IDENTIFICATION OF CELLS FOR TRANSPLANTATION

Field of the Invention

This invention relates to the identification of cells suitable for transplantation into a vertebrate brain. More particularly, this invention relates to the identification of multipotent neural cells which are able to repair neural damage following transplantation into the brain.

Background to the Invention

There is an increasing awareness that damage to a vertebrate brain can be repaired using cell transplantation technology. Typically, the cell transplanted into a damaged brain will be a neural stem cell, e.g. a pluripotent neuroepithelial stem cell which is capable of differentiating into a cell with a neural cell phenotype.

For example, Sinden *et al.*, Neuroscience (1997) 81:599-608, discloses that conditionally-immortalised hippocampus neuroepithelial stem cells can be used to improve spatial learning after transplantation into the ischaemia-lesioned hippocampus. See also WO-A-97/10329.

However, it has been found that not all neural stem cells can be transplanted for successful repair of neural damage.

For example, while MHP36 cells (Sinden *et al.*, *supra*) do aid repair, an apparently similar cell line, MHP15, fails to repair. This difference emerges despite the fact that both MHP cell lines were generated from the same tissue source (namely hippocampus), and both are multipotent neural precursor cell lines, i.e. both cell lines have the capacity to generate a full complement of brain cell types, including neurons, astrocytes and oligodendrocytes.

Therefore, it would be beneficial if it were possible to identify at an early stage, the cells that were suitable for transplantation, or to have the ability to modify cell lines in order to achieve successful transplantation and repair.

Summary of the Invention

The present invention is based on the realisation that cells that are suitable for transplantation and repair may be identified on the basis of their gene expression profile.

The present invention employs, for example, a technique termed differential display (DD) to investigate the differences between repairing and non-repairing cell lines. Differential display is used to visualise the differences in gene expression between two or more cell lines, and it has been found that cell lines that repair have very similar profiles of gene expression, but very different profiles from those cells that do not repair. This represents a major, unexpected discovery because, apart from their capacity to repair, the cell lines are usually remarkably similar, in morphology, growth characteristics and growth factor responsivity.

According to one aspect of the present invention, a method for selecting a cell suitable for transplantation into a damaged vertebrate brain comprises:

(i) isolating cells that are, or are capable of differentiating into, a cell with a neural cell phenotype;

(ii) obtaining the gene expression profile of the cells;

(iii) comparing the expression profile of the cells with that from a control cell known to be suitable for transplantation; and

(iv) selecting those cells with a similar expression profile to that of the control.

In one embodiment of the invention, the control cell is from the MHP 36 cell line. Step (ii) may be carried out by differential display.

The present invention is not only useful for identifying suitable cells for transplantation, but may be used to identify genes or gene products that may be involved in determining whether or not a neural cell can repair, or not.

According to a further aspect of the present invention, a method for identifying a gene involved in determining whether or not a neural cell can aid repair on transplantation into a damaged brain, comprises:

(i) isolating cells that are, or are capable of differentiating into, a cell with a neural cell phenotype;

(ii) obtaining the gene expression profile of the cells;

(iii) comparing the expression profile of the cells with that from a control cell line known to be suitable for transplantation; and

(iv) isolating those genes that are the same (or different) from those expressed by the control.

Using this method, it has been possible to identify a number of genes that are expressed by repairing cell lines but not non-repairing cell lines (and vice versa).

According to a third aspect, a method for selecting a cell suitable for transplantation into a damaged brain, comprises determining the presence of any of the gene sequences identified herein as SEQ ID NOS. 1 to 5.

Description of the Invention

The present invention provides a convenient method for identifying cells that may not be used in transplantation and repair in a damaged vertebrate brain. Using differential display techniques, a cell line can be screened against a control, and a suitable cell line selected for further development.

Although other techniques are known for obtaining gene expression profiles, a differential display technique is preferred. In summary, the technique relies on isolating mRNA from a population of cells, and using this to determine the levels of expression of particular genes. This is most often achieved by using reverse transcription to obtain the copy DNA (cDNA), which is co-amplified using specific and semi-quantitative primer sequences. The results are then compared to a control to evaluate differences in gene expression.

Kits for carrying out differential display are available commercially.

The cells used in the method should be capable of differentiating into a cell with a neural cell phenotype, i.e. the differentiated cell should adopt either a neuron, astrocyte or oligodendrocyte phenotype. Preferably, the cells in the undifferentiated state are multi-potent, i.e. they have the capacity to develop into at least two of the neural cell phenotypes above. Multi-potent cells are known, and procedures for obtaining such cells will be apparent to the skilled person.

