Survival of foetal spinal allografts into acute lesions of the adult dog spinal cord

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Summary

In order to compensate the loss of motoneurons caused by neurodegenerative disease or spinal cord injury, solid pieces of foetal canin spinal cord from embryos at 30, 35 or 45 days of age were transplanted into focal aspiration lesions of the lumbar spinal cord of adult dogs. Concomitantly, a 10-15 cm autologous peripheral nerve graft was transplanted adjacent to the foetal transplant to stimulate and guide axogenesis of the transplanted neurons. This preliminary study shows that foetal spinal neurons can survive for at least four months when grafted in the spinal cord of adult dogs. However, despite immunosuppression to prevent rejection, viable allografts were observed in only three out of 8 grafted dogs. The results obtained allowed us to conclude that embryo tissue taken between 30 and 35 days of gestation permits successful transplantation. The results of this experiment also suggest that for transplantation to work, the trauma caused by surgery to the spinal cord must be as minimal as possible.

Key-words: spinal cord injury - transplantation - peripheral nerve - embryonic transplant - graft - dog.

Introduction

The loss of motoneurons results either from spinal cord traumatic injury or from neurodegenerative disease. This loss leads to severe functional deficits caused by partial or total denervation of muscles [38]. A denervated muscle shows extensive changes in its morphological, biochemical and physiological features [39, 40]. However, these changes are reversible if the muscle is reinnervated by motoneurons [12, 14, 45]. When motoneurons are destroyed, muscle function can be restored only if reinnervation comes from an alternative motoneuron source. The use of embryonic neuron grafts has been advocated to compensate the loss of motoneurons and to promote muscle reinnervation. However, many questions still remain to be answered before a clinical application of this new therapeutic strategy can be used: 1) are these grafts able to survive and differentiate into spinal cord of higher mammalians? 2) can they integrate into the host spinal and supraspinal circuits? 3) are they able to reinnervate denervated muscles?

In the past decade, many experiments have been conducted in order to answer these questions. These studies showed that embryonic motoneurons could survive, differentiate [29, 31, 34, 41] and migrate in the host spinal cord of adult rodents [9, 7, 41]. Grafted neurons were able to extend axons into autologous peripheral nerve grafts [15] and into reimplanted ven-
tral roots [28]. They were shown to be able to reinnervate denervated skeletal muscles which are transferred close to the spinal cord and connected by its own nerve to the spinal cord [6, 7, 27].

Furthermore, the transplants could prevent the retrograde cell death of host neurons [26, 42]. They partially inhibited the post-lesional cavity and glial scar formation [17, 35]. Transplants have showed their ability to enhance locomotion in neonatal kittens [1] and in neonatal rats [10, 11, 20, 24] in which the spinal cords have been transected.

Most of these experiments have used isografts and have been carried out in rodents [3, 4, 15, 17, 16, 29, 31, 34, 37, 41, 42] or occasionally in cats [1, 35, 46, 47], but foetal spinal cord transplantation is poorly documented in large mammals such as dogs which exhibit, like humans being, major vascular edema and post-lesional functional loss after spinal cord trauma.

The aims of our study were: 1) to investigate the survival abilities of foetal spinal cord transplants (FSCT) in the spinal cord of adult dogs, which is a good clinical model for spontaneous spinal cord trauma in which clinical studies and clinical evaluation can be carried out, 2) to determine the appropriate period of gestation in which dog embryos have to be removed for successful grafting, 3) to investigate whether transplanted spinal neurons could grow lengthy axons into a peripheral nerve bridge.

For this purpose, solid pieces of FSCT and a peripheral nerve graft (PNG) were grafted into focal aspiration lesions of the adult dog spinal cord in order to replace the lost neurons and to stimulate and guide axogenesis of transplanted neurons.

Materials and methods

The surgical, behavioral and electrophysiological procedures were all approved by the scientific committee of the National Veterinary School of Maisons Alfort, France.

SURGICAL PROCEDURE

Eleven adult beagles dogs, weighing 12-15 kg, were used in the present experiment.

