Intracerebral Transplantation of Porcine Choroid Plexus Provides Structural and Functional Neuroprotection in a Rodent Model of Stroke

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Background and Purpose—Choroid plexus (CP) secretes a cocktail of neurotrophic factors. In the present study, CP from neonatal pigs was encapsulated within alginate microcapsules for in vitro and in vivo neuroprotective studies.

Methods—In vitro studies involved serum deprivation of rat embryonic cortical neurons and treatment with a range of concentrations of conditioned media from CP. For in vivo studies, rats received a 1-hour middle cerebral artery occlusion followed by intracranial transplantation of encapsulated or unencapsulated CP, empty capsules, or no transplant. Behavioral testing was conducted on days 1 to 3 after transplantation. Cerebral infarction was analyzed using 2,3,5-triphenyl-tetrazolium chloride staining at 3 days after transplantation.

Results—Conditioned media from CP produced a significant dose-dependent protection of serum-deprived cortical neurons. Enzyme-linked immunosorbent assay confirmed secretion of GDNF, BDNF, and NGF from CP. Parallel in vivo studies showed that CP transplants improved behavioral performance and decreased the volume of infarction. Both encapsulated and unencapsulated CP transplants were effective; however, more robust benefits accompanied encapsulated transplants.

Conclusions—These data are the first to demonstrate the neuroprotective potential of transplanted CP and raise the intriguing possibility of using these cells as part of the treatment regimen for stroke and other neurological disorders. (Stroke. 2004;35:000-000.)

Key Words: cerebral ischemia

Stroke is the third leading cause of death and a leading health care burden in developed countries. Although the incidence of stroke has declined over the past decades, there are no effective treatments for mitigating the neuronal loss after stroke. Neural transplantation may be one way of repairing the stroke-ravaged brain, as validated in numerous laboratory studies. Initial clinical trials of neural transplantation therapy for stroke have also been initiated with generally encouraging results. The majority of these laboratory and clinical studies are attempting to integrate transplanted cells into the brain after the initial trauma for reconstructive purposes. Delivery of therapeutic molecules via cell transplantation soon after stroke can also be used to reduce or prevent the disease pathology. One intriguing source of transplantable cells is the choroid plexus (CP), which secretes numerous neurotrophic factors and thus might provide a novel means of delivering potentially therapeutic factors for diseases, including stroke. Although few studies have explored the use of CP as a source of transplantable neurotrophic factor-secreting cells, a recent study showed that when CP was transplanted into the damaged spinal cord of rats, a robust regeneration of damaged axons occurred proximal to the grafted tissue. Moreover, preliminary in vitro studies (Skinner et al, unpublished data, 2001) revealed that conditioned media from neonatal porcine CP exerted significant, dose-dependant neurite outgrowth and dopamine uptake on cultured cells. These data support the contention that molecules secreted from CP are biologically active across experimental paradigms and cell types.

The current studies evaluated the neuroprotective potential of neonatal pig CP in vitro and in vivo. CP was encapsulated into alginate microcapsules and analyzed for secretion of glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF); prevention of the death of cultured embryonic cortical neurons exposed to serum deprivation; its ability to reduce the behavioral and anatomical consequences of stroke after transplantation in rats; and its biocompatibility after transplantation.
Methods

All procedures adhered to National Institutes of Health and Society for Neuroscience guidelines for use of research animals and were approved by the Animal Ethical Care and Use Committee of Diatranz/LCT, Ltd. All surgical procedures were conducted under aseptic conditions. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Animals

Adult male Wistar rats (University of Auckland, New Zealand) ~3 months of age and weighing 250 to 350 g were housed in a temperature-controlled (22±1°C) and humidity-controlled (50±5%) environment with free access to food and water throughout the study, except for 4 hours before surgery.

Isolation, Culture, and Encapsulation of Pig CP

Neonatal pigs (large White/Landrace cross, both sexes, 7 to 10 days old and 3.5 to 5.5 kg; PIC Ltd, Maramarua, New Zealand) were anesthetized with ketamine (500 mg/kg) and xylazine (0.15 mg/kg) and euthanized by exsanguination. The brain was dissected through the midline and the CP was removed and placed in Hanks balanced salt solution (HBSS) (0°C to 4°C) supplemented with 2% human serum albumin and processed as follows: (1) the tissue was minced, allowed to settle, and the supernatant removed; (2) collagenase (Liberase; Roche; 1.5 mg/mL, in 5 mL HBSS at 0°C to 4°C) was added and the tissue allowed to sediment before removing the supernatant; (3) fresh collagenase (1.5 mg/mL, in 15 mL HBSS at 0°C to 4°C) was added, warmed to 37°C, and stirred for 15 to 20 minutes; (4) the digested material was mixed with an equal volume of Roswell Park Memorial Institute (RPMI) supplemented with 10% neonatal porcine serum (prepared at Diatranz/LCT), triturated gently, and passed through a 200-μm stainless steel filter; (5) the suspension was centrifuged (500 rpm, 4°C for 5 minutes); the supernatant was removed, and the resulting cell clusters (50 to 200 μm in diameter) were gently resuspended in RPMI supplemented with 10% neonatal porcine serum; and (6) blood cells were removed by sedimentation for 25 minutes at 0°C to 4°C and removing the supernatant. The final preparation was adjusted to ~3000 epithelioid clusters/mL in RPMI with 10% neonatal porcine serum and placed in nonadherent petri dishes. Half of the media was removed and replaced with fresh media after 24 hours and again after 48 hours.