The differential display technique may be carried out on the cells in the differentiated or undifferentiated state. It is however preferred if the cells are maintained in the undifferentiated state during the technique. Conditions to maintain the cells in the undifferentiated state are known in the art, and include

culture methods making use of growth factors, and recombinant DNA techniques to insert, for example, oncogenes or conditionally-inducible oncogenes into the cells.

The present invention provides a convenient way to identify cells that may be used in transplantation and repair in a damaged vertebrate brain. Using the method of the invention, cell lines can be screened against a control, and a suitable cell line selected for further development.

Genes that are differentially expressed in non-repairing cell lines may be identified. Modification of the genes by, for example, site-directed mutagenesis, may by carried out to inactivate the genes to provide further cell lines that may be suitable for transplantation. For example, disruption of the Pax6 or 3R2C genes may be carried out to prepare cells for transplantation. Methods for modifying or inactivating the genes will be apparent to the skilled person.

Modified cell lines, or cell lines identified by the method of the invention as suitable for transplantation, may be used in conventional transplantation methods. For example, Sinden *et al*, *supra*, shows the preparation of stem cells for transplantation into the mouse brain.

The present invention encompasses the use of cells according to the invention, in the manufacture of a composition for the treatment of damage to the vertebrate brain. Suitable formulations for delivery of the cells to the brain will be apparent to the skilled person having regard to the nature of the cell for therapeutic use. Appropriate amounts of cells for therapeutic use, in addition to suitable excipients, diluents or carriers, will be apparent to the skilled person based on conventional formulation methods.

The following Example shows a differential display experiment to identify the gene expression profile of the cell lines MHP 36, MHP 15, MHP 3 and SVE 10.

Example

The cell lines were grown at 33°C, in standard MHP medium containing bFGF, in 175 cm^3 flasks. Cells were allowed to become 90% confluent before being transferred to 39°C, and cultured for a further seven days in the absence of interferon.

The cells were washed for five minutes with 20 ml of HBSS (Gibco BRL). The cells were lysed by adding 20 ml of Trizol (Life Technologies), and incubating at 37°C for ten minutes. The lysate was then transferred to a 50 ml Falcon tube (Stardts) and 5 ml of chloroform added. The tubes were mixed vigorously for one minute and the phases separated by centrifugation at 4,000 rpm for 15 minutes. The upper aqueous phase was transferred to a fresh 50 ml tube and 7 ml of isopropanol added. RNA was precipitated by placing the tubes at -20°C for 1 hour. The RNA was pelleted by centrifugation at 4,000 rpm for 20 minutes. The pellet was washed in 70% ethanol (made up in DEPC treated water), centrifuged as described above and allowed to air dry at room temperature for ten minutes. The pellet was then resuspended in 439 µl of DEPC-water and transferred to an RNase free Eppendorf tube.

To remove any contaminating DNA from the RNA sample, the following was added to the resuspended RNA solution; $MgCl_2$ to a final concentration of 5 mM; DDT to 100mM; RNase inhibitor, 500 units, and DNase I, 700 units. The tubes were then incubated at 37°C for one hour and the RNA purified by the addition of an equal volume of acid phenol/chloroform/isoamyl alcohol (125:24:1) with vortexing for one minute and centrifugation at 14,000 rpm (4°C) for 6 minutes. The upper aqueous phase was transferred to a fresh Eppendorf tube and the phenol/chloroform extraction repeated until the interface became clear. 8 M LiCl was added to the final aqueous layer to a final concentration of 2.5 M and the tubes placed overnight at -20°C.

The RNA was pelleted by centrifugation at 14,0000 rpm (4°C) for 15 minutes; the pellet was washed with 1 ml of 70% ethanol (DEPC) and allowed to air dry for five minutes. The RNA was then resuspended in 100 µl of DEPC water. The concentration and purity of RNA was determined by measuring the

absorbance at 260 nm and 280 nm. The RNA was then diluted to give aliquots at a concentration of 200 ng/µl which were stored at -70°C.

Differential Display Analysis

All 3' anchoring and 5' arbitrary primers were obtained from the Genomyx Hieroglyph mRNA profile kit. The differential display procedure can be divided into three steps: cDNA synthesis, PCR amplification and gel electrophoresis.

First strand cDNA synthesis was carried using the Qiagen Omniscript reverse transcriptase.

A 20 µl reaction using one of the 12 Hieroglyph oligo (dT) anchored 3' primers and 400 ng of total RNA was used to generate enough cDNA for duplicated differential display PCR (DD-PCR) with the same oligo (dT) primer in pairwise combination with all four Hieroglyph arbitrary 5' primers.