Anesthesia and preparation

Prior to surgery, the dogs were given cephalexine (Rilexine, Reading, 30 mg/kg) and prepared for an aseptic procedure. General anesthesia was induced with an intravenous injection of diazepam (Valium, Roche, 0.2 mg/kg) and thiopental (Nesdinal, Mérial, 15 mg/kg) and maintained with oxygen (98 %)-isoflurane (2 %) mixture (Forane, Abbot) using a Bain inhalator.

Preparation of FSCT

The embryos at 30, 35 and 45 days of age were removed from three cross-breed pregnant females and preserved by hypothermia. Their thoraco-lumbar spinal cord segments were carefully dissected out using microdissecting instruments and an operating microscope. After removal of the meninges and ganglia, each FSCT was immediately transferred to a covered Petri dish containing sterile Hams F-10 nutrient mixture at 4°C ready for grafting.

Preparation of the autologous peripheral nerve graft (PNG)

For PNG removal, the dogs were secured on the table in lateral recumbancy. The saphenous nerve was identified, removed (10-15 cm) and stored in a saline solution at 4 °C before grafting (see reference 25 for detailed procedure).

Cavitation of the host spinal cord and transplantation

After exposure of the vertebrate column, an hemilaminectomy of vertebrae L1 and L2 was performed. A 8-10 mm longitudinal incision of the dura was made on the left side of the spinal cord, allowing unilateral aspiration of ventral horn gray matter. Bleeding was stopped with Gelfoam (Upjohn), the resulting cavity, approximately 5-7 mm in length, was filled with solid pieces of FSCT. Eight dogs (group 1) received FSCT and autologous peripheral nerve segment (PNG) and they were kept alive 4 months after surgery. Three dogs (group 2) received only FSCT and were kept alive 48 h, 72 h and one week after surgery. In group 1, one end of the PNG was positioned in close apposition to the foetal transplant. ThePNG was secured to the dura mater with two 10/0 non-absorbable sutures (Prolene®, Ethnor SA, France). To prevent reinnervation of PNG from its distal end, this extra-spinal end was ligated twice with 5/0 non-absorbable sutures (Prolene®, Ethnor) and tied to nearby subcutaneous tissue. Closure of the surgical wound was performed in layers.

POSTOPERATIVE CARE AND IMMUNOSUPPRESSION

Following anesthetic recovery, ketoprofen (2 mg/kg IM) was given for pain control.

Cyclosporine A (Sandimmun®, Sandoz, 10 mg/kg) was given for immunosuppression two days prior grafting and throughout all the study. Methyl prednisolone acetate (Medrol®, Upjohn, 3 mg/kg) was also administered for 15 days after surgery. The dogs were examined daily by a veterinarian for post-operative complications caused by the spinal cord injury and the immunosuppression treatments.

RETROGRADE AXONAL TRACING WITH HORSE-RADISH PEROXYDASE (HRP)

Four months after grafting, three dogs from group 1 were anesthetized again, using the anesthetic procedure described above. The subcutaneous tip of the PNG was dissected from surrounding tissues and transected. A small Gelfoam pledget, soaked in 30 % HRP solution (Sigma), was left in contact with the cut proximal end of the PNG for one hour. The tip of the PNG was placed on a Parafilm sheet and surrounded with petroleum jelly to avoid contamination of the neighboring tissues. The exposed area was then washed several times with saline.

The three dogs were sacrificed by barbiturate overdosing after sedation with ketamine and immediately perfused through the heart with an isotonic heparinized saline solution, then with 10-12 liters of 3 % glutaraldehyde in 0.1 M...
phosphate buffer. The spinal cord segments containing FSCT were removed and placed in 30 % saccharose solution for 48 hours. The HRP activity was revealed on 40 µm cryostat cross-sections of the spinal cord according to the tetramethyl benzidine technique [23].

IMMUNOCYTOCHEMISTRY

The five remaining dogs from group 1 and the three dogs from group 2 were sacrificed as describe above and were perfused through the heart with Bodian 2 (90 parts of 80 % ethanol, 5 parts of 30 % formalin and 5 parts of pure acetic acid). The spinal cord segments, including the transplants, were dissected out, postfixed in the same Bodian solution, embedded in paraffin and cut into 10 µm serial cross sections. From each grafted animal, some sections were rehydrated and stained with cresyl violet for a general histological study.

