Various Methods of Pukinje Cells Transplantation and their Functional Response in Lurcher Mutant Mice

Schmidtmayerová B., Cendelín J., Korelusová I., Vožeh F.
Department of Pathophysiology of the Faculty of Medicine in Pilsen, Charles University in Prague, Czech Republic

Received January 18, 2005, Accepted February 1, 2005

Abstract: Embryonic cerebellum was transplanted to adult Lurcher mutant mice affected with hereditary olivocerebellar degeneration and with resulting cerebellar ataxia. Grafts were applied as solid pieces of tissue or as cell suspensions. The aim was to replace Purkinje cells lost by the neurodegeneration with embryonic cells and to observe the effect on motor symptoms of cerebellar ataxia. Success rate of the two methods was also compared. Motor skills were tested before and in week intervals after the transplantation. The results were compared with sham-operated controls. When the solid graft was transplanted, the success rate was two times higher as compared with the cell suspension method. Fibre sprouting and cell migration from the graft to the host tissue was observed. Insignificant amelioration of motor skills was found in mice after the solid cerebellar tissue transplantation, while the cell suspension application had no effect.

Key words: Cerebellar ataxia – Cerebellar degeneration – Embryonic cerebellum transplantation – Lurcher mutant mice

This work was supported by COST EU Program B10.10/1998 Brain Damage Repair.

Mailing Address: Assoc. Prof. František Vožeh, MD., PhD., Department of Pathophysiology of the Faculty of Medicine in Pilsen, Lidická 1, 301 66 Plzeň, Czech Republic, Phone +420 377 593 361, Fax +420 377 593 369, e-mail: frantisek.vozeh@lfp.cuni.cz

Introduction

Lurcher mutant mice are a natural model of olivocerebellar degeneration caused by a mutation in the δ2 glutamate receptor gene [1]. The gene is expressed specifically in cerebellar Purkinje cells [2]. In heterozygous individuals (+/Lc), the Lurcher mutants, Purkinje cells die postnatally by excitotoxic apoptosis triggered by glutamate excitotoxicity [3]. Purkinje cell loss is a direct effect of the mutation and it is complete by postnatal day 90. There is also a substantial secondary decrease of the number of cerebellar granule cells and inferior olivary neurons number. Approximately 10% of granule cells and 30% of inferior olivary neurons survive [4]. Lurcher mutant mice suffer from cerebellar ataxia which limits their motor skills [5, 6]. Wild type littermates (+/+) of Lurcher mutants are completely healthy while the homozygous combination of mutated gene (Lc/Lc) is lethal.

Similar cerebellar degeneration as affects Lurchers is known also in human pathology. That is why Lurcher mutant mice represent an appropriate model of treatment possibilities of this disease. In last years therapy of CNS diseases with embryonic or stem cell transplantation is intensively investigated. For cerebellar transplantations pcd mutant mice are often used, however Lurcher mutants seem to have some advantages [7].

We transplanted embryonic cerebellar tissue into the cerebellum of adult Lurcher mutant mice in the form of a solid graft or as a cell suspension. The aim was to replace Purkinje cells, lost by the degeneration, with embryonic cells. We compared the success rate of these two methods and their functional effect on motor skills of Lurchers.

Materials and methods

Donor embryonic cerebellar tissue was obtained from 12 days mouse embryos producing green fluorescent protein (GFP). Fluorescence of GFP was later used to identify the graft derived tissue. These embryos did not carry Lurcher mutation, so that their Purkinje cells did not degenerate by excitotoxic apoptosis. Pregnant female was overdosed with Thiopental at the 12^{th} gestation day. Embryos were removed and pooled in a cold solution (0.9% of sodium chloride and 0.6% of glucose). The cerebella were dissected and pooled in the same solution (0°C).

Cell suspension was prepared by mechanical dissociation of embryonic cerebellar tissue (from 6–8 embryos) in the vehiculum (0.9% of sodium chloride and 0.6% of glucose) without using proteolytic enzymes. Concentration of cells was checked. The cells were separated in the centrifuge. Supernatant was removed. The concentration of the cell suspension was adjusted to 50000 cells/μl by resuspending in adequate volume of vehiculum.

