Video Article

Characterization of the Interaction of Primary Cells from the Rat Inner Ear with Polymer Films As Coatings for Cochlear Implant Electrode Surface

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Abstract

The spiral ganglion (SG) cells prepared from the inner ear of postnatal rats represent one of the key cell culture models in hearing research. Numerous projects in hearing research aim at improving nerve-electrode-interactions by inhibiting the formation of connective tissue following cochlear implant insertion, *i.e.*, by coating the carrier material with ultrathin polymer films for selective cell attachment. Here, we established scanning electron microscopy (SEM) and immunocytochemical (ICC) staining to enable the characterization of the interactions of fibroblasts, glial cells, and spiral ganglion neurons (SGN) growing on polymers, *i.e.*, poly(N,N-dimethylacrylamide) (PDMAA), poly(2-ethyloxazoline) (PEtOx), and poly([2-methacryloyloxy)ethyl]trimethylammoniumchloride) (PMTA). For this purpose the primary cells dissociated from the SG of postnatal rats were cultivated for 48 h on the polymer films. ICC was used to demonstrate the preferences of cell adhesion on the polymer coatings. It could be shown that glial cells and SGN mainly adhered on PMTA monolayers forming long processes, but not on PDMAA and PEtOx films. Also, SEM imaging showed that only PMTA enabled SG neuron survival and neurite outgrowth. In conclusion, the ability of the SGN to survive and to form neurites was associated with glial cell adhesion on different coatings.

Introduction

Numerous projects in hearing research aim at improving nerve-electrode-interactions by inhibiting the formation of connective tissue following cochlear implant insertion by either application of drugs like dexamethasone[1,2,3,4], coating the carrier material with ultrathin polymer films[5,6,7,8], or nano structuring of the carrier materials for selective cell attachment[9,10,11,12]. To characterize biocompatibility, protein binding affinity, and effects on cell morphology, adhesion, and motility, *in vitro* cell culture assays represent the method of choice prior to *in vivo* experiments in laboratory animals.

Even though homogenous cell lines provide reliable experimental data, they do not represent the complex tissue reactions. In contrast, primary cells such as dissociated cochlear SG cells in culture may mimic the natural tissue environment. So far, SG cells from both mice and rats are well-established in *in vitro* cell culture systems to examine the signal pathways that protect the auditory neurons against apoptosis-inducing agents and that influence neuronal interactions with growth factors[13,14,15,16,17,18,19,20,21]. However, the investigations described herein have been confined to the SGN and did not include the biological activities of other cell types such as glial cells, satellite cells, and fibroblasts. Due to their differentiated character, primary cells may require refined techniques to study their behavior on different surfaces as well their intercellular interactions.

This study presents fast, simple, and reliable ICC techniques that enable the examination of SGN interactions with non-neuronal cells (such as Schwann cells and fibroblasts) not only on standard tissue culture plates, but also on modified surfaces like ultrathin polymer layers. The polymers PDMAA, PEtOx, and PMTA were manufactured according to Prücker *et al.*[22]: a photoactive benzophenone derivative was coupled with a chlorosilane anchor for immobilization onto the glass surface. The subsequent spin-coating of the polymers PDMAA, PEtOx, and PMTA on the photoactive group and exposure to UV light resulted in a photochemical attachment of the polymers onto the substrate. As follows, the primary cells were dissociated from the SG of postnatal rats and cultivated for 48 h on the polymer films. ICC methods and SEM were used to demonstrate the preferences of cell adhesion on the polymer coatings.

Protocol

Neonatal Spraque-Dawley rats (P3-5, n = 18 per experiment, n = 5 independent experiments) were used for SG dissection in accordance with the institutional guidelines for animal welfare of Hannover Medical School following the standards described by the German "Law on protecting animals" (Tierschutzgesetz).

1. Dissection of the Cochlear SG from Neonatal Rats [13,14]

1. Sacrifice the postnatal rats, which should not be older than a maximum of 5 days, decapitating the heads quickly using a scissor with pointed tips.
2. Fix a head with a curved standard pattern forceps by inserting it into the nose of a rat.
3. Remove the mandible and pull the skin, starting at the side of the neck, by using Adson-Brown forceps.
4. Open the skull along the midline and separate it into two halves using a scissor with pointed tips.
5. Scrape away the brain by using the handle of one of the forceps and transfer the two head halves into a Petri dish with ice-cold phosphate buffered saline (PBS) tablets.
6. Extract the cochlea out of the temporal bone by using the forceps Dumont #5 and #3c under microscopic view with 4x magnification.
7. Open the bony capsule, carefully exposing the cochlear parts of the membranous labyrinth by using the forceps Dumont #5 and #3c.
8. Remove the organ of Corti and the stria vascularis from the modiolus by using the forceps Dumont #5 and #3c.
9. Separate the entire SG from the modiolus of each head half and place them in ice-cold Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS).

