Amitotic Neuroblastoma Cells Used for Neural Implants in Monkeys

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The potential utility of cultured neuroblastoma cells as donor tissue for neural implants into the mammalian brain has been examined. Cells from a human neuroblastoma cell line, IMR-32, were labeled with [^{3}H]thymidine and chemically rendered amitotic. These differentiated IMR-32 cells were grafted into the hippocampi of five adult African Green monkeys, and graft survival was evaluated for up to 270 days after transplantation. Autoradiographically labeled grafted cells were identified in four animals. Processes from grafted cells could be followed for distances of up to 150 micrometers into the host brain. No evidence for neoplastic growth of the transplant was found. Thus, grafted neuroblastoma cells can survive for prolonged periods in the primate brain and may serve as a practical source of donor tissue for neural implants.

AN INCREASING NUMBER OF INVESTIGATORS over the past decade have successfully used techniques of neural grafting to study development, regeneration, and function in the mammalian brain. Experiments with rodents have shown that developing nervous tissue from the brains of fetal donors can survive and develop relatively normal properties when placed into a host's central nervous system (*1*). In rodents, this grafted neural tissue can moderate neurological disorders, including neuroendocrine dysfunctions (*2*), cognitive deficits (*3*), and motor abnormalities (*4*).

These successes with transplants in rodents have led to suggestions that neural implants might have clinical applications in treating human neurological disorders, particularly neurodegenerative ones, such as Parkinson's and Alzheimer's diseases (*5*). Before neural implants can be used clinically, practical sources of donor tissue must be identified. Thus, our group and others (*6, 7*) have examined the properties of cultured cell lines to determine their suitability as donor tissues in neural implants.

We used a human neuroblastoma cell line, IMR-32 (*8*), for our studies because its properties in culture (*9*) indicate that it might be suitable for use in neural implants. The IMR-32 cells synthesize several neurotransmitters including acetylcholine, norepinephrine, dopamine, and serotonin. The cells can be induced to differentiate in vitro when treated with chemicals that affect cyclic adenosine 3′,5′-monophosphate (cAMP) levels or DNA synthesis (*9, 10*). The differentiated cells become amitotic and exhibit many of the characteristics of mature neurons, including the continued synthesis of neurotransmitters and the development of long neurites.

Three female and two male adult African Green monkeys (*Cercopithecus aethiops*) ranging in weight from 4.0 to 6.1 kg were maintained on a 12:12 light-dark cycle with food and water provided ad libitum. Surgery was conducted in the experimental animal surgical facility at the University of Rochester Medical Center (*11*). In four monkeys, a 5- to 10-mm section of the rostral fimbria-fornix was resected, and the fifth monkey served as a sham lesion control. Twenty days later, all five animals received stereotaxic implants (*12*) into the right hippocampus–temporal lobe region. Four transplants, spaced 3 mm apart and consisting of 80,000 to 120,000 differentiated IMR-32 cells each, were made into the right hippocampus of each animal. The human neuroblastoma line IMR-32 was obtained from the American Type Culture Collection and maintained according to described procedures (*9*). Four to five days before the cells were transplanted [^{3}H]thymidine (15 μCi/ml, 46 Ci/mmol) was added to the culture medium to label cell nuclei. Two days later the cells were rendered amitotic by either mitomycin C and 5-bromodeoxyuridine (BrdU) treatment or prostaglandin E_1 (PGE_1) and dibutyryl cAMP treatment. For transplantation, the cells were removed from the culture dishes by the use of trypsin and resuspended in ice-cold culture medium.

At selected intervals, ranging from 51 to 270 days after transplantation, the host monkeys were anesthetized and transcardially perfused with a fixative solution containing 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1*M* phosphate buffer at *p*H 7.2. The temporal lobes of the brain were removed, frozen, and cut in 40-μm sections. Alternate series of sections were stained for Nissl substance, processed for acetylcholinesterase (AChE) histochemistry (*13*), or processed for light microscopic autoradiography by standard procedures with minor modifications (*14*).

