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Genetic profiling of the peripheral nervous system

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cAMP is involved in the differentiation of human terayocarcinoma cells

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Abstract
The generation of fully differentiated post-mitotic human neuronal cells from stem cells (human teratocarcinoma (hNT2) cells) might enable the development of a co-culture model of human neurons with human Schwann cells (SCs). This co-culture model is an important tool to study formation of myelin sheaths. However, the thin processes of the post-mitotic human neuronal cells formed under known culture conditions do not provide a good substrate for human SCs to start myelination. We optimised the culture conditions of these cells to obtain axons with a larger diameter. Western blotting and immunofluorescence studies were performed to confirm the neuronal status of the cells and diameter of the processes. In this study, we show that addition of cAMP-inducing factors to hNT2 cells resulted in rapid morphological changes including the development of processes with a larger diameter.
Rapid signaling of neurons in the vertebrate nervous system depends on intact and compact myelin, a sheath of stacked membranes that enwraps segments of axons and increases nerve conduction velocity [1]. In the peripheral nervous system (PNS) axons are myelinated by Schwann cells (SCs). Axonal regulation of the SC phenotype is well established and axons are thought to play an important role in the differentiation of proliferating SCs towards a fully differentiated myelin-producing cell [2, 3]. From rat studies it is known that myelination of PNS axons is probably initiated by an axonally derived signal and is associated with the axonal diameter [4, 5]. Furthermore, maintenance of PNS myelin usually depends on physical contact between SCs and viable axons [6].

CAMP has been suggested to be involved in myelination as the second messenger. Agents that induce elevated levels of cAMP stimulate expression of myelin-related genes and downregulate the expression of genes that are upregulated after axotomy [7-9]. Thus, the cAMP pathway could play a role in the communication between axons and SCs. Artificial elevation of cAMP mimics some aspects of axon-SC contact [10].

We examined the possibility of the human teratocarcinoma cell line (hNT2) as neuronal substrate for a model to study human axon-glial cell interactions. Addition of retinoic acid differentiates the hNT2 cells into terminally differentiated post-mitotic neurons [11] with functional axons and dendrites after extensive outgrowth. Co-culture of these neurons with human SCs could provide an excellent tool to study cell-cell interactions, gene expression and the role of growth factors in (re)myelination.

Our previous attempts to co-culture these cells resulted in overgrowth of Schwann cells. As mentioned above, SCs need axons with a certain diameter to start myelination. The diameter of processes formed by hNT2 cells under known culture conditions are about 1 μm and needs to be increased to induce adhesion of the Schwann cells to the axonal processes and myelination. Here we describe the optimisation of the culture conditions of differentiated hNT2 cells into cells with larger diameter processes by manipulation of intracellular cAMP levels. Besides the proposed role of cAMP in interactions between axons and SC interaction, cAMP might also induce phosphorylation of the neurofilaments and thereby increase the axonal diameter [4, 12]. Therefore, we tested in this study whether elevated levels of intracellular cAMP could increase the axonal diameter.
Material & Methods

hNT2 cells were cultured as described previously [11]. Briefly, hNT2 cells were passaged twice a week in Opti-MEM (Gibco BRL, Grand Island, NY, USA) containing 5% FCS (BioWhitaker Inc. Europe, Verviers, France). hNT2 cells were differentiated in Dulbecco’s modified Eagle medium (DMEM; Gibco BRL) containing 10 μM all-trans-retinoic acid (RA) (Sigma, St. Louis, MO, USA). This NT2 medium was renewed twice a week for 4 weeks. The cells were replated after RA treatment. Two days later the cells were manually dislodged and replated on matrigel coated coverslips. The cells were cultured alternately for 2 weeks on medium containing anti-mitotics (10 μM uridine, 10 μM fluorodeoxyuridine (Fluka Biochemicals, Bornem, Belgium) and 1 μM cytosine D-arabinofuranoside (Sigma) and in medium without these anti-mitotics. The cells were then used for further experiments and remained vital for at least another 4 weeks.