2 µl of the 200 ng/µl RNA solution was added to a 0.2 ml thin-walled PCR tube along with 2 µl of Hieroglyph T7 (dT12) anchored primer (AP) (2 µM). This was then heated at 70°C for five minutes before being placed on ice. To the denatured RNA/primer solution the following was added: 10xOmniscript reverse transcriptase (RT) buffer, 2 µl; 5 mM dNTP's, 2 µl; 0.1M DTT; 0.5 µl RNASEOUT RNase inhibitor (40µ/µl)(Gibco BRL); 1 µl Omniscript (1 unit/µl) and DEPC-water to 20µl. The reaction mixtures were then incubated at 42°C for 1 hour.

Differential Display PCR for each sample was then carried out in duplicate. DD-PCR reactions were performed using the Hieroglyph system (Genomyx), Clontech Taq polymerase mix, and [α-^{33}P]dATP (3,000 Ci/mmole, Amersham).

For each cDNA sub-population, a PCR core mix containing the appropriate reverse transcriptase (RT) mix and matching anchored primers (AP) prepared in a volume sufficient for the number of reactions needed. The core mix included all the DD-PCR components with the exception of the arbitrary primers to be used, which were aliquoted separately into the appropriate tubes.

Each individual DD-PCR tube contained the components shown in Table 1.

Table 1

DD-PCR component	[Stock]	1xreaction Vo.(20µl)	[Final]
Water	–	9.35 µl	–
PCR Buffer	10x	2 µl	1x
dNTP (1:1:1:1)	250 µM	2 µl	20 µM
5' ARP primer	2 µM	2 µl	0.2 µM
3' AP primer	2 µM	2 µl	0.2 µM
cDNA	–	2 µl	–
Taq	50x	0.4 µl	1x
[α-^{33}P]dATP	10 µCi/µl	0.25 µl	0.125 µCi/µl

Following DD-PCR, radiolabelled cDNA fragments were electrophoretically separated on a polyacrylamide gel under denaturing conditions. This involved using the Genomyx LR sequencer and LR-optimized HR-1000 polyacrylamide gel formulations. 4.5% and 6% HR-1000 gels were prepared according to manufacturers instructions; 4.5% gels were used to resolve fragments in the size range of 700 bp to 2 kb, while 6% gels were used to separate fragments in the size range of 100 bp to 600 bp.

7 µl of each DD-PCR sample was mixed with 4 µl of sample loading dye (Genomyx) and heated at 95°C for 3 minutes before being chilled on ice. 3 µl of this heat-denatured sample was then added to a gel lane. Duplicate reactions were loaded in adjacent lanes and samples generated with the same primer pairs in consecutive lanes. 6% gels were used for 6 hours at 2,500 V, 100 W, at 50°C; and 4.5% gels were used for 16 hours at 1,500 V, 100 W, at 50°C.

Following electrophoresis, the gel was dried on the glass plate in the Genomyx-LR sequencer according to the manufacturer's protocols. An autoradiograph of the gel was produced by placing a piece of BioMax MR (Kodak) ultra-high resolution film in contact with the gel, for 16 hours.

The autoradiograph showed bands corresponding to genes expressed in the non-repairing cells SVE 10 and MHP 15, but not expressed in the repairing cell lines MHP 36 and MHP 3. There were also bands corresponding to genes expressed in the repairing cell lines but not in the non-repairing cell lines.

Before excising bands from the gel, the autoradiograph was washed in 90% ethanol and allowed to dry. The autoradiograph was aligned on top of the gel with the aid of autorad markers (Stratgene). The autoradiograph was secured along one long edge with tape. Differentially expressed transcripts (DET) were identified and the bands excised according to the Genomyx protocol. The excised bands were placed into 100 µl of elution buffer (EB, 10 mM Tris:HCl, pH 7.5) and the DNA allowed to elute at room temperature for six hours. The eluted DNA was stored at -20°C.

Single Strand Conformation Polymorphism (SSCP) gel analysis was used to eliminate false positives that may arise due to the co-migration of fragments of identical size but different sequences. This procedure involved a limited reamplification of the isolated fragments (SSCP-PCR) followed by the separation of the products of this amplification on a SSCP gel. PCR conditions were similar to those used for DD-PCR, with the exception that the number of cycles were reduced to 2 at the low annealing temperature (50°C), and to 10 cycles at the high annealing temperature (60°C). 4 µl of the SSCP-PCR reaction was added to 10 µl of SSCP-loading buffer (80% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol, 1 mM EDTA, 10 mM NaOH) and denatured at 95°C for 10 minutes before being loaded onto an agarose gel. Samples were electrophoresed for 16 hours at 8 W in 0.6x tris borate EDTA buffer (TBE) on the Genomyx-LR sequencer. Following autoradiography, areas of the gel corresponding to candidate differentially expressed transcripts were excised and placed into 100 µl of elution buffer (EB).