Cells labeled with [^{3}H]thymidine were found in or near the implantation tracts in four monkeys (Fig. 1A and Table 1). An apparent angiogenesis was associated with grafts, and the autoradiographically labeled cells within the parenchyma of the brain were usually found within a 125-μm radius of the needle tract, often clustered around blood vessels. Sites where the implantation tract had passed through natural cavities within the brain (such as subarachnoid space in the hippocampal sulcus and the lateral ventricle overlying the hippocampus) often contained large numbers of autoradiographically labeled cells. The nuclei of the labeled cells, as identified by a light Nissl counterstain, were heterogeneous in size and resembled the nuclei of cultured IMR-32 cells (*9*). The reliability of [^{3}H]thymidine labeling as an index of grafted cells has been demonstrated by studies on rodents (*15, 16*). Lindsay and Raisman (*15*) have shown in their studies with [^{3}H]thymidine-labeled neural grafts that if the transplant died, the host glia and other cells were unlabeled even though the death of the implant resulted in the release of all its [^{3}H]thymidine into the

Table 1. Survival of grafted IMR-32 cells. NA, not applicable; autoradiography was not conducted on this animal. −, Zero cells; +, 1 to 10 cells; ++, 10 to 100 cells; +++, 100 to 1000 cells; ++++, >1000 cells.

Animal	Lesion	Days after transplantation	Cell treatment	Autoradiographically labeled cells	AChE-positive cells
A	Fimbria-fornix	51	PGE_1/cAMP	NA	−
B	Fimbria-fornix	155	PGE_1/cAMP	++	−
C	Fimbria-fornix	210	PGE_1/cAMP	++	−
D	Sham	270	Mitomycin C/5-BrdU	+++	+++
E	Fimbria-fornix	240	Mitomycin C/5-BrdU	++++	++++

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brain transplantation site. We have found no significant passive transfer of [^{3}H]thymidine from dead grafted neuroblastoma cells into the host rat brain (*16*).

Acetylcholinesterase histochemistry was conducted on sections adjacent to those used for autoradiography. Grafted AChE-positive cells (Fig. 1B) could be identified only in the two animals that received neuroblastoma cells differentiated by mitomycin C/5-BrdU treatment. In these two animals, AChE-positive fibers could be identified running from grafted cells as far as 150 μm through the host tissue. In many instances, implanted cells were closely associated with blood vessels. In the ventricular and subarachnoid spaces, the organization of the AChE-positive cells was easier to discern (Fig. 1C); as many as several hundred cells could be identified in a cluster of which approximately 90% of the cells exhibited a positive staining reaction for AChE. Protoplasmic protuberances, sprinkled with AChE-positive patches, connected the cell bodies.

Several factors may contribute to the long-term survival of the human-derived IMR-32 cells in the African Green monkey brain. The major human transplantation antigens (HLA-A, -B, and -C) are expressed only weakly by many neuroblastoma cell lines, including IMR-32 cells (*17*). Also, as numerous investigators have noted (*18*), the immunologically privileged nature of the brain, while not absolute, is important for promoting the survival of neural implants.

On the basis of studies of IMR-32 cells in culture and in transplants in rodents, the possibility that the implanted cells in the primate hosts had reverted to a mitotic state seems unlikely. In culture, undifferentiated IMR-32 cells show a doubling time of 48 hours (*9*); grafted into the hippocampi of rats with septo-hippocampal lesions, they form distinct, rapidly growing tumors within 7 days (*19*). No evidence of mitotic activity in the grafted cells (tumor formation or cells in mitosis) was found in the monkeys receiving differentiated cell implants.

The expression of AChE seems to be variable in grafted IMR-32 cells. In vitro, only a portion of undifferentiated and differentiated IMR-32 cells (Fig. 1D) can be histochemically stained for AChE (*9*). The activity of other proteins has similarly been demonstrated to be variable in clonal lines of neuroblastoma cells (*20*). Also, certain compounds, such as BrdU, can induce enzymatic activity in IMR-32 cells (*21*). Our results suggest that the expression of AChE by implanted IMR-32 cells depends, at least in part, on the treatment used to induce differentiation.

Thus, grafted neuroblastoma cells can survive for prolonged periods in the primate brain and may serve as a practical donor tissue in neural transplants. It is not yet known whether grafted neuroblastoma cells are capable of reversing neurological deficits. The specific cell line we used, IMR-32 cells, exhibited some of the properties desirable for donor tissue; the cells survived in four of the five host animals and gave no evidence of reverting to a mitotic state in any of the graft recipients. However, structural integration with the host brain was limited, and the heterogeneity of AChE expression suggests a greater variability of phenotypic expression than is desirable. It will be possible to use rodents as graft recipients to survey the properties of other neuroblastoma cell lines in order to select the best candidates for future functional transplantation studies.

