data not shown), and calretinin (CR) (data not shown), as well as markers of projection neurons such as calbindin (CB) (Fig. 2 C and D). P-zone areas represented an average of 58.4% of the grafted tissue in patient 1 (see Fig. 1). Graft P-zone volumes in patient 5 averaged 49% of total graft volume (data not shown).

Grafted P-zones had a decreased density of neurons in comparison to both similar P-zones evaluated 18 months posttransplantation (Fig. 2 E and F) as well as host striatal P-zones (see Fig. 2 C and D). Surviving grafts did not exhibit the morphology of healthy cells in comparison to grafts 18 months posttransplantation (25). Morphological changes typical of neuronal degeneration in HD were observed in nearly all medium spiny-projection neurons labeled with CB (see Fig. 2). These cells appeared rounded, vacuolated, and lacked structural integrity of the cytoplasm and dendritic arborization. On H&E staining, abnormal chromatin organization could be seen (see Fig. 1F). CB immunoreactivity within the graft was also diminished (despite nickel-intensified DAB enhancement used in this study) in comparison to CB immunoreactivity in control regions of this HD brain (see Fig. 2C,
Inset) as well as in similar grafts at 18 months posttransplantation, where intensification was not necessary (see Fig. 2 A and B). Caspase-3, a marker of apoptosis, was similarly present in both the graft (see Fig. 1 J) and the host neurons (see Fig. 1 K). In comparison, grafted interneurons had a healthier appearance with retained dendritic arborization, and maintained good immunohistological staining (see Fig. 2 G).

Graft-Induced Inflammatory and Immune Responses. Cluster of differentiation 8 (natural killers and cytotoxic T cells, CD8) (Fig. S3 a and b), cluster of differentiation 4 (T helper cells, CD4) (Fig. S3 c and d), and human leukocyte antigen (HLA-DR) (data not shown), were positively identified within the graft, indicating an ongoing immune response at the time of death.

Although astrogliosis typical of HD was observed in the host striatum, the edge of the graft was demarcated by a strong astrocytic reaction (Fig. 3 A), where GFAP-positive cells had a morphological appearance of reactive astrocytes. Within the graft itself, the more rarely encountered GFAP cells were recognizable by their nonactivated state (Fig. 3 B). The pattern of astroglial response respected the boundaries of the solid transplants (Fig. 3 C).

In contrast to the astrocytic response around the border of the graft, the microglial response differently recognized the P- and non-P-zones within the graft (Fig. 3 D). In particular, there was activation of microglia within and surrounding those components of the grafts containing striatal markers. Grafted regions lacking striatal markers, which contained immunologically identical donor tissue, were relatively free of microglial activation (see Fig. 3 D). Activated microglia were frequently intermingled with neuronal nuclei- (NeuN) positive cells and were periodically seen to engulf neuronal elements of the graft, more so on the edge of P-zones than in the host striatum suggestive of potential phagocytosis (Fig. 3 E–G).

The absence of protein aggregates (ubiquitin) (see Fig. S3 e) or of abnormal huntingtin protein (EM48) (Fig. S3 g) within the graft suggest that the transplant was spared from the primary pathological effect of the abnormal huntingtin gene. However, ubiquitin (Fig. S3 f and g) and EM48 (Fig. S3 h) were expressed within the host striatum. Additionally, EM48 expression was also pronounced in layer 5 of the cortex (Fig. S3 i and j).

Host Projections to the Graft. Synaptophysin immunoreactivity was demonstrated in patient 1 (Fig. 4 A–D), suggesting establishment of synaptic contacts by transplanted neurons. Host-derived dopaminergic fibers grew into the transplant in both cases, as demonstrated by tyrosine hydroxylase (TH) immunohistochemistry (Fig. 4 E and F). In the striatum, vesicular glutamate transporter 1 (vGlut1)+ axonal varicosities (terminals), found specifically in cortico-striatal axonal projections (43), were observed closely apposed to CB+ neurons in both the host striatum and the transplant (Fig. 4 G). Electron microscopic examination of the graft P-zone in patient 1 revealed asymmetrical synaptic contacts established by vGlut1 immunolabeled axon varicosities (terminals) (Fig. 4 H). Technical difficulties in achieving adequate preservation of the fine structure precluded a complete ultrastructural morphological analysis. Of note, the regions within the grafts receiving glutamatergic input colocalized exactly with the regions expressing microglial activation.

