PROTEIN GENE PRODUCT 9.5 EXPRESSION IN RETINAL TRANSPLANTS

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ABSTRACT

To investigate the epigenetic factors on the differentiation of retinal neurons, embryonic rat retinas were transplanted to the midbrain or vitreous body, and the pattern of organisation of protein gene product 9.5 (PGP 9.5), a specific neuronal marker, was examined 1-5 weeks after transplantation. In retinal transplants well-defined neuronal populations were visualised and the transplants survived and differentiated much better if they were grafted to the midbrain of young recipient animals as compared to those grafted to the vitreous body in mature animals. The overall pattern of distribution of neuronal populations in different retinal layers was comparable to that observed in the normal retina. The laminar pattern of synaptic organisation in the plexiform layers in the intracranial transplants was also well preserved, and the time course of development was comparable to that of normal developing retinas. These findings suggest that factors such as the site of transplantation are important for determining the survival and differentiation of chemically defined retinal cells, and that the time course of expression of specific molecules, i.e. PGP 9.5, in transplanted retinal cells seems to be determined by programmes intrinsic to the retinal tissue. The expression of PGP 9.5 in the transplants also indicates that this protein plays an important role in the development of retinal cells.

Degeneration of neurons in the mammalian CNS frequently occurs as a result of aging, trauma or diseases, and replacement of degenerated neurons in the brain offers one possible way of repairing the damage or restoration of function. Extensive research has been focused on transplantation of central and peripheral nervous tissues, and a large quantity of data concerning the role of neural transplants has been generated. While the use of neural transplants as a treatment for various neurological diseases has become more and more controversial, animal studies have provided a wealth of useful information with regard to factors that affect the survival and differentiation of neuronal transplants, and the establishment or regeneration of functional connections between the transplant and the host brain (2, 14). For instance, Lund and co-workers have shown that fetal retina transplanted to developing rat brain can establish functional

connections with visual targets in a highly specific manner (5, 16, 17, 19). Several groups of investigators have also used retinal transplant as an experimental model for studying chemical differentiation of neurons after being grafted to the eye or various parts of the brains (1, 6, 8, 12).

In order to further investigate the epigenetic factors on the differentiation of retinal neurons, this study aims directly to compare the development of chemically distinct populations of retinal cells after transplantation to partially denervated midbrain in neonatal rats and the vitreous body of adult animals, by examining and comparing the expression of a specific neuronal marker, protein gene product 9.5 (PGP 9.5). PGP 9.5, a carboxyl-terminal hydrolase isozyme for ubiquitin, is a cytoplasmic protein and known to label a number of neuronal and neuroendocrine tissues at early development and adult stages (13, 20, 22). Our previous studies have also

indicated that PGP 9.5 are present in the developing retina very early and distributed in certain specific populations including retinal ganglion cells, amacrine and horizontal cells (4). The fact that nearly the entire population of ganglion cells is PGP 9.5 immunoreactive suggests that this protein can be used for assessment of changes of these cells during normal or abnormal development (3, 4). In view of these findings, and our interest in intrinsic and extrinsic factors that affect the development or survival of retinal neurons, we decided to investigate the PGP 9.5 neurons in the transplanted retina by addressing several fundamental questions. First, does the PGP 9.5-immunoreactive distribution pattern develop subsequent the retina grafted to an abnormal environment; and second, how and when is the specific pattern present; and three, does the PGP 9.5-immunoreactivity maintain in the transplanted retina? To answer the question of specificity, we transplanted embryonic retina into the midbrain of newborn rats and the vitreous body of mature animal and examined the expression of PGP 9.5 in the transplants. The results can allow direct comparison of development between different neuronal populations under the experimental condition.