Before encapsulation, the cell clusters were washed by sedimenting 3 times in 2% (0.75 μmol/L) human serum albumin in HBSS at room temperature. The cells were encapsulated in alginate according to previously published protocols. Empty capsules were processed under identical conditions. Encapsulated cells were maintained in culture for 7 days before transplantation. Conditioned media was removed after 24 hours and stored at −20°C for in vitro analysis.

Stroke Surgery

Rats were anesthetized using equithesin (1 mL/kg intraperitoneally). Transient unilateral focal ischemia was produced using a well-established middle cerebral artery (MCA) occlusion model. Based on our extensive experience with the MCA occlusion model, physiological parameters including blood gases of animals undergoing such surgical procedure remain within normal limits.

Transplantation Surgery

Within 10 minutes of MCA occlusion, individual animals were placed in a stereotaxic apparatus (Kopf Instruments). A craniotomy was performed over the predicted location of the cerebral infarction (medial-lateral 3.0 mm to 5.0 mm and anteroposterior 1.0 mm to −2.0 mm). Animals were then randomly assigned to 1 treatment group (N=16 per group): (1) stroke only (MCA plus craniotomy but no transplant); (2) stroke plus control transplant (empty capsules); (3) stroke plus CP-loaded capsules; and (4) stroke plus unencapsulated CP. For rats receiving transplants, the dura was excised and 50 to 55 microcapsules/CP suspended in 30 μL of isotonic saline were placed into the craniotomy, resulting in a bed of alginate capsules/CP directly overlying the cortex. A small piece of surgical cellulose prewetted with RPMI was placed over the capsules/CP before suturing the incision closed.

Behavioral Testing: Motor Asymmetry

The elevated body swing test was used to evaluate the functional consequences of the MCA occlusion and to quantify improvements in motor function produced by the CP transplants. Animals were tested once on days 1, 2, and 3 after surgery. The elevated body swing test reliably detects stable motor asymmetry at these early time points.

Neurological Evaluation

Animals were tested for neurological function using the Bederson test on day 3 after surgery. Using previously described methods, a neurologic score for each rat was obtained using 3 tests that included contralateral hindlimb retraction, beam walking ability, and bilateral forepaw grasp. All 3 tests were combined to give an average neurologic deficit score.

Histology

After behavioral testing on day 3 after stroke, animals received a lethal dose of equithesin (500 mg/kg, intraperitoneally), were perfused with 100 mL of ice-cold saline, and the brains were harvested. Quantitative determinations of infarct volume were performed on a subset of animals (N=10 per group) using standard 2,3,5-triphenyltetrazolium chloride staining and quantitative image analysis as previously described.

Host Tissue Reaction to Choroid Plexus Transplants

The host tissue reaction to the alginate capsules and CP transplants was evaluated in a subset (N=6 per group) of animals. Using previously published protocols, 40-μm-thick cryosections were taken throughout the craniotomy and were processed for immunohistochemical visualization of gliosis via a standard avidin biotinylated enzyme complex method using anti-GFAP antibody.

In Vitro Biological Activity

Conditioned media or nonconditioned media (from empty capsules processed identically) was placed onto primary day 15 embryonic cortical neurons, and neuronal survival was quantified after serum deprivation. The techniques used for preparing and maintaining primary cortical neuronal cultures were as previously published. Brains were removed from Wistar rats on embryonic day 15, and homogeneous suspensions of cortical neurons were prepared and kept in a humidified incubator under 5% CO2 and 95% air at 37°C for 4 days. On day 4, cells were replated in 24-well plates, and over the next 2 days, a subset of cells was cultured without serum and with a range of concentrations of conditioned or unconditioned media (0% to 30%). On day 6, cell viability was analyzed using trypan blue. All studies were conducted in triplicate.