2. Enzymatic Dissociation of the SG and Cell Cultivation Set-up [23]

1. **Preparation of the enzyme solution and the cell culture medium**
    1. Prepare the enzyme solution with 0.1% trypsin and 0.01% DNase I in Ca^{+2}/Mg^{+2}-free HBSS and keep it at 37 °C in the incubator.
    2. Prepare 15 mL of cell culture medium with neuro medium supplemented with 25 mM HEPES, 30 U/mL penicillin, 0.15% glucose, 8.75 µg/mL insulin, and 1x N2-supplement.

2. **Dissociation procedure**
    1. Incubate the SGs in 2 mL of the enzyme solution at 37 °C for 8 min, shake the cells gently, and incubate for an additional 8 min at 37 °C.
    2. Stop the enzymatic activity by adding 200 µL fetal bovine serum (FBS).
    3. Discard the supernatant by using a pipette. Wash the cell clusters three times with cell culture medium by short pulse centrifuging (4,000 rpm for 3 s) and removal of the supernatant by pipetting at room temperature.
    4. Disrupt the cell clusters carefully by pipetting up and down the suspension using 1,000 µL and 200 µL filter tips, consecutively, until any clusters are seen.
    5. Determine the cell yield by using the Neubauer cell count-chamber following staining of a 1:5 or 1:10 dilution of the cell suspension with 10% trypan blue to exclude apoptotic cells from the cell count.

3. Cell Seeding

1. **Coating of the glass plates**
    1. Place the glass plates (8 mm x 8 mm) with and without polymer coating (*e.g.* PDMAA, PEtOx, PMTA) with a curved standard pattern forceps into the wells of a 48 well-microtiter plate.
    2. Pipette 100 µL of 70% ethanol into the wells and incubate all glass plates for 10 min each and rinse them with PBS, pH 7.5, at room temperature using a pipette. Repeat this wash two more times.
    3. Coat the glass plates without polymer for the positive control (PosCtrl) with 100 µL of 0.1 mg/mL poly-DL-ornithine for 1 h at room temperature. Then rinse them once with PBS, pH 7, at room temperature.
    4. As follows, coat PosCtrl with 100 µL of 0.01 mg/mL laminin at 37 °C for 1 h and rinse them once with PBS, pH 7.5, as described in step 3.1.3.

2. **Experimental set-up**
    1. Add 250 µL/well of the cell suspension containing 2 x 10^4 cells in serum-free culture medium, for each polymer coating, positive control, and negative control (glass plate without polymer and ornithine/laminin coating) by using a pipette. Use for each assay n = 5 wells.
    2. Add 250 µL of serum-free culture medium with 20% FBS to each well by using a pipette (total volume now is 500 µL).
    3. Cultivate the cells in the 500 µL culture medium in a humidified incubator at 37 °C and 5% CO_2 for 48 h.

4. ICC Staining

1. **ICC detection of cell specific antigens to examine the population composition of the SG following cultivation on the polymer films**
    1. **Fixation of the cells**
        1. Remove the PBS (pH 7.5), by pipetting and fix the cells by adding 200 µL/well of methanol.
        2. Incubate for 10 min at room temperature and rinse 3x with 250 µL PBS, pH 7.5, by using a pipette.

    2. **Preparation of the permeabilization and antibody dilution buffers**

1. Prepare the permeabilization buffer with Triton X-100 0.1% (w/v) to PBS, pH 7.5 (PBSTx).
2. Prepare the antibody dilution buffer with a final concentration of 1% bovine serum albumin (BSA) in PBS.

3. **Antibody incubation**
   1. Permeabilize the cells with 0.1% PBSTx for 5 min, remove the permeabilization solution, and wash three times with PBS, pH 7.5, by pipetting.
   2. Dilute the primary antibodies in the antibody dilution buffer as described in **Table 1** and prepare a master mix containing enough of the respective diluted primary antibody.
   3. Pipette 100 µL of the desired antibody solutions onto the glass plates and incubate them for 1 h at room temperature.
   4. Remove the antibody solution by pipetting and rinse the glass plates 3x with PBS, pH 7.5, for 5 min each.
   5. Prepare a 1:400 dilution of the fluorescently labeled secondary antibodies in antibody dilution buffer (**Table 2**) and prepare a master mix containing enough of the respective diluted secondary antibody.
   6. Pipette 100 µL of the desired secondary antibody solutions onto the glass plates, incubate for 1 h at room temperature protected from light, and wash the glass plates 3x with PBS, pH 7.5, as described in step 4.1.3.4.
   7. Pipette 20 µL of the mounting gel containing 4,6-Diamidino-2-phenylindole (DAPI) onto the glass plates, remove them from the microtiter plate, and place them upside down on coverslips (24 x 60 mm^2).
      NOTE: The control experiments to verify the specificity of the immune staining have been performed in parallel by incubating the fixed cells with secondary antibodies only. Hereby, the primary antibodies were exchanged with 1% BSA in PBS.