MATERIALS AND METHODS

Experimental Animals

Sprague-Dawley rats were used in this study. A total of 105 rats of both sexes, including 40 donors and 65 recipients. Briefly, the host animals were divided into two groups. In the first groups of neonatal rats ($n = 45$) which were anaesthetised with ether, embryonic day (E) 14 retinas were transplanted to the left side of the midbrain of newborn or postnatal day (P) 0 recipients immediately after intraorbital transection of their right optic nerve, a procedure that was used to denervate the left superior colliculus and to enhance the growth of optic axons from the retinal transplants (7, 8, 11, 12). In the second group of young adult rats ($n = 20$) anaesthetised with ether, E14 retinas were injected into the left vitreous body of young adult rats. In some cases, the retinal transplants were prelabelled with the fluorescence vital dye DiI for distinguishing the graft from the host tissue. After recovery, the host animals were allowed to survive for 1–5 weeks before they were anaesthetised again and killed by perfusion. However, a large number of newborn recipients disappeared one or two days after transplantation presumably

eaten by apparently aggressive mother rats and only 38 animals with transplants survived. Among those host rats that survived, thirty animals had fetal retinas transplanted to their midbrains.

Immunocytochemistry

All animals were perfused with vascular rinse followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The eyes or brains with transplants were dissected out and post-fixed for 1 h before they were transferred to 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 30% sucrose at 4°C for overnight, before they were sectioned at 15 μm on a cryostat. The sections were then collected on poly-L-lysine coated slides, and processed for immunocytochemistry for PGP 9.5 (Ultraclone, diluted to 1:4,000). Control sections were incubated with primary antibodies replaced by normal rabbit or goat serum, or PBS. Retinal and brain sections were then reacted by avidin-biotin-complex and developed by the glucose oxidase nickel-diaminobenzidine enhancement method. No positive immunoreactivity was observed in control sections. In some cases, adjacent sections were stained with cresyl violet for revealing the cytoarchitecture of the retinal tissue and the brain.

RESULTS

Intracranial Transplants

As observed in previous studies (11, 12, 15), the transplanted retinas were frequently organised in the form of rosettes, reflecting the disruption during transplantation, though relatively well preserved sheets of retinal tissue were observed in some transplants. Three or five weeks after transplantation, the transplants had grown considerably with the largest measured 2.0 mm $\times$ 0.8 mm in size (Fig. 1A). Examples are shown in Fig. 1 which show retinal transplants 3 or 5 weeks after being grafted to the host brain. As shown in this figure, all retinal layers and major cellular populations have already been generated and the pattern is comparable to that in normal control retina at the same age. The main difference with regard to structural organisation is that the inner nuclear layer (INL) can be subdivided into two laminar zones (Fig. 1, B and C), a feature which seems to be characteristic of retinal transplant but not observed in the normal retina. In sections reacted for PGP 9.5, distinctly labelled cells similar to those observed in the normal developing retinas (4)

Fig. 1 Cresyl violet-stained intracranial retinal transplants showing well-laminated retinal grafts. (A) is a low power view showing a 3-week-old transplant (T) located on the surface of the host brain (H). (B) shows a portion of a 3-week-old transplant at a higher magnification. The three cellular and two plexiform layers are clearly identifiable at this stage. The arrowheads indicate the GCL. (C) shows a 5-week-old transplant. Note an increase in the thickness of the transplant and that the INL can be divided into two sublaminae. Scale bars: A, 40 μ m; B and C, 10 μ m

were clearly identifiable as described below.

One week after transplantation, a distinct population of moderately to darkly stained cells and numerous immunoreactive fascicles of fibers were

observed in presumptive ganglion cell layer (GCL) and nerve fiber layer at the age corresponding to normal E21 rat (Fig. 2A). A large number of lightly to moderately immunoreactive cells were

Fig. 2 Photomicrographs showing PGP 9.5 immunoreactivity in intracranial retinal transplants 1 to 5 weeks after grafting. (A) shows a portion of a retinal transplant 1 week after transplantation. Immunoreactive neurons are observed in the presumptive GCL (large arrowhead), and within the NBL. In addition, immunoreactive fascicles of fibers presumably in the nerve fiber layer (small arrowhead) are observed on the surface of the transplant. (B) Two weeks after transplantation, PGP 9.5 neurons are located in the INL and GCL which are separated by the lightly stained IPL. Immunoreactive cells resembling horizontal cells are observed in the outer part of the retinal transplant. Note the newly formed OPL indicated by the open triangle. (C) shows a P21 transplant in which the three cellular layers can be seen readily. Immunoreactive neurons are located mainly in the GCL, inner part of the INL (double asterisks), and horizontal cell layer. (D) shows a retinal transplant 5 weeks after grafting. Note that the thickness of the retinal tissue and the density of immunoreactive cells are reduced in this transplant, but the overall pattern of immunoreactive cells is similar to that in the normal retina. Magnification is the same for all photomicrographs. Scale bar, 50 μ m

observed in regions corresponding to the INL and outer part of the neuroblast layer (NBL), though these layers were not clearly recognisable in most regions at this stage.