Enzyme-Linked Immunosorbent Assay Determinations of Trophic Factor Secretion

Enzyme-linked immunosorbent assay was used as described previously. The conditioned media from encapsulated cells, nonconditioned media from empty capsules, and basic culture media (containing human serum albumin) were assayed for GDNF, BDNF, and NGF using mouse monoclonal antibodies as capture antibodies and biotinylated goat antibodies as detection antibodies. All studies were conducted in triplicate.

Results

Encapsulation of Pig CP

The encapsulation procedure produced alginate microcapsules ~400 to 550 μm in diameter that contained 1 to 4 clusters of CP, typically in a spherical, ovoid, or branched
Host Tissue Reaction to CP Transplants

A minimal gliotic reaction was produced by the craniotomy surgery with only scattered reactive astrocytes observed within the cortex. The local reaction was not altered after the transplantation of either empty capsules or CP-loaded capsules. However, a marked increase in gliosis was produced in the transplant site of the unencapsulated CP compared with encapsulated CP, empty capsules alone, or craniotomy alone (Figure 1). No surviving unencapsulated CP cells were noted.

Behavioral Testing: CP Grafts Reduce Stroke-Induced Motor Deficits

Repeated measures of ANOVA revealed a significant interaction effect over the 3-day poststroke period (F(6,120) = 36.44, \( P < 0.0001 \)). Whereas the performance of animals receiving encapsulated CP transplants at day 1 after surgery did not reach statistical significance (\( P > 0.05 \)). Bonferroni post-hoc \( t \) tests confirmed that compared with craniotomy alone or empty capsules, CP transplants significantly ameliorated (\( P < 0.01 \)) motor asymmetry (Figure 2A) at days 2 and 3 after surgery (\( > 16\% \) and \( > 23\% \), respectively). The resulting average motor asymmetries of 74% and 62% on days 2 and 3 after surgery were less than the conventionally accepted 75% criterion for stroke rats to be considered significantly impaired. Animals receiving unencapsulated CP also displayed significant attenuations of motor deficits (\( P < 0.05 \)) at days 2 and 3 after surgery, but such benefits were less than that achieved with encapsulated CP and resulted in average motor asymmetries of 78% and 81%, indicating continued significant motor asymmetries. No significant changes occurred in either craniotomy or empty capsule group.

Similar benefits were observed on neurologic impairment as assessed by the Bederson test (ANOVA, \( F_{3,60} = 71.31, P < 0.0001 \)) (Figure 2B). Relative to craniotomy or empty capsule group, rats receiving encapsulated CP transplants were improved by 35% to 40% (\( p < 0.0001 \)). Unencapsulated CP produced a modest 20% improvement in neurologic score (\( P < 0.001 \)). There were no differences between the craniotomy and empty capsule group (\( P > 0.10 \)).

Histology: CP Grafts Reduce Stroke-Induced Cerebral Infarcts

Consistent with previous studies, MCA occlusion produced a cerebral infarct encompassing much of the striatum in control animals (Figure 3). The attenuation of behavioral deficits after CP transplants was accompanied by significant reductions in cerebral infarction (ANOVA, \( F_{3,60} = 13.94, P < 0.0001 \)). This effect was comparable between encapsulated and unencapsulated CP transplants with the infarct volume reduced by \( \approx 30\% \) relative to control animals.

In Vitro Biological Activity: Conditioned Media From CP Protect Neurons Against Serum Deprivation-Induced Cell Death

Cortical neurons deprived of serum exhibited significant cell death (\( \approx 90\% \)) compared with cells maintained in serum-containing media (Figure 4). In vitro, the molecules secreted from CP promoted the survival of cortical neurons (overall ANOVA, \( F_{3,6} = 109.01, P < 0.0001 \)). This protective effect was dose-dependent, with maximal effects obtained when...
serum-deprived neurons were cultured with 10% to 30% conditioned media ($p<0.0001$). At these concentrations, neuronal survival was 60% to 85% and did not differ significantly from serum maintained cells ($P>0.05$). Nonconditioned media from empty capsules did not reduce serum deprivation-induced cell death ($P>0.10$).

**Enzyme-Linked Immunosorbent Assay Analyses: CP Secretes Trophic Factors**

Conditioned media collected from CP (~8 million cells per mL conditioned media) contained detectable amounts of GDNF (68 pg/mL per 24 hours), NGF (22 pg/mL per 24 hours), and BDNF (31 pg/mL per 24 hours). These concentrations fall within the range previously found to decrease deprivation-induced cell death ($P<0.0001$ versus $P<0.05$). Nonconditioned media from empty capsules did not reduce serum deprivation-induced cell death ($P>0.10$).