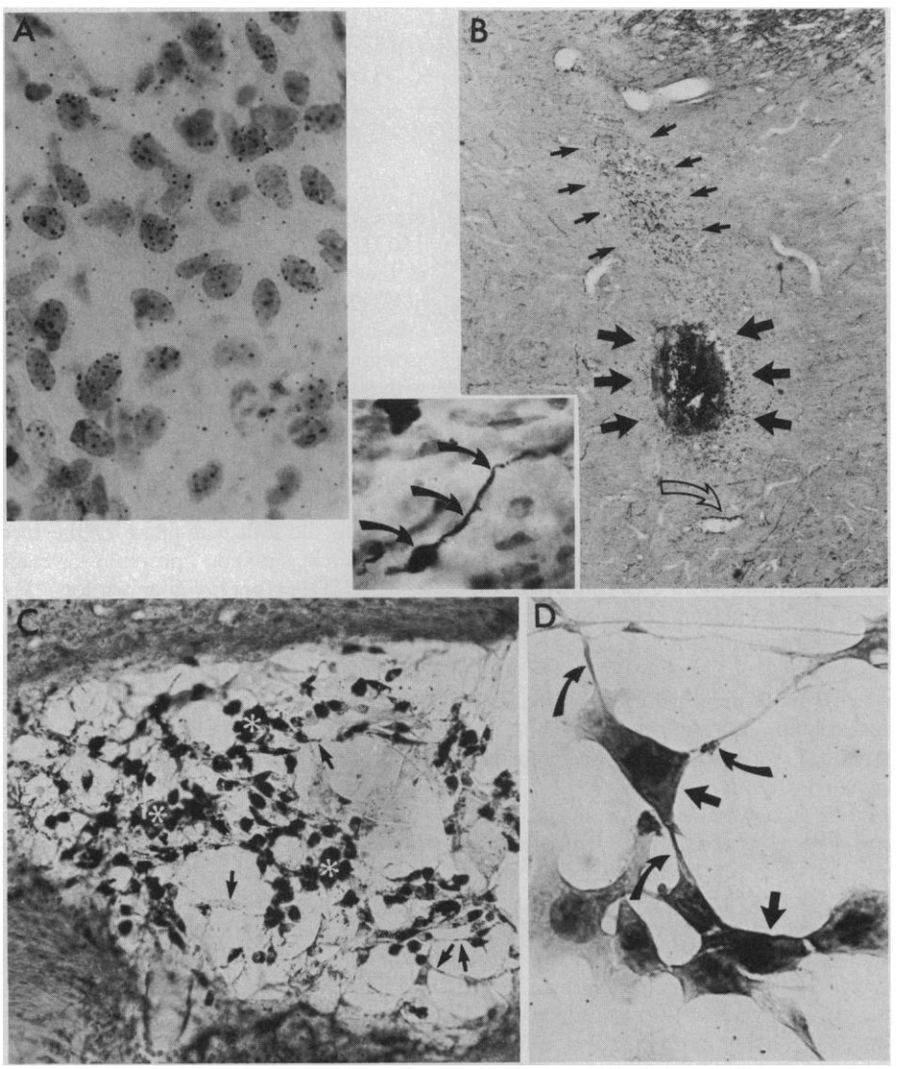

Fig. 1. (A) Autoradiograph of [^{3}H]thymidine-labeled cells in the right lateral ventricle near the right hippocampus. Autoradiograph with Nissl counterstain, ×850. (B) A bolus of grafted AChE-positive cells (large arrows) seen in a needle tract that terminated in the hippocampus. AChE-positive cells and processes are found scattered along the path of the implantation cannula (small arrows) and around nearby blood vessels (open arrow). AChE histochemistry, ×50. (Inset) A high-power magnification of a grafted AChE-positive cell and its processes (arrows) in a needle tract. AChE histochemistry, ×800. (C) AChE-positive cells (asterisks) in the subarachnoid space adjacent to the hippocampus. The nuclei and perikarya are often darkly stained, and the cell processes (arrows) contain dark patches of AChE staining. Compare with (D), which shows the staining pattern of an IMR-32 cell in culture. AChE histochemistry, ×320. (D) IMR-32 cells in culture. The staining intensity for AChE varied among cultured cells. Here are a cluster of darkly stained cells (straight arrows) and their processes (curved arrows). The perinuclear region often showed the most intense staining reaction for AChE. AChE histochemistry, ×1000.

