Running title: Adult human retinal progenitor cells.

The Effect of Postmortem Time, Donor Age and Sex on the Generation of Neurospheres from Adult Human Retina.

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Abstract

Background / aim: Post-mortem adult human retina contains pluripotent progenitor cells capable of forming neurospheres with different retinal cell types. We address whether this is the case at all ages and at different post-mortem times.

Methods: Adult human post-mortem retina-derived cell suspensions generated neurospheres in fibroblast growth factor 2 (FGF2) and N2 supplement. The yield of neurospheres from limited dilution or single cell cultures is very low so we studied cells generated per $10^5$ viable cells from a cell suspension derived from whole retina. Retinal tissue from donors aged 18 -91 at various post-mortem times (between 23-44 hours) was studied in the context of generation rate and time for neurospheres.

Results: The potential to generate neurospheres from adult human retina remains throughout life. Neurosphere cellular components were not affected by donor age or post-mortem time (they contained nestin$^+$, GFAP$^+$ and Neurofilament$^+$ cells). On average of 34.36 neurospheres were generated per $10^5$ viable cells. After a few days in culture neurospheres begin to form. The time for this to occur was independent of donor age but prolonged at longer post-mortem times. No significant effect of donor sex was found.

Conclusion: Neurosphere-forming retinal progenitor cells are found in adult human retina throughout life. This cell population are a potential target for therapeutic intervention to influence repair and regeneration of the retina.
Introduction

Recovery of function in the central nervous system (retina, brain, and spinal cord) is an increasingly accepted phenomenon. It is likely that an inherent regenerative capacity is present throughout life. This may occur through a number of mechanisms including cellular replacement, synaptic plasticity and architectural tissue reconformation. The recognised potential of progenitor cells to generate new cells supports this concept. Neural progenitor cells are present in the prenatal, developing and postnatal human retina.[1-3] Retinal neural progenitors are present in the adult human retina in reduced numbers compared to developing tissue.[4-7] Retinal neural progenitor cells generate neurospheres and differentiate into several cell types including glia, neurons and photoreceptors.[1-6] The presence of these cells is potentially important for tissue repair as the retina has the framework to sustain the migration of these cells.

Cell suspensions from adult human post-mortem retina generate neurospheres from cells capable of division and differentiation.[6] This work not only demonstrated progenitor cells but also could be derived from post-mortem tissue and passaged in vitro (unpublished data,[6]). We describe the potential to generate neurospheres from donors of different age and sex, as well as the effect of post-mortem time.

Methods

Cell preparation and culture

Donor tissue from the National Corneal Transplant Service (NCTS) Eyebank (University of Bristol, Bristol Eye Hospital) was obtained with research consent and ethical approval (Central and South Bristol Research and Ethics project number E5866). Eyes were obtained from donors without known concomitant ocular disease. The lens was removed with the iris; the ciliary body and pars plana were dissected, followed by removal of the vitreous. As the vitreous was removed from the eyecup, in its entirety, the often detached retina could be stripped from the hyaloid face and remained whole within the eyecup attached to the optic nerve. The whole retina was removed en bloc by dissecting it from the optic nerve head. Retinal pigment epithelium, choroid and ciliary body remained within the eyecup. This permits selective dissection of the whole retina. The retina was placed in a petridish to expose all areas and rinsed with DMEM (GIBCO) to remove any retinal pigment epithelial cells that may have remained attached. Cell suspensions were prepared from whole neural retina following enzymatic digestion at 35°C (with trypsin, DNAse and collagenase, SIGMA, UK) and mechanical disruption (trituration with 19, 21 & 23g needles). Cell suspensions were washed and passed through a 40µm sieve to remove debris. Cells were suspended in DMEM:HAMS F12 (GIBCO, UK) at a density of 2 x 10^6 cells/10ml. Culture medium was supplemented with FGF-2 (SIGMA, UK) at 20 ng ml⁻¹ and 1% neural supplement (N2 supplement, GIBCO, UK). Cell suspensions were cultured for 1 month and neurosphere generation was quantified. Neurospheres were confirmed by noting retinal lineage phenotype, as previously described.[6] Cell suspensions generated from 50 pairs (25 male and 25 female) donors were compared to generate the data presented. Donor age was between 18 – 91 years with up to 48 hour post-mortem times. Neurospheres were counted in culture using phase-contrast microscopy. Cellular lineage was confirmed with immuno-cytochemistry.[6] Neurospheres were counted in suspension at various time points in vitro. The number of neurospheres per 10^5 vital cells was used as an index of the ability of a cell...
suspension to generate neurospheres (NSph / $10^5$ cells). The time for neurospheres to begin to form was also recorded.