Two weeks after transplantation, the age of the retinal transplants corresponding to P7 normal retinas, well-developed retinal grafts were observed in some cases. PGP 9.5 immunoreactive neurons appeared to be distributed mainly in the GCL, inner part of the INL and in the newly formed outer plexiform layer (OPL) where a single row of

stained cells with morphology similar to the horizontal cells was observed in the normal retina (Fig. 2B). The staining intensity in the inner plexiform layer (IPL), however, was less than that of normal developing retina, and no sign of sublamination was detected in this region.

Three weeks after transplantation, the transplants corresponding age at E14 had grown considerably as compared to those in earlier stages (Fig. 1, A and B). In certain regions, the three populations of cells described above were clearly identifiable,

though the number and staining intensity were generally lower than in normal retinas at corresponding age. Nevertheless, cells with moderate to high PGP 9.5 activity were clearly observed in many parts of the transplants. Some of the cells in the GCL had relatively large somata ($> 15 \mu\text{m}$), indicating that they are ganglion cells. In addition, immunoreactive fibers were frequently found within or on the surface of the transplants. The OPL was readily identified as indicated by the distinctly immunoreactive horizontal cells and their processes (Fig. 2C). PGP 9.5 immunoreactivity was also located in the IPL, and relatively distinct sublaminae were also observed in some regions.

Five weeks posttransplantation, the retinal grafts corresponding to a mature retina in most cases studied were still large in size. The retinal tissue was highly convoluted, but a well laminated pattern was clearly recognisable as confirmed by the characteristic histological features of the retina (Fig. 1C). However, the retinal tissue was generally thinner as compared to transplants observed 3 weeks after transplantation, and the number of PGP 9.5 immunoreactive cells appeared to be reduced substantially, particularly in the GCL. Nevertheless, a laminated pattern with immunoreactive cells distributed mainly in the horizontal cell layer, INL, and GCL was identifiable (Fig. 2D). Some large somata with high PGP 9.5 activity and immunoreactive fibers were observed in certain parts of the transplants, and above background immunoreactivity was still detected in the IPL.

Intravitreal Transplants

In all 15 rats included in this study, the histological features and immunostaining pattern of the host retinas on the side with retinal grafts appeared to be comparable to the normal control retinas, despite some obvious distortion of the tissue caused by the grafting procedure in some cases. By contrast, the retinal transplants were generally small in size and the histological features of the tissue appeared to be much disrupted. The immunostaining was relatively poor though in some cases, distinctly labelled cells organised in a laminar fashion were observed. For instance, Fig. 3A shows a retinal transplant 2 weeks after grafting to the host eye. The GCL was still identifiable, and many immunoreactive cells, some resembling horizontal cells were located in regions corresponding to the INL. By 4 weeks posttransplantation, however, all intravitreal transplants showed

sign of severe degeneration or regressive changes. Histological features characteristic to the normal retinal tissue were no longer recognisable, and the graft was invariably small. PGP 9.5 immunoreactivity in the remnant tissue was greatly reduced and no cellular populations in organised manner were observed. One of the transplants that had the best preserved morphology is shown in Fig. 3B.

