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An antibody surface for selective neuronal cell attachment

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ABSTRACT

An optimal surface for culturing human embryonic stem cell (hESC)-derived neuronal cells is of high interest. In this study, a specific antibody to a neural cell adhesion molecule (NCAM) was immobilised on a solid surface of polystyrene and used as a selective matrix for culturing of hESC-derived neuronal cells. Thereafter, hESC-derived neurospheres were seeded on the matrix. The neurospheres did not attach to the NCAM antibody containing matrix whereas individual neuronal cells did. The neuronal cell attachment was depended on the NCAM antibody concentration. The neuronal cells were viable on the NCAM antibody containing matrix during an 8 day follow-up and exhibited typical bipolar morphology of immature neurons. Specific binding of the NCAM antigen to an immunoglobulin-polymer coated surface was verified by surface plasmon resonance (SPR) measurements. This study is to our knowledge the first demonstrating the use of an antibody layer as a selective surface for hESC-derived neuronal cells.

Keywords: antibody, neural cell adhesion molecule, neuronal cells, stem cells, surface plasmon resonance

1. Introduction

The growth and maturation of human embryonic stem cell (hESC)-derived neuronal cells requires a supporting matrix to which cells can adhere. The most used matrixes for this purpose are extracellular proteins like laminin, collagen, or fibronectin (Flanagan et al., 2006; Whittemore et al., 1999; Rappa et al., 2004). HESCs are pluripotent cells typically derived from poor quality embryos donated by couples undergoing *in vitro* fertilization treatments. In theory, hESCs can be differentiated into all cell types of the human body including neuronal cells (Skottman et al., 2007). Indeed, hESCs have been differentiated into neural precursor cells and further into different neuronal subtypes and glial cells (Guillaume and Zhang, 2008). Since current differentiation protocols produce not entirely homogenous populations containing both neural and non-neural cells, a selective surface supporting only neural cell attachment, growth and maturation is highly desirable.

Examples of more selective growth matrices for cells can be found: oriented surfaces of an epidermal growth factor have been used with rat fetal neural stem cells (Nakaji-Hirabayashi et al., 2007) and specific terminal peptide sequences of laminin (pentamer IKVAV) or fibronectin (tetramer RGDS) have also been utilized in recent publications in culturing of among others human umbilical vein endothelial cells (Jung et al., 2009). Antibodies for defined cell-surface targets would offer an even more selective attachment and growth surface. The neural cell adhesion molecule (NCAM) is a binding glycoprotein expressed on the surface of neurons, glia, skeletal muscle, and natural killer cells. NCAM has been implicated in having a role in cell-cell adhesion and neurite outgrowth (Ditlevsen et al., 2008). Thus, an NCAM specific antibody might be used as a supportive and selective matrix for binding of neuronal cells.

Our aim in this study has been to clarify if surface immobilised antibodies to a defined target on the cell surface can be used to alleviate cell attachment. We have previously immobilised antibody Fab´-fragments site-directly onto gold through the free thiol groups and included hydrophilic polymers in between the proteins to hinder non-specific binding (Vikholm-Lundin and Albers, 2006; Vikholm-Lundin et al., 2007). The role of the polymer on the surface is to provide a hydrophilic surrounding for the antibodies and to preserve the native-like water-surrounded environment. First, we studied the interaction of NCAM antigen with binary monolayers composed of anti-NCAM antibodies physisorbed on gold and post-treated with a non-ionic hydrophilic polymer *N*-[tris(hydroxymethyl)methyl]-acrylamide (pTHMMAA). Secondly, neuronal cells derived from hESCs were allowed to attach on layers composed of only the polymer, or anti-NCAM antibodies and the polymer on polystyrene, which is the surface normally used for culturing of cells. To our knowledge this is the first study demonstrating the use of a selective antibody surface for attachment of hESC-derived neuronal cells and could be a very useful technique also for other researchers working in this field.

2. Materials and methods

2.1. Materials for surface construction and SPR measurements

The anti-NCAM antibodies developed by P.W. Andrews (Andrews et al., 1990) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, IA). The NCAM antigen was purchased from Abcam (Cambridge, UK) and was specified as "Recombinant fragment, corresponding to amino acids 20-220 of Human NCAM", which covers the first two extracellular N-terminal Ig-like domains of the protein. Buffers used were 10 mM HEPES-buffer containing 150 mM NaCl, pH 6.8 and phosphate-buffered saline (PBS) composed of 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.5. HEPES (N-[2-hydroxyethyl] piperazine-N´-[2-ethanesulfonic acid]) (minimum 99,5%) was purchased from Sigma-Aldrich (Steinheim, Germany), Na_2HPO_4 was

purchased from Merck, NaH_2PO_4 and NaCl from J. T. Baker. The polymer of *N*-[tris(hydroxymethyl)methyl]-acrylamide (pTHMMAA) (Fig. 1A) was prepared as previously described (Vikholm-Lundin et al., 2007). Polystyrene well plates were purchased from Nunc, Thermo Fisher Scientific, Rochester, NY. Hydrogen-peroxide (30%) was bought from Merck KGaA and ammonium hydroxide (28-30% NH3) and bovine serum albumin (BSA, minimum 98% purity) from Sigma-Aldrich.