Discussion

We have demonstrated that graft survival in patients with HD is attenuated a decade following transplantation. Multiple surviving
Inflammatory responses to grafts. Both astrocytosis and microgliosis were evaluated and found to be consistent in all 3 hemispheres; representative photomicrographs of patient 1 are illustrated here. (A) The astrocitic response is significant in the host but minimal within the graft, as investigated by GFAP staining. The edge of the graft is demarcated by a particularly strong astrocytic response, characterized by activated astocytes. A few nonactivated astocytes are observable within P-zones (B). Abundant GFAP fiber staining is seen within the non-P-zone (C). These nonactivated astocytes expressed fibers that extended throughout the non-P-zones but did not significantly invade P-zone. (D) Low-power photomicrograph of double immunostaining for the neuronal nuclear marker NeuN (brown; DAB chromogen) and the microglial marker Iba-1 (black; nickel-DAB chromogen) at the interface of a P-zone and non-P-zone and at the interface of the graft and the host. A pronounced microglial response is demonstrated cuffing the edge of the P-zone as well as within the P-zone. Microglial cuffing is seen predominantly at the interface between P-zones and non-P-zones, as opposed to the interface between the non-P-zone portion of the graft and the host. (E) Higher magnification of NeuN/Iba-1 staining at the interface graft/host. (F and G) Higher power photomicrographs depicting examples of grafted neurons intermingled with microglial cells. Of note, microglial cells were often observed engulfing neuronal elements, resembling an ongoing phagocytic event. (Scale bars: A, 25 μm; B, 20 μm; C, 100 μm; D, 300 μm; E and F, 25 μm; G, 12.5 μm.)

Grafts were observed in only 2 of our 3 patients. Case 3 (see ref. 32) had only 1 of 16 surviving grafts, which is unusual for any PD or HD transplant recipient using similar techniques (3, 13, 14, 37, 38). Caudate grafts did not survive in any of these HD patients, similar to what we reported 18 months after transplantation (25). Because the caudate displays the greatest neuronal degeneration and astroglisis in HD, the current findings suggest that this region is a poor transplant niche in this disease. Long-term putamenal grafts had decreased cellularity in comparison to those at 18 months posttransplantation. Grafted cells appeared unhealthy, particularly projection neurons within the grafts. The expression of intraneuronal caspase-3 suggests that striatal cell loss was induced, at least in part, by apoptosis. These results differ significantly from 18-month and 6-year posttransplantation cases where healthy graft survival was reported (25, 36). Neural degeneration at 10 years of neuronal transplant in HD patients is widespread and potentially incompatible with any long-term graft-derived benefit. These results differ from similar case reports in patients with PD (37–39). In those cases, 2 to 8% of transplanted cells demonstrated disease-specific markers (Lewy bodies) (37, 39) and 40 to 80% of dopaminergic neurons and cytoplasmic α-synuclein (38, 39). Despite this, cells were viable, metabolically active (38) and remained differentiated with neuritic reinnervation of the host striatum and with long-term clinical efficacy in individual anecdotal cases (37, 38).

There was diminished cellular viability in all grafted cells at 10 years (vs. 18 months) posttransplantation. Importantly, poor cellular viability was profound in the grafted striatal projection neurons in particular, as opposed to grafted interneurons that still showed distinct cellular cytoarchitecture and polarity but with fewer dendritic extensions than was observed at 18 months posttransplantation. The preferential degeneration of grafted medium-spiny neurons in comparison to interneurons recapitulates the pattern of

Fig. 3. Graft connectivity. (A) Low-power photomicrograph of synaptophysin immunoreactivity inside the graft (dotted line). The density of synaptophysin reaction product is depicted in (B), in the form of an optical density plot taken along the line shown in (A). (C and D) High magnification of synaptophysin staining in (C) a P-zone of the graft and (D) in the host striatum. (E) Photomicrograph of TH immunostaining of the grafted P-zone (dotted line). (F) Numerous host-derivered TH+ fibers are observed with seamless penetration of the graft border. (G) vGlut1 axon varicosities (black dots; arrows within the neuropile) were found closely apposed to cell bodies labeled for CB (brown, arrowheads) in both the host striatum and the transplant. (H) Example of a vGlut1-labeled axon varicosity (asterisk) within the transplant P-zone exhibiting an asymmetrical synaptic contact (between small arrows). (Scale bars: C and D, 25 μm; E, 100 μm; F, 25 μm; G, 20 μm; H, 500 nm.) av, axon varicosity; sp, spine.
the clinical symptoms of HD (23). However, meaningful neuritic outgrowth was not demonstrated in either the present study or previous studies of grafts transplanted into the striatum (26) as well as widespread degeneration of cortical afferents projecting to the grafts (2–4, 35). However, meaningful neuritic outgrowth was not demonstrated in either the present study or previous studies of grafts surviving 18 months (25) or 6 years posttransplantation (36). The volume of striatal loss in our transplant recipients is similar to what has been described in other patients with HD (42). Therefore, the present study failed to demonstrate that embryonic striatal grafts slowed degeneration of the surrounding striatum via either neuritic outgrowth or graft-derived neurotrophic expression (35). However, the present study demonstrated that glutamatergic cortico-striatal projections terminate onto transplanted striatal components of the grafts. The functional significance of this interaction remains unknown. Rather than the grafts positively influencing the cortex, the pathology within the cortex appeared to induce neuronal degeneration within the grafts.