For immunocytochemistry, rehydrated sections were placed in a 3 % hydrogen peroxide solution for 15 min at room temperature to reduce endogenous peroxidase activity. After washes, sections were incubated with a range of primary antibodies diluted in 0.01 M PBS containing 10 % triton X-10 and 10 % normal goat serum (overnight, 4°C). Primary antibodies used in this study included polyclonal rabbit anti-glial fibrillary acidic protein antibody to identify astrocytes (anti-GFAP ; 1 : 100 ; Dako, Glostrup, Denmark), monoclonal mouse anti-neurofilament to identify axons (anti-NF, clones NR4 and 2F11, 1 : 100, Dako, Glostrup, Denmark), polyclo- nal rabbit anti-microtubule associated protein 1b to label axons and neurons (anti-MAP-1b, 1 :100, Boehringer-Mannheim). The next day, sections were washed and incubated for two hours at room temperature with appropriate biotinylated secondary antibodies (goat anti-rabbit for GFAP and MAP-1b immunohistochemistry and goat anti-mouse for NF immunohistochemistry ; Interchim Montluçon, France) diluted at 1 : 200 in 0.01 M PBS containing 1 % triton X-100 and 10 % normal goat serum. After washes, sections were transferred to a solution containing the avidin-biotin-peroxidase complex (1 : 100 in PBS, Dako, Glostrup, Denmark) for 1 hour. After extensive washing, the sections were exposed to 0.4 % 3,3′-diaminobenzidine (Sigma St Quentin Fallavier, France) containing 0.01 % hydrogen peroxide for a few minutes. After immunohistochemistry, some sections were counterstained with cresyl violet. Control sections that did not receive primary antibodies were used to distinguish specific binding.

Results

CLINICAL EVALUATION

Immediately following transplantation, the grafted dogs displayed various degrees of functional impairment. However from 1 to 28 weeks postoperatively, the dogs from group 1 showed a progressive improvement in motor function. While a locomotor deficit still remained in five dogs 4 months postoperatively, a full motor recovery was observed in the other three dogs in which the graft was found to have survived.

In three dogs, a FSCT was found at 4 months (Fig 1). These transplants were removed from dog embryos at 30 and 35 days of age. They appeared well integrated within host spinal cord and exhibited intimate fusion with adjacent host gray and white matter. At the host-transplant interface, GFAP-immunopositive astrocytes were thus packed in dense layers, or loosely arranged at places where fusion of host and transplanted tissues was apparent (Fig 1). Close to the transplant, numerous mononuclear cells could be found (Fig 1). In other areas, these cells surrounded the transplants.

The transplants were highly vascularized and contained a large neuronal population of varying sizes and shapes. However, the normal architectural arrangement of adult neural tissues was not observed. Nevertheless the transplanted neurons developed typical longs intermingling processes and numerous MAP1b immunoreactive fibers which form a thick network without any specific orientation (arrowheads). High magnification of the interface between TR2 area of the transplant (TR) and the host ventral horn (H). It contains numerous immunoreactive neurites and is not well-defined. Scale bars = 200 µm in A, 50 µm in B and 100 µm in C.

The other five dogs from group 1 did not show any viable graft tissue on histological examination. The transplanted foetal dog tissue did not survive and was subsequently replaced by large cavities. Moreover, necrosis and cystic cavitation were seen within the host spinal cord. However, the spinal cord anatomy was not usually disturbed beyond 0.5 cm from the transplant. The histological examination of spinal cords from group 2, 72 h, 48 h and 7 days post-transplantation, showed that the transplants were rapidly transformed into an homogeneous mass of cellular debris and revealed severe rejection of the graft with significant lymphocytic infiltration and spongiform degeneration (not shown).

PNG INTEGRATION AND RETROGRADE LABELING

The PNG appeared to be well integrated in the host spinal cord. Numerous immunoreactive NF fibers extended from the host spinal cord into the PNG (Fig 3). HRP labeling revealed 20 and 35 labeled neurons located in the spinal cord of two dogs close to the transplantation site at the immediate vicinity of the intraspinal tip of the PNG. No FSCT was found in these dogs.

Discussion

The results of this investigation demonstrate the feasibility of grafting FSCT into acute lesions of the adult dog spinal cord. Moreover, this study suggests that the survival of the FSCT depends upon several factors including: 1/ the age of the donor fetus, 2/ the recipient bed condition, 3/ the immune reaction to the graft.