As hosts young adult Lurcher mutant mice were used (2–4 month old). Host mouse was anaesthetized with ketamine (100 mg/kg) and xylazine (16 mg/kg). Fur hairs were trimmed at parietal and occipital area of the head. The mouse was fixed in a stereotaxic holder. The operating field was disinfected. The skin was cut in the

midline in parietal and occipital area (the cut length approximately 1.5 cm). The muscles were removed laterally. Atlantooccipital membrane was perforated with injection needle. In the occipital bone just below the tuberositas occipitalis externa a hole was drilled (1 mm in diameter). Through it 2–3 pieces of the embryonic cerebellum were applied in 10 μl of vehiculum (0.9% sodium chloride, 0.6% glucose) to 26 Lucher mutant mice using a glass microcapillary. To 15 Lurchers 5 μl of cell suspension through a microcapillary were applied. The wound was sutured in one layer and finally disinfected. To control mice only vehiculum was applied by the same procedures (12 mice sham-transplantation of solid tissue, 10 mice sham-transplantation of cell suspension).

The motor skills were examined onetime a week before the transplantation and from the second to the fifth week after the operation. Animals were tested with a set of three methods: horizontal bar, ladder and rotating cylinder. We measured time that the animals were able to stay on the bar, ladder or cylinder and assessed criterion meeting to stay there for 60 s or leave the apparatus actively. If the animal fell down within 60 s, the trial was considered as unsuccessful. All procedures were repeated four times in each session. Results of animals with transplanted cerebellar tissue were compared with sham-operated control mice. Only animals with histologically proven graft were used for the statistical analysis. Individuals with unsuccessful transplantation (those where the graft was not found by histological examination) were excluded from the set. In animals sacrificed earlier than 4 weeks after the transplantation motor abilities were not assessed. Finally, motor skills were analysed in 9 mice that received solid graft and in 4 animals that the cell suspension was grafted. For statistical analysis Mann-Whitney test was used.

Finally, 2–9 weeks after the operation the mice with transplanted cerebellar tissue were deeply anaesthetized with thiopental and transcardially perfused with phosphate buffer (pH 7.4) and 4% paraformaldehyde. Brains were removed and

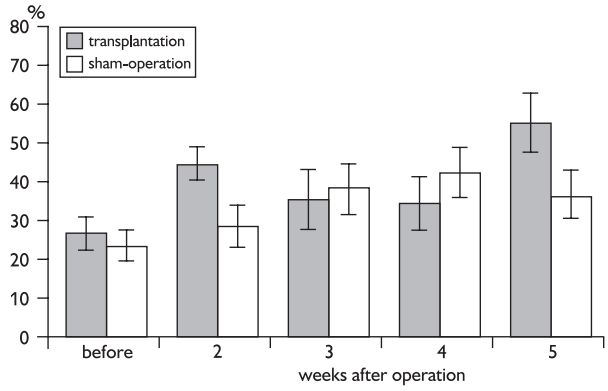

Figure 1 – Comparison of motor skills in mice that was transplanted the solid cerebellar graft and sham-operated controls. Mean criterion meeting in % in all tests (horizontal bar, ladder, rotating cylinder) before and 2, 3, 4 and 5 weeks after the operation. Error bars represent standard error of the mean.

postfixed in 4% paraformaldehyde for at least 2 hours. The cerebellum and the brainstem were sectioned in the frontal plain with vibratome (50 μm).
Grafted and graft derived tissue was detected according to its green fluorescence. Localisation and size of the graft as well as the number, localisation and fibres of grafted or graft derived cells were examined. Then the slices were stained according to Nissl and detailed morphology of the graft was assessed in light microscope. Nissl staining was performed after examination using fluorescent microscope because the staining procedure destroys natural fluorescence of GFP.

Results

In mice with transplantation of a solid piece of the embryonic cerebellum green fluorescent tissue indicating successful transplantation was found in 18 of them, it means in 69.2%. Typical structure of cerebellar cortex was well developed and vascularisation of the graft was visible (Colour fig. 13, 14). Sprouting of nerve fibres from the graft to the host tissue was observed. In some cases graft derived cells migrated from the graft and colonised surrounding host cerebellar cortex (Colour fig. 15).

Success rate of cell suspension was lower. Green fluorescent cells were found in 5 from 15 mice treated with this method, it means in 33.3%. In 2 animals the cells were dispersed around the place of application (Colour fig. 16). In other 3 dispersed cells were not present. Only small clusters of insufficiently suspended tissue survived. The clusters were usually localised on the surface of the host cerebellum or brain stem. The percentage of mice with detected graft did not depend on the latency between the transplantation and histological examination. Motor tests showed only statistically insignificant differences between mice with the solid cerebellar graft and sham operated controls. Individuals with the graft were more successful than controls in the second and the fifth week after the transplantation (Fig. 1). In a mice that the cell suspension was applied there were no differences (Fig. 2).