DISCUSSIONS

In general, the results from this study indicate that retinal transplants can survive and continue to grow and differentiate when they are grafted to the host midbrain which was partially denervated by removal of optic input. By contrast, retinal transplants in the vitreous body of host eyes did not seem to develop properly, and in many cases, especially those with longer survival time appeared to undergo degeneration. These observations suggest that the eye where the retina is normally located is not a favourable environment for maintaining the retinal graft as compared to the midbrain that contains the superior colliculus which is the major subcortical visual target in the rat. The differences observed may simply reflect the amount of trophic substances available to the retinal grafts. In cases where retinal tissue was grafted to the host eye, the supply of trophic substances could be very limited because of the relatively closed environment of the eye. Nevertheless, the relatively well preserved retinal structures in 1–2 weeks intravitreal transplants, as reflected by PGP 9.5 immunostaining in the GCL and INL suggests this molecule is required in the retinal development for certain populations. Further, the fact that the transplants develop and differentiate in a foreign environment also indicates that the trophic substances provided by the host eye and the retinal transplant itself may help to maintain the survival of the transplanted tissue, and allows the cells to differentiate at least for period of time.

The results from the intracranial transplantation have shown that PGP 9.5 somata were first identified one week after graft, and the specific interneurons and plexiform layers became immunoreactive 2 and 3 weeks later respectively. The overall pattern of immunoreactivity and the time course of development or maturation process of PGP 9.5 neurons in the transplanted tissue is on the whole very similar to that of normal rat retina (3, 4). In principle, results from this study provides strong support of previous studies (15–17, 19) showing

Fig. 3 Photomicrographs showing PGP 9.5 immunoreactivity in intravitreal retinal transplants. (A) shows a transplant 2 weeks after grafting. Note that immunoreactive cells in an organised manner can still be recognised in the GCL and INL (double small asterisks). (B) shows a retinal graft 4 weeks after transplantation to the vitreous body. This transplant clearly shows sign of severe degeneration, and no organised pattern and retinal layers can be observed. Scale bar, 50 μ m

that neurons in retinal transplants can not only survive but also continue to grow and differentiate for a reasonably long period of time after they are grafted to the brain or region near to the normal visual targets. The expression of PGP 9.5 in specific populations of cells in the retinal transplant at the right time and right place, and in a manner similar to that in the normal developing retina, suggest that cells in the retinal transplant develop according to a genetically determined programme. The fact that the expression of PGP 9.5, as well as other specific molecules studied previously (7, 8, 11, 23), following a timetable determined by the age of the donor tissue, implies further that retinal cells in the transplants develop according to programmes which are intrinsic to the retinal tissue, and not determined by the host animals.

However, the site or environment where the transplants are grafted to the host animals seems to have an important role in determining the survival and differentiation of transplanted cells. For example, as mentioned above, retinal transplants do not grow well in the eye or far away from the target regions. This may simply be due to a lack of target tissue or trophic substances available in the intraocular environment. Similarly, the obvious reduction in cell density in the GCL in intracranial transplants can also be explained as a consequence of lack of trophic support from target structures because it is likely that not all transplants are connected properly with the denervated superior colliculus. This is in keeping with previous studies showing that the survival of retinal ganglion cells depends on the availability of target tissues (9, 10).

On the other hand, the fact that many ganglion cells in the GCL are immunoreactive for PGP 9.5 suggests that the metabolic activity of these cells is comparable to that in ganglion cells in the normal retina.

In addition to labelling ganglion cells and amacrine cells, PGP 9.5 is shown to be useful for labelling developing young and mature horizontal cells under normal or experimental conditions. The finding that PGP 9.5 is present early in the outer nuclear and plexiform layers before synapses are generated indicates this molecule can be a marker for studying the developmental sequence (4, 13) and local circuits in the retinal populations (18). Both the present and previous studies have demonstrated a close correlation between the formation of a horizontally organised network of processes of PGP 9.5-positive cells in the horizontal interneurons and synaptogenesis in the OPL, which occurs at the end of the first and the beginning of the second postnatal week (21). Together with the observation that PGP 9.5-positive reaction appears in the IPL before synaptogenesis begins in this region has also implicated a function role of the PGP 9.5 neurons and their processes in relation to development of connections in the retinal sublayers. However, the PGP 9.5 staining in the IPL of transplant appeared to be slightly lighter and the sublaminae became unclear compared to that in normal retina. This may reflect a down-regulation in PGP 9.5 activity in the IPL region, and a process that seems to be dependent of influence of the environment.

This work was supported in part by a grant to S. T. Chen studying in London from the National Science Council of Republic of China.

Received 25 October 1996; and accepted 16 December 1996

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