**Statistical Analysis**

Data were analysed with Spearman Correlations, ANOVA and Mann-Whitney tests, using StatView® from the SAS Institute Inc (USA).

**Results**

The generation of neurospheres from adult human retina is a consistent phenomenon, working with post-mortem tissue generates some variability. The rate at which this occurs is recognised to be low.[2] These data attempt to identify factors contributing to this variability in rates of neurosphere generation.

**Neurospheres generated from post-mortem adult human retina.**

Retinal cell suspensions were cultured in the presence of FGF2 and N2 supplement for 1 month. Neurospheres were identified under phase-contrast as free floating balls of cells.[6] Neurospheres contained: (i) nestin$^+$ cells (ii) cells of various retinal lineages including glial, neuronal and photoreceptors.[6] The neurospheres increased in size and number *in vitro* with time. In addition to cellular phenotypes previously demonstrated within spheres, BrdU incorporation and increasing cell counts showed cell numbers were increasing.[6] Retinal cellular phenotypes were confirmed in these cultures.

**Effect of donor age on neurosphere-generating ability of retinal cell suspensions**

Our investigations have studied retina from donors aged 18 to 91 years. Retinal cell suspensions of all donor ages generated neurospheres,[6] at an average of 34.36 neurospheres per $10^5$ viable cells. With increasing donor age, the number of neurospheres generated per $10^5$ viable cells, increased slightly, this correlation was found to be statistically significant (Spearman Rho = 0.334; $P = 0.0193$) (fig 1A). The time to generate neurospheres *in vitro* showed no statistically significant correlation with donor age (Spearman Rho = 0.114; $P = 0.4263$, non significant (NS), fig 1B). Thus neurospheres formed at similar time intervals *in vitro* regardless of donor age.

**Effect of post-mortem time on neurosphere generation.**

Several non-human mammalian studies have found that neurospheres can be generated from the CNS with prolonged post mortem times.[8-10] This is possible with adult human retina. Statistical analysis of post-mortem time versus the number of neurospheres generated per $10^5$ viable cells (fig 2A) found no statistically significant correlation (Spearman Rho = -0.24; $P = 0.8679$, ns). Thus although there was a slight decrease in the generation of neurospheres from tissue with longer post-mortem times, this failed to reach statistical significance up to 48 hours post-mortem. There were few post-mortem times below 24 hours, reflecting the time to arrange consent, transport and screening of human donor tissue. There was a statistically significant (Spearman Rho = 0.597; $P < 0.0001$,) increase in the time to generate neurospheres *in vitro* with increasing post-mortem time (fig 2B). Neurospheres formed significantly more slowly *in vitro* from tissue with longer post-mortem times.

**The effect of donor sex on neurosphere generation.**

Analysis of variance found no statistically significant difference between the number of neurospheres generated per $10^5$ viable cells between male and female donors ($F_{1,48}$...
In this data set, the mean time to generate neurospheres was shorter for males (mean = 12.5, SEM = 2.072, range 7 – 60 days) than females (mean = 28.2, SEM = 3.844, range 7 – 60 days) and this difference was statistically significant (ANOVA F_{1,48} = 12.956; P = 0.008, significant). However, Mann-Whitney testing found that the mean post-mortem time for male donors (21.42 hours) was significantly different from that for female donors (29.58 hours) (U = 210.5, Z-value = -1.979, P = 0.048). Given the above described effect of post-mortem time on the time for neurospheres to form in vitro, the effect of donor sex on the time to generate neurospheres can be attributed to this confounding variable in the data (an unequal distribution of post-mortem times between male and female donors).