5. Distribution and Morphology of the Adherent SG Cells on the Uncoated and Coated Glass Plates[24]

1. **Fixation of the cultivated cells**
   1. Remove the culture medium with a pipette.
   2. Using a pipette gently load the glass plates with the adhering cells with 2.5% glutardialdehyde in 0.1 M sodium cacodylate, pH 7.3. Incubate for 1 h at room temperature.

2. **Dehydration of the cells**
   1. Remove the solutions with the pipette the wells with the containing glass plates and fill them with 30% acetone in 10 min. Repeat twice.
   2. Dehydrate the cells twice with 50%, 70%, 90% acetone in 10 min, respectively, as described in 5.2.1.
   3. Rinse the cells with 100% acetone 6x within 30 min.
   4. Put the specimens in the critical point dryer (CPD) and exchange 8x acetone against fluid CO_2.
   5. Deflate the CO_2 at the pressure of 7.3 MPa and the temperature of 31 °C in the CPD.

3. **Mounting and preparation of the samples for SEM**
   1. Transfer the glass plates containing the dried cells with standard forceps and fix them with fluid carbon in xylol (*e.g.* Leit-C) on top of the aluminum holder.
   2. Put the aluminum holder on the desk of the high-resolution sputter coater.
   3. Evacuate the chamber to 9 x 10^{-2} mbar.
   4. Flush with argon 3x by opening the exit valve and closing the valve, followed by floating with argon from the gas cylinder.
   5. Keep the voltage at 1 kV; a current of 20 mA will be induced by the floating argon.
   6. Ionize the gold target for 120 s to produce a 20 nm thick gold layer on the specimens.

4. **Analysis of the specimens with SEM**
   1. Analyze the specimens at 10 kV with low magnification (x156) and higher magnification (until x1,250).
   2. Save micrographs of the cells in all regions of the uncoated and coated glass plates with the SEM software (Gebert and Preiss)[24].

Representative Results

The aim of establishing the ICC protocol was the differentiation of the cell types accompanying the adhesion and neurite outgrowth of the SG neurons. As shown in **Figure 1A-H**, the method detected not only the expression of the neurofilaments in SGN, but also vimentin in both fibroblasts and glial cells on the polymer films (**Figure 1A, C, E, G**). Double staining with anti-vimentin and glial cell specific anti-p75 NGFR antibody enabled the identification of the glial cells adhering on PMTA, PDMAA, and PEtOx (**Figure 1B, D, F, H**). Whereas both the positive control and PMTA allowed attachment and growth of all cell types of the SG, the PDMAA and PEtOx presented mainly fibroblasts rather than glial cells (**Figure 1F, H**). Moreover, specific staining of the glial cells and the fibroblasts allowed the verification of the inhibition of the glial cell attachment on both PDMAA and PEtOx films by relating the number of fibroblasts and glial cells to the total number of DAPI stained cell nuclei[7]. Additionally, SEM revealed not only the distribution of the different cell types but also their interactions. On the PMTA coatings the SG cell types demonstrate their typical morphology compared to the control (**Figure 2A, B**). In contrast, flat cells with broad lamellopodia are connected to the PEtOx film (**Figure 2C**). The PDMAA surface is cell-free (**Figure 2D**).

Figure 1: Representative fluorescence microscopic view of the SGN and non-neuronal cells following cell specific labeling. The SG cells were double-labeled either with anti-neurofilament (NF, yellow) and anti-Vimentin (Vim, red) antibody, or anti-p75 neurotrophic growth factor receptor (p75-NGFR, denoted as p75, yellow) and anti-Vim antibody, which demonstrated the adherence of all cell types on the glass plates (**A**),**PMTA (C**), PDMAA (**E**), and PEtOx (**G**). Positive staining of the p75-NGFR antigen clearly demonstrated glial cells attaching to glass plates (**B**), PMTA (**D**), PDMAA (**F**), and PEtOx (**H**). This figure has been modified from Hadler *et al.*[7] Please click here to view a larger version of this figure.