Sequencing and Identification of DETs

The recovered differentially expressed cDNA was reamplified by PCR to provide significant template material to allow for direct sequencing. The PCR reactions contained the components shown in Table 2.

Table 2

PCR component	[Stock]	1xreaction Vo. (100 µl)	[Final]
Water	--	49 µl	--
PCR Buffer	10x	10 µl	1x
dNTP (1:1:1:1)	250 µM	10 µl	20 µM
5' ARP primer	2 µM	10 µl	0.2 µM
3' AP primer	2 µM	10 µl	0.2 µM
SSCP-cDNA	--	10 µl	--
Taq	50x	1 µl	1x

The PCR products were then purified using PCR purification kit (Qiagen), according to the manufacture's protocols. The purified products were eluted in 60 µl of elution buffer. Purified PCR products were sequenced using the Thermosequenase radiolabelled terminator cycle sequencing kit (Amersham). The reactions were performed using the M13 reverse primer (-48) (Genomyx) according to manufacturer's protocols. Sequencing products were loaded on to a 6% Long Ranger (FMC), 8 M Urea, 1xGTB (glycerol-tolerant buffer) gel and electrophoresed for four hours at 2,500 V, 100 W, 50°C in 1xGTB. Following autoradiography, the sequencing ladders were read manually and the resulting sequence data was used to search the GenBank databases (both published and EST).

Table 3 shows the gene expression profile of the cell lines used in the experiment. MHP 36 and MHP 3 are cells that are suitable for transplantation into a damaged brain. It is clear that the expression profile in MHP36 and MHP3 is similar. Likewise, the profile in MHP15 and SVE10 is similar. However the profiles of MHP36/3 and MHP15/SVE10 are markedly different. This illustrates the difference in function of the two sets of cells as MHP15 and SVE10 cells do not have the ability to repair.

Table 3

	Cell Line				Homology	
	MHP 3	MHP 15	MHP 36	SVE 10 Clone23	Gene	%
DD-Band						
SEQ ID No. 1 A	+++++	-	+++++	-	Human EST	88% over 68bp
SEQ ID No. 2 B	+++++	-	+++++	-	Glogin-245	100%
SEQ ID No. 3 C	+++++	-	+++++	-	Mouse EST	95% over 175bp
SEQ ID No. 4 D	+++++	-	+++++	-	Mouse EST	99% over 438bp
SEQ ID No. 5 E	+++++	-	+++++	-	Heavy Chain immunoglob'	94% over 108bp
SEQ ID No. 6 F	-	+++++	-	+++++	Mouse EST	91% over 84bp
SEQ ID No. 7 G	-	+++++	-	+++++	Neuroprotective Protein, Adnp	100%

Analysis of the expression products revealed that the non-repairing MHP15 cells expressed a gene termed Pax6 (Gotz *et al*, Neuron (1998), 21;1031-1044), while cells from MHP36 did not express this gene.

Therefore, neural cells that express the Pax6 gene are not expected to be able to repair brain damage, while neural cell lines that do not express this gene are able to aid repair.

Pax6 is believed to be a positional specification gene, playing a role in determining the position an embryonic cell is fated to adopt in the developing brain (Fernandez *et al*, Development (1998) 125: 2099-2111).

Further analysis revealed that the non-repairing, non-multipotential cells expressed the gene 3R2C (GeneBank Accession No. D25216). The MHP 36 cells did not express this gene when cultured under permissive conditions.

CLAIMS

1. A method for selecting a cell suitable for transplantation into a damaged vertebrate brain, comprising:

(i) selecting cells that are, or are capable of differentiating into, a cell with a neural cell phenotype;

(ii) obtaining the gene expression profile of the cells;

(iii) comparing the expression profile of the cells with that from a control cell known to be suitable for transplantation; and

(iv) selecting those cells with similar gene expression profiles to that of the control.

2. A method for identifying a gene, the expression of which can determine whether or not a neural cell can repair a damaged brain, comprising:

(i) selecting cells that are, or are capable of differentiating into, a cell with a neural cell phenotype;

(ii) obtaining the gene expression profile of the cells;

(iii) comparing the expression profile of the cells with that from a control cell known to be suitable for transplantation; and

(iv) isolating those genes that are the same (or different) from those expressed by the control.

3. A method according to claim 1 or claim 2, wherein the control cell does not express the gene Pax6 or 3R2C.

5. A method according to any preceding claim, wherein step (ii) is carried out by differential display.

4. A method according to any preceding claim, wherein the control cell is MHP36.

6. A method for selecting a call suitable for transplantation into a damaged brain, which comprises determining the presence of any of the gene sequences identified herein as SEQ ID NOS. 1 to 5.

SEQUENCE LISTING

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