2.2. Antibody-polymer layers on gold for SPR measurements

Studies on binary monolayer formation of antibodies and polymer on gold were carried out on gold *in situ* with surface plasmon resonance (SPR) (Biacore 3000, GE Healthcare). Thin glass slides were coated with a 50 nm thin gold layer in-house by RF magnetron sputtering. The gold surfaces were always cleaned in a boiling solution of hydrogen-peroxide –ammonia in water (1:1:5) and rinsed with water prior to surface assembling. Instantly after the cleaning step, the slides were mounted in a plastic chip cassette by double-sided tape and inserted into the Biacore 3000 SPR instrument. First, the antibody was allowed to physisorb on the pre-cleaned gold surface for 15 minutes, followed typically by 10 min wash with PBS buffer. Next, the pTHMMAA polymer at a concentration of 200 μg/mL was post-adsorbed on the surface. Non-specific binding was measured by running BSA at a concentration of 500 μg/mL on the surface. The NCAM antigen binding was measured in HEPES-buffer by injecting increasing concentrations of antigen (0.0001-10 μg/mL) over the surface and rinsing with HEPES-buffer.

2.3. Human embryonic stem cells and neural differentiation

The hESC lines used were Regea 06/040 or 08/023, derived and characterized at Regea - Institute for Regenerative Medicine, University of Tampere, Finland (Skottman, in press; European Human Embryonic Stem Cell Registry, www.hescreg.eu). Regea has the approval from the Ethics

Committee of the Pirkanmaa Hospital district in Finland to derivate, culture, and differentiate new hESC lines from surplus embryos after obtaining signed informed consent from donating couples undergoing *in vitro* fertilization treatment. Briefly, hESCs were cultured in an undifferentiated stage in Knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % Knockout serum replacement, 2 mM GlutaMax, 0.1 mM 2-mercaptoethanol (all from Gibco Invitrogen, Carlsbad, CA), 1 % non-essential amino acids (Cambrex Bio Science, East Rutherford, NJ), 50 U/mL penicillin/streptomycin (Lonza Group Ltd., Switzerland), and 8 ng/mL basic fibroblast growth factor (bFGF R&D Systems, Minneapolis, MN) on top of a human feeder cell layer (CRL-2429, ATCC, Manassas, CA). The undifferentiated stage of hESCs was assessed daily by morphologic analysis and periodic immunostaining for hESC-markers Nanog, OCT-3/4, SSEA-4, and Tra-1-60. In addition, karyotyping was performed and indicated that the hESC lines maintained normal karyotype. All cultures were tested mycoplasma-free.

The differentiation protocol (Hicks et al., 2009) was further developed from Nat et al. (2007). For neural differentiation the hESC colonies were manually dissected into small clusters containing ~3 000 cells which were transferred into 6-well ultra low attachment plates (Nunc), and cultured as floating aggregates, hereafter called neurospheres, for 6 weeks prior to plating on the NCAM antibody matrix. The neural differentiation medium consisted of 1:1 DMEM/F12: Neurobasal media supplemented with 2mM GlutaMax, 1×B27, 1×N2 (all from Gibco Invitrogen), 25 U/mL penicillin and streptomycin (Lonza Group Ltd.) and 20 ng/mL bFGF (R&D Systems). The neurospheres were manually dissected into neural aggregates approximately 300 μm in diameter when they were seeded on the NCAM antibody pTHMMAA polymer matrix. At the time of seeding and for the follow-up period bFGF was withdrawn from the medium to further induce the neuronal differentiation of neural aggregate cells.