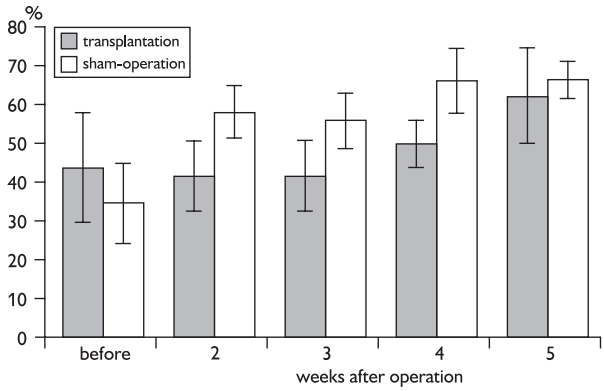

Figure 2 – Comparison of motor skills in mice that was transplanted the cell suspension and sham-operated controls. Mean criterion meeting in % in all tests (horizontal bar, ladder, rotating cylinder) before and 2, 3, 4 and 5 weeks after the operation. Error bars represent standard error of the mean.

Discussion

Solid grafts developed in the host body. Individual cells migrated out of the graft. Fibre sprouting indicated some interactions between the graft and the host tissue, which are necessary for functional effect of the transplantation.

The success rate of solid graft transplantation was approximately two times higher than in cell suspension application. It can be explained in several ways. First, the cell suspension method is more time consuming and there is longer time between extraction of the embryo from the uterus and application of the graft into the host cerebellum. Viability of the tissue decreases during this time. The second cause can be a destruction of the embryonic cells during mechanical dissociation, since we did not use proteolytic enzymes to prepare the suspension. The third possibility is an influence of the microenvironment. Neurogenic properties of the tissue surrounding the graft are very important for its fate [8]. Individual cells of the suspension are in closer contact with the host tissue, while the solid graft creates its own microenvironment, which can be more suitable for embryonic cell development and proliferation. The conditions inside the graft can be more similar to those in real embryonic cerebellum. On the other hand, the host cerebellum is changed by neurodegenerative process (hosts were Lurcher mutants). Injury, neurodegeneration and excitotoxic affection (all present in Lurchers, some injury is made by graft application) negatively influence neurogenic properties of the tissue [9, 10].

Also the functional effect was observed only in solid graft transplantation, nevertheless it was insignificant.

Conclusion

Solid graft transplantation was in Lurcher mutant mice more successful as compared with cell suspension application. At present it seems to be a more suitable method for experimental studies of the transplantation effects on the deteriorated functions in the olivocerebellar degeneration of Lurcher mutant mice. The work did not prove unambiguous effect of transplantation on motor patterns of the cerebellar ataxia. Effects were only moderate and insignificant.

References

1. PHILLIPS R. J. S.: 'Lurcher'. A new gene in linkage group XI of the house mouse. *J. Genet.* 57: 35–42, 1960.
2. ARAKI K., MEGURO H., KUSHIYA E., TAKAYAMA C., INOUE Y, MISHINA M.: Selective expression of the glutamate receptor channel delta 2 subunit in cerebellar Purkinje cells. Biochem. *Biophys. Res. Commun.* 197: 1267–1276, 1993.
3. ZUO J., DE JAGER P. L., TAKAHASI K. J., JIANG W., LINDEN D. J., HEINTZ H.: Neurodegeneration in Lurcher mice caused by mutation of δ2 glutamate receptor gene. *Nature* 388: 769–773, 1997.
4. CADDY K. W. T., BISCOE T. J.: Structural and quantitative studies on the normal C3H and Lurcher mutant mouse. *Phil. Trans. Roy. So. Lond. B.* 287: 167–201, 1979.

5. KŘÍŽKOVÁ A., VOŽEH F.: Development of early motor learning and topical motor skills in a model of cerebellar degeneration. *Behav. Brain Res.* 150: 65–72, 2004.
6. LALONDE R., BOTEZ M. I., JOYAL C. C., CAUMARTIN M.: Motor abnormalities in Lurcher mutant mice. *Physiol. Behav.* 51: 523–525, 1992.
7. HECKROTH J. A., HOBART N. J. H., SUMMERS D.: Transplanted neurons alter the course of neurodegenerative disease in Lurcher mutant mice. *Exp. Neurol.* 154: 336–52, 1998.
8. ROSSI F., CATTANEO E.: Neural stem cell therapy for neurological diseases: dreams and reality. *Nature Rew. Neurosci.* 3: 401–409, 2002.
9. MACKLIS J. D.: Transplanted neocortical neurons migrate selectively into regions of neuronal degeneration produced by chromophore-targeted laser photolysis. *J. Neurosci.* 13: 3848–3863, 1993.
10. SNYDER E. Y., YOON C., FLAX J. D., MACKLIS J. D.: Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. *Proc. Natl. Acad. Sci. USA*. 94: 11663–11668, 1997.