Human SCs were obtained from nerve biopsies and were cultured in Iscove’s modified Dulbecco’s Medium (IMDM, Gibco BRL) containing 10% FCS, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX, Sigma), 0.5 μM forskolin (ICN, Costa Mesa, CA, USA), 2.5 μg/ml insulin (Sigma) and 10 nM β-heregulin (a gift from Genentech, San Francisco, CA, USA) as described by Hanemann et al. [13]. This medium is referred to as human Schwann cell medium (HSCM).

To study the effects of cAMP-inducing factors on differentiation of hNT2 cells various factors were added to the standard NT2 medium (DMEM, 10% FCS, 0.2M L-glutamate (Roche) and 100 Units/ml penicillin (Gibco BRL) and 100 μg/ml streptomycin (Gibco BRL). The different concentration and combination of factors added to the NT2 medium were: 0.5 mM IBMX; 0.5 μM forskolin; 2.5 μg/ml insulin; 10 nM β-heregulin; IBMX and forskolin or IBMX and forskolin and insulin. The hNT2 cells were also cultured in HSCM and Human Schwann Cell Conditioned Medium (HSCCM) which was HSCM that had been in contact with SC for 5 days. HSCCM was filtered over a 0.2 μm filter (Millipore, Bedford, MA, USA). The cells were cultured in the presence of these factors for at least 5 days. After incubation, cells were either used for immunocytochemistry or Western blotting.

For the immunocytochemistry study, cells were washed with PBS, fixed in PBS containing 4% paraformaldehyde for 10 min at room temperature, washed with PBS, permeabilized with methanol for 10 min at -20°C and washed with PBS/10% FCS. Coverslips were overlaid with the primary antibody for 30 min at 37°C and washed with PBS/10% FCS. A monoclonal antibody recognizing both NF-H and NF-M in neurofilaments, J47 (gift from Dr D. Dahl [14, 15], Harvard University, Boston, MA, USA), was used. The secondary antibody (anti-mouse coupled to FITC (Sigma)) was then applied for 30 min at 37°C. After washing with PBS, water and 100% ethanol the
coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing propidium iodide (100 ng/μl, Sigma). The coverslips were viewed with a Vanox (Olympus, Melville, NY, USA) microscope equipped with fluorescence optics. Incubation with the secondary antibody alone was used as a negative control. After immuno-staining, images were captured and analysed using a CCD camera (Diagnostic Instruments, Sterling Heights, Michigan) mounted on an Axiotron microscope (Zeiss, Oberkochen, Germany). The length, width and number of processes were measured from the stem of the cell body using Cytovision software (Applied Imaging, Newcastle Upon Tyne, UK). Per treatment between 26 and 31 cells were measured. All data were analysed with the Kruskal-Wallis test (with Dunn’s multiple comparison test for post-hoc analysis), assuming a non-normal distribution of the data. Differences between mean values of treatment groups variables were tested with the Mann-Whitney test. Only two-tailed significance is reported. A P-value of P<0.05 was used for significance in all comparisons. All analyses were done with SPSS 10.7 software.

The Western blots were obtained after the cells were washed with PBS, lysed in buffer containing 20 mM Tris-HCl, 6% glycerol, 0.4% SDS and 5 mM DTT and homogenized by flushing through a syringe (26 3/8 gauge). The homogenates were boiled for 5 min. Protein extracts were separated by SDS-PAGE using gels containing 6% polyacrylamide and transferred to nitrocellulose filters. The nitrocellulose filters were pre-incubated in Tris-HCl containing 0.5% Tween-20 (TBST) and 5% non-fat dry milk powder. Blots were incubated overnight at 4°C with the primary antibody, PAb anti-CREB-Phos (New England Biolabs) recognizing cAMP-converted phosphorylated cAMP response element binding protein (CREB) in TBST containing 5% non-fat dry milk. Then, membranes were washed in TBST and incubated with horseradish peroxidase-conjugated secondary anti-mouse antibody (1:3000 dilution; Dako, Glostrup, Denmark) for 2 h. After three washes in TBST, immuno-reactive bands were detected using enhanced chemiluminescence with LumiLightPlus (Roche, Basel, Switzerland).