2.4. Construction of antibody-polymer layers onto polystyrene for cell attachment

Anti-NCAM antibodies were allowed to physisorb on polystyrene by applying concentrations of 0, 25, 50, 75, or 100 μg/mL onto the well plates for 15 minutes. The wells were rinsed with PBS buffer and post-treated with the pTHMMAA polymer (200 μg/mL) for an additional time of 15 minutes. The wells were thereafter rinsed again with PBS buffer. An improvement of the non-fouling properties of binary monolayers composed of antibodies and polymer have previously been noticed if the layers are allowed to stand for a few days (Vikholm-Lundin and Albers, 2006). Thus, the treated well plates were let to stabilize in the buffer for 2 days at +4°C before cell seeding. Next, the wells were rinsed once with neural differentiation medium without bFGF after which 800 μL of the same medium was added to each well. The well plate and the medium were pre-warmed at +37 °C and then the neural aggregates were applied on the surface for attachment. The cells were cultured on the matrix for 8 days and half of the medium without growth factors was changed every second day. At days 3 and 8 the cells were imaged using an Olympus microscope (IX51, Olympus, Finland) to assess the cell types attached and the cell growth. Thereafter, the cells were fixed for immunocytochemical analysis using 4 % paraformaldehyde for 20 min at room temperature. Altogether two parallel wells of each NCAM concentration for both hESC line-derived neural cells were prepared.

2.5. Staining of the cells for imaging and analysis

The fixed cells were stained with polyclonal rabbit anti-microtubule associated protein (MAP-2, 1:400, Chemicon, Temecula, CA) for neuronal cells or for mouse anti-human OCT-3/4 (Millipore, Billerica, MA) or monoclonal mouse anti-Tra-1-60 (Chemicon) for undifferentiated hESCs. Briefly, the cells were blocked against non-specific antigen binding with 10 % normal donkey serum, 0.1 % Triton X-100, and 1 % BSA in PBS for 45 min and washed with 1 % normal donkey serum, 0.1 % Triton X-100, and 1 % BSA in PBS. The primary antibody was diluted with the washing solution, added to the cells, and incubated overnight at +4 °C. The next day, the cells were washed with 1 %

BSA in PBS and incubated for 1 h at RT with the same solution containing Alexa Fluor-488 or -568 (1:400, Invitrogen) conjugated anti-rabbit or anti-mouse secondary antibody. Thereafter, cells were sequentially washed with PBS and phosphate buffer, mounted with Vectashield with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Peterborough, UK), and cover-slipped. When primary antibodies were omitted (negative control), no positive labelling was detected. Stained neuronal cells were imaged and counted using an Olympus microscope (Olympus) equipped with a fluorescence unit and camera (DP30BW, Olympus). For statistical analysis, neuronal cell samples derived from two hESC lines were pooled together, thus the number of samples was 4 for each NCAM antibody concentration. A non-parametric Mann-Whitney *U*-test and an SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL) were used for statistical analysis. A p-value less than 0.05 was considered significant.

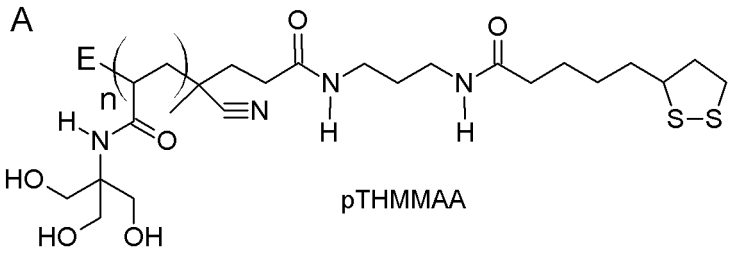
3. Results and discussion

3.1. Binding of NCAM antigen to antibody-polymer layers immobilised on gold

The binding of NCAM antigen to a mixed NCAM antibody and pTHMMAA monolayer was first evaluated with SPR. A fast increase in response was observed when antibodies at a concentration of 50 μg/mL were physisorbed on the gold surface (data not shown). The antibody layer formation showed a response of 1900 ± 200 RU corresponding roughly to 190 ng of antibodies/cm^2 (Vikholm-Lundin and Albers, 2006). Next, the pTHMMAA polymer at a concentration of 200 μg/mL was post-adsorbed on the surface with a response of 200 ± 40 RU. The polymer intercalates on the surface to sites not coated by antibodies and has previously been used in immunoassays for reducing the non-specific binding of interfering molecules (Vikholm-Lundin and Albers, 2006). The non-specific binding was measured by running BSA at a concentration of 500 μg/mL on the surface. Non-specific binding of BSA was 60 ± 40 RU corresponding only to 6 ± 4 ng/cm^2, which suggests that the polymer is intercalated between the antibodies and effectively shielding them.

Non-specific binding has otherwise been noticed to take place in the vicinity of the antibodies (Vikholm et al., 1999).