THE AGE OF FSCT

Fourty-five day-old FSCT did not survive transplantation whereas survival of the transplants was obtained with donor tissue taken from fettuses between 30-35 days of age. Thus, our results are consistent with other studies showing that the surviving of FSCT declines as donor age increases in rat [8, 33] and in cat [1, 35]. The best embryonic stages for grafting were obtained with donor tissue taken from fettuses ranging between 12 to 15 days of gestation in the rat, with 22 to 25 days embryos in the cat [1, 35]. This most favorable period for grafting corresponded with the second trimester of gestation in the dog (30 to 35 days) as already observed in rat and cat.

RECIPIENT BED CONDITION

The severity of injury could also influence the survival rate of the FSCT. Two 35 day-old FSCT did not survive 4 months after transplantation. Immediately after transplantation, these dogs showed a much more dramatic functional loss than the three dogs in which a surviving graft was observed at 4 months post-operation. Furthermore, this locomotor deficit lasted until the end of the study. Our short-term studies (group 2) showed that FSCT can be rapidly destroyed after the transplantation. This destruction could result of acute rejection and/or of the spinal cord trauma. Oedema and secondary reaction resulting from severe injury could produce a deleterious tissue environment in the host CNS. Moreover, the revascularisation of the grafted tissue, which is vital for transplant survival, could be more difficult in traumatized tissue [5, 30]. This finding is in agreement with other experiments [1]. The graft could produce a protective effect on the host spinal cord. The transplant could be acting as a source of neurotrophins, promoting survival of damaged host neurons [26, 42] and could also inhibit partially the post-lesional cavity and glial scar formation [17, 35]. But the expression of these advantages is only possible when the recipient bed allows graft survival. The intramedullary injection of cell suspension containing motoneurons may help to reduce the spinal cord injury and promote graft survival.

THE IMMUNE REACTION TO THE GRAFT

Even under immunosuppression, some regions of the transplant which had survived for four months post-transplantation, included numerous mononuclear cells. Such observations must be considered as an imminent host-graft immune response [21] as previously described in the cat [1, 35]. The use of pregnant females from a breed other than that of the recipient dogs provides a clinically relevant model of spinal cord allograft which probably produce a more important graft rejection than in a rat model in which the same breed is generally used for the donor and the recipient. The immune reaction also seems to depend upon the stage of foetal graft development. When FSCT was removed from cat embryos at 22 to 25 days of age, immunosuppression was not essential for graft survival, whereas it became indispensable for 28 days and 38 days grafts survival [1, 35]. It has been supposed that the host immune response becomes more robust and that some antigens can be expressed with embryo maturation.

The CNS has been considered an immunologically privileged site [2, 13] but this privilege is not absolute [22, 32, 43, 44]. Immunosuppression is, therefore, widely used in patients receiving grafts. Without immunosuppression, rejection of the FSC allografts occurred in rats after the initial period of growth and maturation of the transplant [44]. The cyclosporin dose used in our experimentation is the same as that used in dogs for kidney and lung transplantations [36]. However, the dose used in the present experiment did not seem to be sufficient to induce a total immunosuppression. This could result from a modification of the cyclosporin pharmaco-kinetics by spinal cord trauma [19].

Conclusion

The present experiment confirms the capacity of intrinsic neurons of the damaged spinal cord of the adult dog to form and grow axonal extensions into PNG that have been implanted into the spinal tissue [25]. However, subsequent studies are necessary to investigate if transplanted neurons can grow axons into a PNG and contribute significantly to the reinnervation of denervated muscles. This study also indicates that allografts of foetal spinal cord tissue can survive and integrate within the spinal cord of a large mammal which exhibits, like man, major vascular oedema and post-lesional functional loss after spinal cord trauma. In addition, it allowed the determination of an adequate stage of gestation at which perform successful foetal spinal cord transplantation in the adult dog. The difficulties encountered in this experimentation (neurological deficits, rejection and destruction of the transplants) show that use of bigger and/or higher (on the evolution scale) animals other than rodents appears to be a necessary step in studies aimed at proposing therapeutic approaches in mankind.

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