**Discussion**

The present sets of experiments are the first to our knowledge to demonstrate the in vivo and in vitro neuroprotective effects of CP. Conditioned media from encapsulated CP contained detectable amounts of several trophic factors including GDNF, NGF, and BDNF and produced a clear and dose-dependent protection of serum-deprived embryonic cortical neurons. Parallel in vivo studies in MCA-occluded rats further demonstrated that transplanted CP significantly reduced the extent of cerebral infarction and associated behavioral deficits. These studies did not optimize the transplant site or the numbers of cells used per recipient. Rather, the CP transplants were placed on the cortex overlying the normally infarcted brain region to provide a fairly stringent test of neuroprotection in that therapeutic molecules from CP would be required to diffuse through several millimeters of cortical tissue. Accordingly, the concentrations of therapeutic molecules reaching the infarcted region would be modest compared with those that might be achieved locally. Even under these less than ideal conditions, a significant structural and functional benefit was produced by the CP transplants. Combined with our in vitro studies that identified several potentially therapeutic trophic factors secreted by CP, these studies suggest a mechanistic link between these molecules and the observed therapeutic benefit. Conceivably, however, other molecules, alone or in combination with the ones reported here, could have contributed to the beneficial effects of CP transplants. Ongoing studies are conducting a more exhaustive analysis of the scope of polypeptides secreted by pig CP as well as evaluating alternative transplant sites, different numbers of transplanted cells, and a range of post-stroke transplant times in this same model.

We directly compared encapsulated versus unencapsulated pig CP transplants. Alginate microcapsules have been routinely used to protect discordant cell transplants from host rejection,10,15–17 thus eliminating the need for immunosuppression and allowing the implanted cells to be obtained from xenogeneic sources (ie, porcine cells used in the current studies). Microencapsulation of cells has also been successfully used for nearly 2 decades to deliver transmitters, growth factors, and other proteins, hormones, and enzymes.10,15–17 In the present studies, microcapsules conferred the additional advantage of facilitating transplantation and localization on the cerebral cortex. Interestingly, unencapsulated CP transplants also reduced infarct volume and improved behavior, although the magnitude of the behavioral effects were less than those achieved using encapsulated cells. These data further confirm that molecules secreted by CP are neuroprotective, but the additional GFAP analysis cautions that simple “naked” xenogeneic cell grafts produce a significant inflammatory host reaction likely caused by rapid rejection. Although the inclusion of an immunosolitary barrier may not be absolutely required, it appears that it provides greater clinical applicability and opportunity for functional improvements without the detriment of poor biocompatibility.

Traditionally, the CP has been associated with the production of CSF and the formation of the CSF–blood barrier.18 However, its broader function is the establishment and maintenance of baseline levels of the extracellular milieu throughout the brain and spinal cord, in part by secreting a wide range of growth factors into the CSF. Studies have confirmed the presence of numerous potent trophic factors within CP, including transforming growth factor-β, GDF-15, GDNF, IGF2, NGF, NT-3, NT-4, BDNF, VEGF, and FGF2.8,18,19 Currently, treatments for stroke are limited and the most effective pharmacotherapy (ie, systemic delivery of tissue plasminogen activator) produces limited therapeutic benefit, even when administered within 3 hours after stroke.20 Central delivery of neurotrophic factors may represent an alternative means of enabling neuroprotection after stroke. Several neurotrophic molecules have been reported to provide varying degrees of neuroprotection in animal models of stroke.6,7,12,21,22 If trophic factors prove to be a worthwhile therapeutic strategy, the method of delivery will certainly be critical. To date, administration of trophic factors has been
limited to intraventricular infusions using pumps or cannulae. An alternative is the implantation of cells that have been genetically modified to produce a specific therapeutic protein. Although the use of immortalized cell lines avoids many of the constraints associated with mechanical forms of delivery, the incorporation of a transforming element, poor stability of protein expression, and safety concerns makes the use of genetically modified cells currently untenable on a widespread basis.\textsuperscript{7,17,23} CP cells, however, are primary cells and do not represent a significant tumorigenicity risk. The endogenous role of CP in growth factor production raises the possibility of providing stable and dose-controlled protein delivery simply by modifying the numbers of cells implanted.

In conclusion, we report here for the first time to our knowledge that transplanted CP has robust neuroprotective effects in a rodent model of acute stroke. Based on these data, further study is warranted to evaluate the use of CP as an alternative graft source for cell-based delivery of growth factors and cell replacement therapy across a range of acute and chronic central nervous system diseases.

Acknowledgments

C.V.B. is supported by VA VISN7 Career Development Award, VA Merit Review, and American Heart Association grant-in-aid Award. We thank the excellent technical assistance of Kathy Schuler, Gail McGuiness, Jonathan Lane, and Guolong Yu.

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Stroke. published online July 29, 2004;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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