Next, the NCAM antigen binding to the layer was studied (Fig. 1B). The antigen binding to the layer increased with concentration, giving a response of 250-350 RU, when the monolayer was spread from an anti-NCAM concentration of 50 μg/mL. The same surface was also constructed with a lower antibody concentration (25 μg/mL; data not shown) resulting in a 60 units lower response at the highest antigen concentration of 10 μg/mL. These results are in agreement with previous studies when binding antigen to binary layers of antibodies and the pTHMMAA polymer (Vikholm-Lundin and Albers, 2006). Next, the cell-growth studies were carried out encouraged by the good characterization results obtained by SPR.



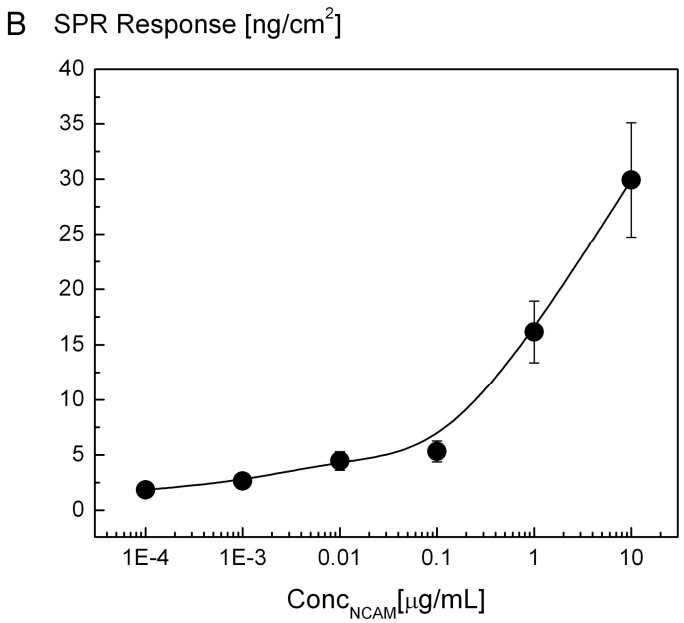


Fig. 1. (A) The structure of the pTHMMAA–polymer. (B) SPR standard curve showing NCAM antigen binding to a layer composed of anti-NCAM antibodies and pTHMMAA–polymer spread from concentration of 50 and 200 μg/mL, respectively.

3.2. Binding of neuronal cells to antibody-polymer layers immobilised on polystyrene

First, the anti-NCAM antibodies at concentrations from 0 to 100 μg/mL were physisorbed on polystyrene and then post-treated with the pTHMMAA polymer (200 μg/mL) to produce a binary monolayer. Neurospheres differentiated for 6 weeks are a heterogeneous cell population containing mostly neural precursor cells. If plated on laminin some non-neural cells can occur (unpublished). In order to verify cell attachment on the antibody/pTHMMAA layer the cells were imaged during culturing and fixed and stained after 8 days on either polystyrene coated with only the polymer or with the polymer (200 μg/mL) and antibodies (25-100 μg/mL). The staining of the cells after fixation verified the phenotypes of attached cells. HESC-derived neuronal cells do not normally adhere on plain polystyrene and this was also observed when only the pTHMMAA polymer was applied on the surface as only MAP-2 negative, non-neuronal cells attached to the wells (Fig. 2 C). If NCAM antibodies, on the other hand, were immobilised on the surface, attachment of MAP-2 positive neuronal cells could be observed (Fig. 2 D and E). In fact, all the attached cells on NCAM antibody matrices of 25 μg/mL or 50 μg/mL were MAP-2 positive neurons as 100% co-localization of MAP-2 and nuclear stain DAPI was observed. The cell counts revealed that significantly higher amounts of neuronal cells were attached when the concentration of the NCAM antibody was increased from 25 to 50 μg/mL on the surface ($p < 0.05$, 52 ± 31 cells vs. 206 ± 69 cells, respectively, Fig. 2 F). This suggests a specific cellular NCAM protein binding to the immobilised NCAM antibodies on the well plate surface. The polymer seems to hinder quite efficiently non-neural cell attachment.

