An investigation into the effect of hyaluronic acid (HLA) polymer size on neuronal and glial cell viability and attachment

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Introduction

HLA is a human endogenous biopolysaccharide discovered by Karl Meyer in 1938, in the vitreous humour of bovine eyes and is a key component of the extracellular matrix (ECM) found in every cell type in the body. It is naturally synthesized by a class of integral membrane proteins called hyaluronan synthases, and degraded by a family of enzymes called hyaluronidases.

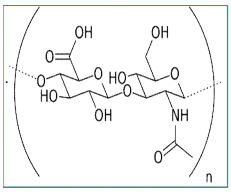

Figure 1.1 Monomer of HLA

Since HLA was first demonstrated to enhance peripheral nerve repair (PNR) *in vivo* over 21 years ago, there has been an increasing focus on the use of HLA in the development of nerve conduits for PNR (**1**). Despite the dynamic range of polymer lengths reported to exist *in vivo* (human blood serum ~100-300kDa vs. human synovial fluid 6000-7000kDa(**2**)), the majority of studies to date have experimented with high molecular weight (HMW) HLA, such as that incorporated into a biphasic nerve guidance conduit (NGC) recently developed by Stejskalova, A. et al (**3**), regardless of the high production costs incurred during commercial product manufacture.

Aims & Objectives

The purpose of this study was to evaluate if low molecular weight (LMW) HLA could positively effect neuronal and glial cell viability and attachment, with a view to the future design of a less expensive neuronal conduit for nerve repair.

Methods

1. Cell models

Two continuous cell lines were used in this study, a glial cell line to represent the Schwann cells found in the PNS and a human neuronal cell line.

Glial cell line:
The RT4-D6P2T cell line is an immortalized Schwann cell line derived from an N-ethyl-N-nitrosourea (ENU) induced rat peripheral neurotumour (**4**)

Neuronal cell line: SH-SY5Y are an immortalized human chatecholaminergic cell line routinely employed as a neuronal model *in vitro* and were used undifferentiated for this study (**5,6**)

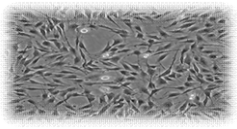

Figure 2.1 RT4 D6P2T cells (4)

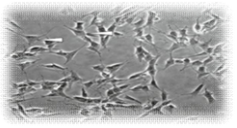

Figure 2.2 SH-SY5Y cells (5)

2. Cytotoxicity testing of LMW HLA

- Cell proliferation in the presence and absence of HLA were detected via UV spectrophotometry, using the viability dyes, MTT and Alamar blue. 24 well plates were coated in HLA-Poly-D-lysine (PDL) overnight followed by a 2 hour dry step before seeding cells at a density of 0.5 x10^6 cells/ml

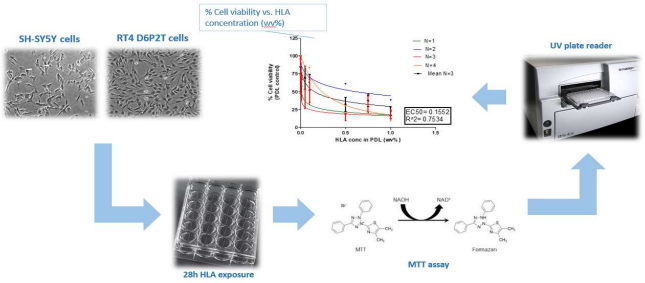

Figure 2.3 Flow diagram of cytotoxicity methodology

3. SH-SY5Y cell attachment assay

- The aim of the cell attachment assay was to evaluate the viability of detached cells following 28 hour HLA exposure
- SH-SY5Y cells were counted in media aliquots throughout various wash steps of the procedure
- Remaining cells containing formazan were assayed via the MTT assay
- All statistical analysis was performed using one way ANOVA in GraphPad Prism version 5.03

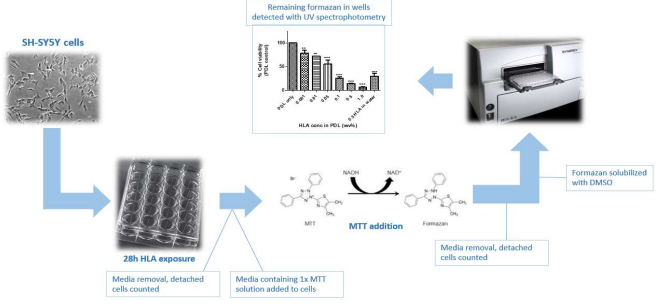

Figure 2.4 Flow diagram of SH-SY5Y cell attachment assay method

Cytotoxicity testing of LMW HLA

LMW HLA (8-50kDa and 30-50kDa) reduces the proliferation of SH-SY5Y cells

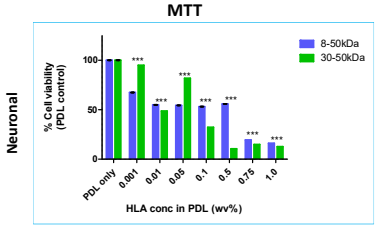

Figure 3.1 The effects of 8-50kDa HLA vs. 30-50kDa HLA in SH-SY5Y cells. Data shown is the mean +/- SEM of 2 independent experiments conducted in triplicate. ***P<0.001 when comparing 30-50kDa to 8-50kDa data using a 2-way ANOVA with Bonferroni post test