Figure 2: SEM of the SG cells adhering on the polymer films and glass plates coated with ornithine/laminin. The SG cells retained their different cell morphology not only on ornithine/laminin coated glass plates (positive control, **A**) but also on PMTA layers (**B**). In contrast, cells with broad flat lamellopodia were predominately represented on PEtOx films (**C**). The adhesion of SG cells was clearly reduced on the PDMAA coated surfaces (**D**). This figure has been modified from Hadler *et al.*[7] Please click here to view a larger version of this figure.

Primary antibody	Host	Description	Cell specificity	Dilution
Neurofilament 200kD, monoclonal	Mouse	Intermediar filament	Neurons	1:400
p75, polyclonal	Rabbit	Neurotrophic growth factor receptor	Glial cells	1:500
Vimentin clone V9, monoclonal	Mouse	Intermediar filament	Fibroblast, glial cells	1:200
Vimentin, polyclonal	chicken	Intermediar filament	Fibroblast, glial cells	1:750

Table 1: Primary antibodies used to determine specific antigens in the SGN, fibroblasts, and glial cells.

Secondary Ab, IgG (H+L)	Host	Description	Abs./em. [nm]
Anti-chicken	Goat	Texas Red (**GaCh TR**)	586/605
Anti-mouse		New Dylight 488 (**GaM 488**)	493/518
Anti-rabbit		Alexa Fluor 594 (**GaRb 594**)	591/616

Table 2: Secondary antibodies used in this study.

Discussion

This study represents for the first time the differential interactions of SGN, glial cells, and fibroblasts on varying polymer films. Staining of the specific intermediary filaments and the neurotrophic growth factor receptor enabled not only a strong distinction between the cell types and their morphology following adhesion, but also the quantitative determination of the interesting cell types. Hereby, as described in Hadler *et al.*[7], the growth of fibroblasts was clearly scaled down on the PMTA layers in comparison to the glial cells. This finding indicates these surfaces as preferential substrate for glial cell attachment and glial cell driven formation of the ECM rather than for fibroblasts. As well, adhesion and survival of the SGN was found to be strongly associated with glial cell growth. It is supposed that the glial cells are not only involved in trophic support and neuronal protection, but also in providing myelin proteins to induce regeneration of the nerve fibers[25,26]. Moreover, the SEM examination revealed that both PDMAA and PEtOx films inhibited the attachment of the glial cells and, in consequence, the adhesion and survival of the SGN.

Possible critical steps in the protocol refer to the dissection of the cochlear SG from postnatal rats, which require strong surgical skills and knowledge of the inner ear anatomy; especially, for the dissection of cochlear parts of the membranous labyrinth under microscopic view due to the rat cochlea fine-structure. Additionally, the postnatal rat should no older than five days, since the bony capsules become hard and, in consequence, the quality of the dissection of the SGs may be degraded. It is critical to ensure that during the enzymatic dissociation procedure the homogenous cell suspension is free of any cell clusters.

Prior to ICC detection of the specific antibody-antigen interactions it has to be considered that the choice of the fixation solution depends not only on the protein structure of the desired antibodies, but also on the chemical structure of the surface for cell adhesion. In general, depending on the cell culture surface the SGN may be alternatively fixed with methanol/acetone and 4% paraformaldehyde (PFA).

There are several reasons to limit the number of SGN for each experiment: (i) in general, it is best practice to limit the number of animals used for any experiment, (ii) the number of postnatal rats is varying and depends on the number of pregnant female rats, (iii) and the extraction of the cochlear tissues is time-consuming and labor-intensive. Thus, flow cytometry techniques[27] may not be useful for cell sorting of the SGN cultures because it requires higher cell numbers.

For the SEM analysis, it is critical that the cells dry in contact with the material. Therefore, the chemical stability of the test material not only during the cell culture but also the chemical resistance of the surfaces in acetone must be tested beforehand. Size and/or light transmissibility is not important, and therefore, complete biomaterials after explantation can be analyzed with a high resolution. Nevertheless, only the cells as well formed extracellular matrices on free or dissected surfaces of the specimens can be evaluated. Cell-to-cell contacts as well as interactions

between the cells and the materials can be precisely documented. If the cell types are difficult to separate, immunochemical surface labeling with gold allows a detailed analysis.

By this consideration, there is no alternative to immunostaining and cell counting under microscopic view. Also, labeling of the antibody-antigen-interaction visualizes morphology, interactions, and function of the cells adhering on the polymer films or other surface types.

ICC staining of specific antigens together with SEM techniques allows not only identification and quantification of primary cell culture assays, but also cellular behavior and interactions with various substrates to characterize their biological effects and toxicity. Furthermore, high-resolution microscopy may reveal new insights of signal pathways and metabolic activity of the cells of interest.

Disclosures

The authors have nothing to disclose.

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