Risks associated with neuronal transplantation in this study are not trivial. Two subjects developed a total of 3 subdural hematomas that required surgical evacuation, out of a total of 14 hemispheric transplants (32). This was likely because of cerebrospinal fluid loss in the setting of brain atrophy leading to the formation of hygromas, which frequently convert to hematomas. This also led to a targeting error in the last needle tract in 1 patient. This safety profile differs dramatically from that observed in transplant recipients with PD, where the same surgeon (T.B.F.) had no surgically relevant subdural hematomas in 66 consecutive transplant procedures (32). Based on these results, it is our impression that any future surgical therapy for HD should intervene in earlier stage patients with less brain atrophy, and minimize cerebrospinal fluid loss to diminish the risks of subdural hematomas and targeting errors.

Clinical efficacy has been postulated to require that greater than 50% of grafted volume is composed of Zones 4 (27, 29) that contain striatal projection neurons. This anatomical threshold was achieved in both the present and our previous study (3). However, clinical efficacy in our patients was mild in magnitude, short-lived, subject to the limitations of small open-label trials (placebo effect, as well as potential investigator and subject bias), and did not prevent the unremitting and fatal progression of HD. Similar clinical outcomes have been reported by others (31), despite the fact that their grafts were derived from a much more nonspecific dissection of the striata in their first autopsy report (53). These similar clinical results, using significantly different dissection methods with a “subthreshold” quantity of Zones within grafts, suggests that clinical benefits, if any, are the result of nonspecific effects of these grafts (19, 20). The combination of an unfavorable risk/benefit profile, short-term and mild benefits at best, and significant disease-like graft degeneration makes future trials of fetal-cell transplantation using these techniques potentially unwarranted. Therapeutic strategies (with or without neural grafts) aimed at altering inflammatory or immune responses, host-derived neurotoxicity, and neurotrophic support in the brain may prove to be more promising for the future treatment of HD.

Methods

Donor Tissue Preparation and Transplantation. Methods for tissue preparation, neuronal transplantation, immunosuppression, location of the transplants, number of donors per patient, and clinical genetic as well as radiologic evaluation have been described previously (3, 23, 25, 32). Solid-tissue transplants measuring 0.5 to 1 mm³ were derived from the far lateral portion of the lateral ventricular eminence to optimize the percentage of tissue of striatal origin (23). All patients received transplants into both the caudate and putamen derived from 5 to 8 striatal primordia per site. The 3 patients in this series represent patients 1, 3, and 5 from our clinical report (32).

Postmortem Tissue Preparation and Histological Evaluation. Postmortem brain tissue was fixed in 10% formalin for 24 h. Brain sections were prepared using 4 μm thick sections for immunohistochemical analysis and 50 μm thick sections for light microscopic analysis. Series of adjacent sections were processed for histochemical and immunohistochemical (see Supplemental Methods: SI Text for protocol details) analyses for the visualization of H&E (brain cytoarchitecture) and various striatal subtypes of interneurons; NADPH-d (marker for nitric oxide containing striatal interneurons),
Three-dimensional reconstruction, Striatal P-Zone Evaluation and Cell Counts

Three-dimensional reconstruction was performed using the Serial Section Reconstruction method provided by the Neurolucida software, version 6.0 (Microbrightfield) on Nissl-stained sections. Volumetric evaluation of graft size (Cavaliere method) as well as P-zone/non-P-zone areas for patients 1 and 5 were explicitly performed using the right hemisphere of both patients 1 and 5. Both grafts and P-zones were delineated using Nissl staining and the Tracing Contours option in the Stereo Investigator software, version 5.0 (Microbrightfield). Areas for either P-zones or non-P-zones were calculated using Contour Measurements option. Cell counts were performed using the Optical Fractionator probe (Stereo Investigator software) based on different immunostaining markers for NADPH (interneurons), H&E (general cell marker), and CB (projection neurons). More detailed assessment of graft volume and estimation of striatal P-zones, and 3-dimensional reconstruction are provided in SI Text.

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