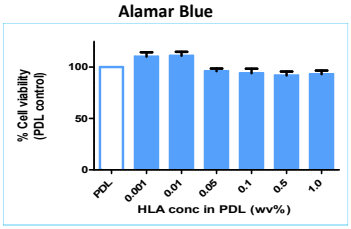

Figure 3.2 30-50kDa HLA has no effect on cell proliferation or viability when assayed using Alamar blue. Data shown is the mean +/- SEM of 2 independent experiments conducted in triplicate

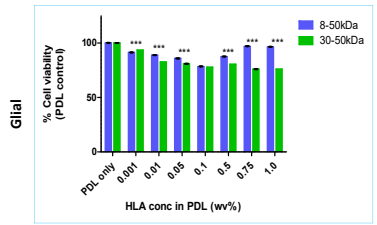

Figure 3.3 30-50kDa has a significantly greater effect than 8-50kDa HLA in RT4 D6P2T cells. Data shown is the mean +/- SEM of 3 independent experiments conducted in triplicate. ***P<0.001 when comparing 30-50kDa to 8-50kDa using a 2-way ANOVA with Bonferroni post test

LMW HLA significantly reduced SH-SY5Y cell viability and attachment when assayed using MTT (figure 3.1/3.2) but not when assayed with Alamar blue.

30-50kDa HLA had a significantly greater effect than 8-50kDa on cell viability and attachment in RT4 D6P2T cells (Figure 3.3)

SH-SY5Y cell attachment assay

LMW HLA (30-50 kDa) reduces SH-SY5Y cellular attachment but not cell viability

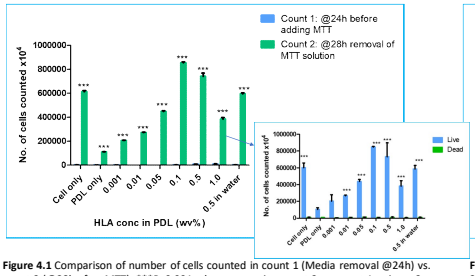

Figure 4.1 Comparison of number of cells counted in count 1 (Media removal @24h) vs. count 2 (@28h after MTT). ***P<0.001 when comparing count 2 vs. count 1 using a 2-way ANOVA with Bonferroni post test

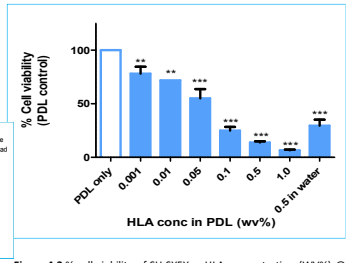

Figure 4.2 % cell viability of SH-SY5Y vs HLA concentration (WV%) @ 28h analysed using MTT assay. . ***p<0.001 when compared against PDL only control using one-way ANOVA with Newman-Keuls post test

Data shown are the mean +/- SEM of two independent experiments, conducted in triplicate.
The cell loss observed in Figure 4.2 correlates with the trypan blue counting assay data shown in Figure 4.1.
LMW HLA significantly reduces cellular attachment in SH-SY5Y cells in a dose-dependant manner.

Discussion

Due to the prohibitive cost associated with the isolation and purification of HMW HLA directly from animal sources, the present study sought to evaluate the effect of LMW HLA on neuronal and glial cell lines as an alternative substrate for the following reasons:

- Recombinant human LMW HLA can be easily purified from bacterial sources due to its smaller chain size
- LMW HLA exhibits less viscosity than HMW, allowing easier cell culture based assays
- LMW HLA costs significantly less to produce at large-scale (**7**)

Although no cytotoxicity was observed in the glial cells, LMW HLA had a significant effect on the cellular attachment/adherence of SH-SY5Y cells. This effect was more pronounced when using the 30-50kDa HLA vs. 8-50kDa HLA at higher concentrations only.

Conclusions and Future Work

This study has highlighted previously undocumented effects of LMW HLA on neuronal cell attachment, although previous literature has indicated the inflammatory nature of LMW HLA in both animal and cell models (**8**). Future experiments will examine the suitability of increasing sizes of LMW - HMW HLA (100kDa to 1MDa) for incorporation into a nerve guidance conduit design for peripheral nerve repair.

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References

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