Discussion

Since the identification of progenitor cells within the adult mammalian eye [4-7] there has been much interest in identifying and characterising progenitor cells within developing and adult human retina.[1-7] We have documented the presence of progenitor cells within post-mortem adult human retina and also the characteristic ability of these cells to generate neurospheres that contain retinal lineage specific cells.[6, 7] The question then arose as to whether this potential remains throughout life. The data presented highlight that adult human post-mortem retina contains cells able to generate neurospheres irrespective of donor age or post-mortem time (up to 48 hours). Neurospheres were generated from donor retinal cell suspensions irrespective of donor age (18-91 years). Donor age did not significantly affect the time (days) to generate an average of 34.36 neurospheres per 10^5 viable cells of whole retina.

Several non-human studies have shown that neurospheres can be generated from the CNS with prolonged post-mortem times.[8-10] In rodents, post-mortem time did not influence neurosphere generation from CNS progenitor cells, when the CNS tissue was stored at room temperature for up to 30 hours or at 4°C for up to 48 hours. Progenitor cell phenotype and ability to differentiate was similar to cells isolated directly from healthy tissue. The data presented from post-mortem human retina corroborate this, even at prolonged post-mortem times, neurospheres can be passaged and differentiated cells can be generated.[6]

For stem cells isolated from peripheral blood, yields of viable cells are greater from male than female donors[11]; our data find that the number of neurospheres generated from donor retina was not sex-dependent. Our finding that neurospheres from male donors formed more rapidly was associated with a skewed distribution of post-mortem times between the male and female donors. This effect of donor sex is attributed to differences in post-mortem time (confirmed as a significant determining factor in neurosphere generation).

The adult human retina generates neurospheres from single cell cultures at an extremely low rate,[2] comparable with the rates which we describe.[6] As a result, it is necessary to culture large numbers of cells in order to study neurosphere generation. We therefore elected to study whole retina, to reduce sampling bias and increase our yield of neurospheres rather than carry out limited dilution experiments. Our major interest was whether neurospheres could be generated and at what rate per 10^5 cells. To justify limited dilution experiments and single cell cultures, enriched and isolated progenitor populations are needed. When possible these experimental strategies will represent a viable experimental approach for analysing growth factor and transcriptional control of progenitor cell fate in this cell population. Understanding and facilitating progenitor cell differentiation will open possibilities of optimising tissue repair and regeneration. It is reasonable to anticipate that, in order to
function in their natural microenvironment progenitor cells must respond to signals, which guide their proliferation, differentiation and integration.[3, 12-14]
Cells that generate neurospheres remain throughout life in the human retina. We have qualitative data to support retinal specific phenotypes in the spheres, but have not quantified differences in differentiation capacity that may exist at different ages or with extended post-mortem times.
The presence of viable retinal progenitor cells throughout life in the human retina and their potential to be cultured from tissue after extended post mortem times is important. These cells are potential targets for therapeutic manipulation, to direct their behaviour in situ or developing transplantation (experimental or therapeutic) paradigms.

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Figure Legends

**Figure 1. Donor age and ability to generate neurospheres.**
(A) With increasing donor age there was a slight increase in the number of neurospheres generated per $10^5$ viable cells (Spearman Rho = 0.334; P = 0.0193). (B) donor age had no effect on the time to generate neurospheres (Spearman Rho = 0.114; P = 0.4263, ns).

**Figure 2. Post mortem time and neurosphere generation.**
(A) There was no significant correlation between post-mortem time and the number of neurospheres generated per $10^5$ viable cells (Spearman Rho = -0.24; P = 0.8679, ns). (B) Extended postmortem time retarded neurospheres generation (statistically significant correlation Rho = 0.597; P < 0.0001, highly significant).
References.
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