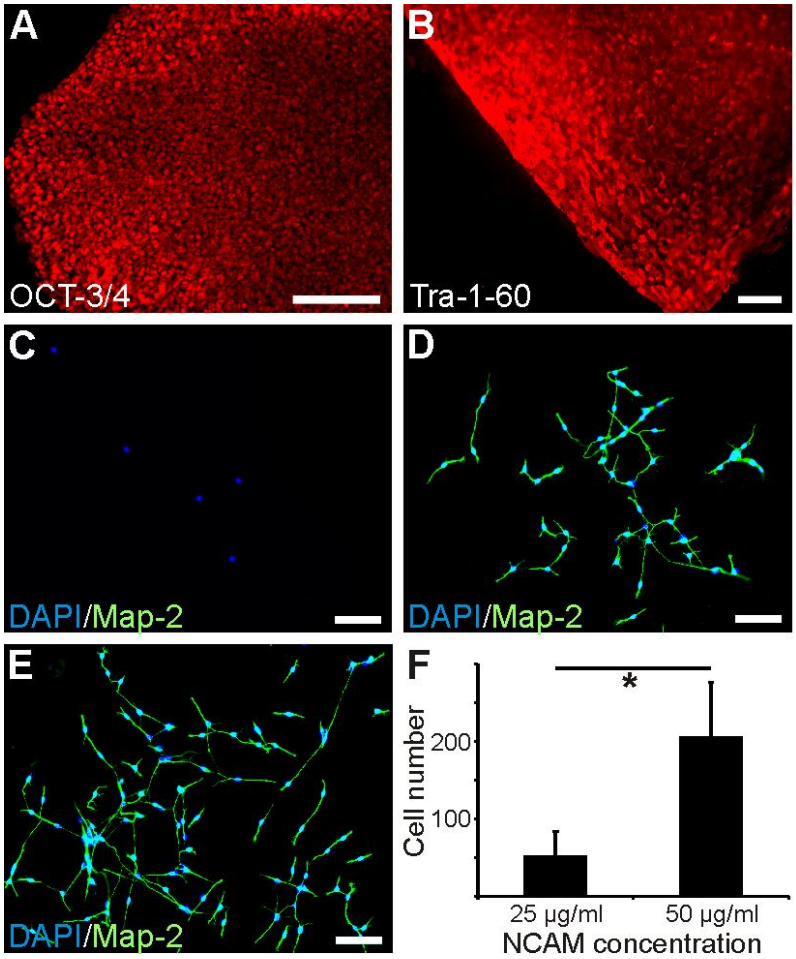


Fig. 2. Human embryonic stem cell (hESC) lines used routinely stained positive for pluripotent markers (A) OCT-3/4 (Regea 06/040) and (B) Tra-1-60 (Regea 08/023). (C) MAP-2 positive hESC-derived neuronal cells did not attach on plain polystyrene surface with pTHMMAA polymer (200 µg/mL) whereas there was concentration dependent attachment on surfaces coated with (D) 25 µg/mL or (E) 50 µg/mL of anti-NCAM antibodies and 200 µg/mL of pTHMMAA. (F) The attachment of MAP-2 positive neuronal cells was significantly higher to surfaces prepared from 50 µg/mL anti-NCAM antibodies compared to surfaces prepared from 25 µg/mL anti-NCAM antibodies ($p<0.05$). Results represented as mean ± standard error of mean (SEM). Scale bar = 200 µm.

At an NCAM antibody concentration of 75 or 100 µg/mL, few MAP-2 negative cells were also observed besides neuronal cells (data not shown). Thus, these concentrations were not considered optimal for neuronal cell attachment and not studied further. This suggests that at high NCAM antibody concentrations the binding selectivity starts to diminish due to a steric hindrance with an increased non-epitopic binding sites of antibodies available on the surface for non-neuronal cell attachment. Corresponding binding characteristics have been observed for antigen binding to

monolayers developed for immunoassays (Vikholm-Lundin and Albers, 2006). The optimum amount of antibodies in the layer is dependent on the size of the antigen. A higher antigen binding and an improved neuronal cell growth could be expected if the antibodies were further site-directly immobilised on polystyrene.

During culturing no significant neuronal cell proliferation or extensive neurite extension was observed (data not shown). Most likely NCAM antibody sites are occupied by attached neuronal cells and thus the pTHMMAA polymer does not support cell proliferation. Also, there was no support for the neurite extension and the cells remained as bipolar immature neuronal cells. Thus, this NCAM antibody–pTHMMAA polymer surface may be used as selective matrix for immature neuronal cells. It remains to be studied whether adding of different antibodies or patterning of the NCAM antibodies to the matrix would enhance neuronal cell maturation.

4. Conclusions

In this paper we have shown that hESC-derived neuronal cells can be attached selectively on polystyrene with the aid of NCAM antibodies embedded in a monolayer of hydrophilic pTHMMAA polymer molecules. The amount of neuronal cells on the surface significantly increased in relation to the increased amount of the antibodies in the monolayer. Plain polystyrene with pTHMMAA polymer alone did not present adhering of the neuronal cells. In the future our aim is to increase the amount of the functional antibodies in the layer by using site-directed immobilisation of the antibodies in order to improve the selective neuronal cell attachment and maturation.

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