The generation of fully differentiated post-mitotic human neuronal cells from stem cells (hNT2 cells) by treatment with RA [11] might enable the development of a co-culture model of human neurons with human SCs. To obtain axonal processes with a sufficient large diameter, a prerequisite for myelination, the culture conditions of these cells had to be optimised.

The first step was to differentiate hNT2 cells into neuronal cells. After applying RA treatment, only a part of the hNT2 cells appeared to differentiate into neurons, determined on the basis of expression of neurofilaments using immunocytochemistry.
As previously described by Pleasure [11], two types of cells could be distinguished on the basis of their morphology after RA treatment. We divided these cells into: type-I cells, consisting of large, round, cytoplasm rich cells without processes and type-II cells, which grow in aggregates and develop long, thin processes (Figure 1A). Type-I cells were negative for neurofilament immunostaining and were different in morphology compared to the non-RA treated cells, which are small and rectangular. We were not able to completely remove these type-I cells from the cultures, even after treatment with anti-mitotics, suggesting that these cells do not divide as rapidly as the undifferentiated parental hNT2 cells. We therefore designated these type-I cells as partially differentiated cells. The long, thin processes of the type-II immuno-stained
positive for neurofilaments (Figure 1B). These cells were classified as differentiated neuronal cells. The width of these processes was 1.2 µm (N=38, SD=0.24).

Since a large process diameter of the neuronal processes is a prerequisite for myelination, these type-II cells were not suitable as a neural input for a human co-culture model. Therefore, we varied the culture conditions of the differentiated cells to achieve an increased axonal diameter. First, we tested whether SCs had an effect on axonal diameter, because cells may secrete a factor that affects axonal diameter [7]. The hNT2 cells were therefore incubated with HSCCM. The conditioned medium was obtained after being in contact with the Schwann cells for 5 days. Treatment of hNT2 cells with HSCCM induced a distinct change in morphology (Figure 1C). Some of the hNT2 cells showed an increase in number of processes and size of processes (Table I). However, it appeared that only the type-I cells showed these morphological changes, whereas the thin processes of the type-II cells remained unchanged. We observed by viewing the cells at a 4 h interval that the amount of type-II cells stayed the same and the type-I cells changed in morphology. These morphologically different cells became positive for neurofilament staining (Figure 1D) indicating that neuronal differentiation had occurred. Since, no significant differences in number or width of processes were observed between HSCM and HSCCM treatment (Table I) it is unlikely that a factor secreted by SCs is responsible for this effect or the concentration of the factor may be too low to measure an effect in our system. As only the length of the processes was significantly longer (P<0.005, Table I) in the HSCCM treated cells, a factor secreted by SCs might be present that elongates the processes.

HSCCM contains growth factors such as IBMX, forskolin, insulin and β-heregulin. To investigate whether the morphological changes induced by HSCCM treatment was due to these factors, they were added individually and in combinations to the hNT2 cells. The addition of IBMX, which upregulates intracellular levels of cAMP, to the cell culture induced similar morphological changes to the type-I cells as the treatment with HSCCM. Again the type-II cells were not affected by IBMX (Figure 1E,F, Table I). The amount of type-II cells remained equal, whereas the morphologically changed cells replaced the type-I cells. Annexin V and TUNEL negative staining demonstrated that the altered morphology of the type-I cells was not due to apoptosis (data not shown). However, when these cells were incubated again in normal NT2 medium lacking IBMX, the cells died, suggesting a dependency on high cAMP levels and the differentiation in a non-reversible state.