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11. All research procedures related to the use of animals (care and maintenance, surgery, and euthanasia) were conducted according to care and use programs accredited by the American Association for the Accreditation of Laboratory Animal Care.
12. The stereotaxic coordinates for each animal were determined by radiographs and computerized tomographic (CT) brain scans; the latter obtained on a General Electric model 8800 CT scanner with a resolution approaching 1 mm. Through the use of a stereotaxic needle carrier, a 50-μl glass micropipette with a pulled tip having an approximate opening of 100 μm was lowered into the brain at the *xyz* coordinates. In each transplantation site, the cells were infused into the brain over a 6-minute period in 4 μl of Eagle's minimum essential medium supplemented with 10% fetal calf serum, gentamicin (50 μg/ml), and Fungizone (2.5 μg/ml). The micropipette was then slowly withdrawn from the injection site.
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Prorenin in High Concentrations in Human Ovarian Follicular Fluid

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Although the kidney is a major source of prorenin, the precursor of renin, there are extrarenal sources for plasma prorenin that have not been identified. The selective increase in plasma prorenin at the time of ovulation suggested that one of these sources might be the ovary. Prorenin was therefore measured in fluid aspirated from 18 ovarian follicles and in plasma collected from three women who were undergoing in vitro fertilization. The follicular fluid contained high concentrations of prorenin that were approximately 12 times higher than plasma prorenin. The prorenin from follicular fluid was immunochemically identical to kidney and plasma prorenin. Thus, the ovary is a likely source for the ovulatory peak of plasma prorenin.

THE ENZYME RENIN AFFECTS BLOOD pressure and electrolyte homeostasis by catalyzing the formation of angiotensin I from circulating angiotensinogen; the active octapeptide, angiotensin II, is then formed by the action of angiotensin-converting enzyme. Angiotensin II causes arteriolar vasoconstriction and stimulates biosynthesis of the adrenal mineralocorticoid aldosterone (*1*). In humans approximately 90% of circulating renin is the large molecular weight, enzymatically inactive, biosynthetic precursor, prorenin (*2–4*). The kidney is probably the only source of circulating active renin because renin disappears from blood after bilateral nephrectomy. The kidney also normally produces close to 90% of plasma prorenin; however, because prorenin persists in plasma after total nephrectomy (*5*), prorenin must also have extrarenal sources. Renin has been identified by biochemical (*6*) and by immunohistochemical techniques (*7*) in several extrarenal tissues. However, no major source of prorenin has been identified in conjunction with the active enzyme in these tissues, with the notable exception of the placenta. Thus, the extrarenal sources of plasma prorenin have remained enigmatic.

Prorenin may be secreted from the female reproductive tract (*8–11*). Thus plasma prorenin, but not active renin, increases acutely during the luteinizing hormone (LH) surge of the menstrual cycle at the time of ovulation (*8*). This increase also occurs in women who receive human chorionic gonadotropin (hCG) to induce ovulation (*9*). Plasma prorenin also increases within a few days after conception (*10, 11*), roughly in parallel with plasma hCG (*11*). We therefore surmised that the ovary might be a source of circulating prorenin and measured the prorenin concentration of human ovarian follicular fluid at the time of ovulation.

Ovarian fluid from 18 different follicles and peripheral venous blood were collected from three women (ages 29 to 32) undergoing in vitro fertilization (*12*). All had received the LH and follicle-stimulating hormone–containing preparation Pergonal, 150 IU per day intramuscularly, from day 3 to day 12 or 13 of their menstrual cycle; hCG (10,000 IU) was administered intramuscularly 36 hours before follicular fluid aspiration on day 13 or 14. One to 4 ml was withdrawn; the follicle was then flushed with 1 to 2 ml of modified Ham F-10 solution; this wash solution was added to the follicular fluid; and the mixture was frozen within 5 minutes of aspiration.

During the follicular phase of the menstrual cycle, plasma prorenin averages 27 ± 8 (SD) ng per milliliter per hour (*8*)—close to ten times the active renin concentration. In these women stimulated with hCG, the concentration of plasma prorenin was elevated markedly (174 to 208 ng per milliliter per hour) and was 20 times that of active renin (Table 1), in agreement with a previous report (*9*). However, follicular fluid prorenin was in turn very much higher than plasma levels, ranging from 730 to 5430 ng per milliliter per hour. This was approximately 100 times the concentration of active renin in follicular fluid. (Fig. 1 and Table 1). The concentration of prorenin in follicular fluid was 11 times greater than that in plasma (ranging from 4- to 26-fold).

Renin substrate (angiotensinogen) in follicular fluid was present at close to 60% of the plasma concentration. In vivo it was probably the same concentration as in plasma because the fluid was diluted approxi-

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