Addition of forskolin, which maintains high cAMP levels in cells, also induced morphological changes in the type-I cells but the effects were less pronounced as compared to those of IBMX (Table I). The number of processes and the length of processes
were less compared to the treatment of IBMX or HSCCM but the width of the processes was significantly wider ($P<0.001$, Table I). This suggests that IBMX is responsible for the increase in process number and forskolin contributes to the width of the processes. However, the addition of both of these factors did not result in optimal process number and width as may be expected. IBMX and forskolin are not complementary in their action. However, they seem to partially reverse each other's effect on the culture (Table I). Addition of insulin to these two factors does not make a significant difference (Table I). Neither, the addition of β-hergulin (Figure 1G), a specific SC growth factor, nor the addition of insulin had an effect on the morphology of the cells (Figure 1H). This strongly suggests that cAMP-inducing factors in the HSCCM are the active components, which change the morphology of the hNT2 cells. The exact mechanism of the cAMP mediated morphological changes is not yet known. However, high intracellular cAMP levels induce and stimulate the production and phosphorylation of neurofilaments [12] and exposure to cAMP analogs has been shown to increase expression of myelin genes in SC [7]. cAMP might be involved in a signaling pathway that is coordinating myelination-associated changes in both neurons and SCs.

Table I. Effect of various culture conditions on the morphology of type-I cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of processes (SD)</th>
<th>Length of processes, μm (SD)</th>
<th>Width of processes, μm (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT2 Medium</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IBMX</td>
<td>5.3(1.2)</td>
<td>147(45)</td>
<td>15.7(6.3)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>3.5(1.0)^a,b</td>
<td>115(49)^a,b</td>
<td>29.9(15.6)^a,b</td>
</tr>
<tr>
<td>IBMX + Forskolin</td>
<td>4.0(0.8)^a,b,c</td>
<td>160(37)^d</td>
<td>16.7(7.4)^a,b,c</td>
</tr>
<tr>
<td>IBMX + Forskolin + Insulin</td>
<td>4.4(0.9)^a</td>
<td>147(37)^d</td>
<td>14(5)</td>
</tr>
<tr>
<td>HSCM</td>
<td>5.9(1.5)</td>
<td>122(43)^a</td>
<td>10.1(3.6)</td>
</tr>
<tr>
<td>HSCCM</td>
<td>5.6(1.2)</td>
<td>155(40)</td>
<td>11.7(4.7)</td>
</tr>
</tbody>
</table>

a significantly different versus HSCCM.
b significantly different versus IBMX.
c significantly different versus IBMX but not versus Forskolin.
d significantly different versus Forskolin but not versus IBMX.

Phosphorylation of CREB was measured in the differentiated hNT2 cells, to test whether IBMX or forskolin did indeed induce elevated levels of cAMP in the cell cultures. An increase in phosphorylation of CREB was observed after addition of the various cAMP-inducing factors to the hNT2 cells (Figure 2), confirming that the cAMP levels were indeed raised.
Therefore, we conclude that culture conditions leading to an elevated level of intracellular cAMP are responsible for the altered morphology of hNT2 cells in these experiments. The activation of CREB, which is involved in the differentiation of the oligodendrocytes as well [16], might also play a role in this system. We were able to culture cells with a larger process diameter, which show immuno-staining with the neurofilament antibodies, suggesting that we have developed neurons with processes that are large enough to start myelination. It is not completely certain whether the morphologically changed type-I cells are indeed the right axonal input for a co-culture system. Studies into the precise character of these cells are needed before starting co-cultures of human SCs and hNT2 cells.

Figure 2. Western blot of CREB phosphorylation. hNT2 cells were incubated for 0 (negative control), 2, 4, 8, 24 and 48 h in the presence of HSCCM. The western blot was incubated with an antibody against phosphorylated CREB. CREB phosphorylation is induced after 2 h of incubation with Schwann cell conditioned medium. Same results were seen using IBMX or forskolin individually. The positive control sample is phosphorylated CREB.

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References