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Establishment of vestibular schwannoma primary cell cultures obtained from cavitron ultrasonic surgical aspirator tissue material

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ABSTRACT

Background: Vestibular schwannoma (VS) is a benign tumor arising from the Schwann cells of the eighth cranial nerve. The complexity in treatment is associated with unpredictable progression of this tumor. Some of the VS do not alter for years, while others rapidly increase in size. The mechanisms behind size progression are not well studied. Furthermore, despite several studies, there is no pharmacological treatment available for sporadic VS. Therefore, in vitro models are essential tools to study the cellular and molecular processes of VS. In addition, patient-derived cell cultures are important for substance screening to investigate pharmacological approaches in vitro.

New method: This study presents a simple and fast method for culturing VS cells from patient tissue material obtained using a cavitron ultrasonic surgical aspirator (CUSA). In addition, the cells were characterized based on the expression of schwannoma markers, growth properties and screened for fibroblast contamination.

Result: We could show that CUSA obtained material is a suitable resource for isolation of VS primary cultures and enables real time analysis on living cells.

Comparison with Existing Methods: To date, only a few protocols are available for culturing VS cells from patient tissue material. A disadvantage of these methods is the relatively large amount of tissue needed to obtain the primary cells, which can be difficult, especially in small VS. By obtaining the cells from the CUSA, there is the possibility to establish a primary culture even with limited material.

Conclusion: This approach could be particularly useful for testing substances that represent candidates for drug therapy of vestibular schwannoma.

1. Introduction

Vestibular schwannoma (VS) is the most common tumor of the cerebellopontine angle and occurs with a rising incidence of approximately 1:100,000 (Goldbrunner et al., 2020; Reznitsky et al., 2019; Stangerup and Caye-Thomasen, 2012). VS cause vertigo, tinnitus, hearing loss and facial paresthesia, due to the tumor localization in the cranial nerve region. The therapeutic options are limited to Wait and Scan, radiotherapy or microsurgical resection by craniotomy. To date, the development and progression of this benign tumor has not been well studied (Goldbrunner et al., 2020). Therefore, it is necessary to establish cell culture models for testing treatment options and investigating tumor pathology. Existing culture models obtained from murine schwannoma tissue are limited in transfer to human schwannoma characteristics. In recent years, the cavitron ultrasonic surgical aspirator (CUSA) has been used for precise intracapsular VS rejection. Based on the work of Richmond and Hawksley (1983), some previous studies showed that CUSA obtained tissue (COT) is vital and suitable for histopathology and cell culturing of medulloblastoma, meningioma and glioma tumor cells (Day et al., 2013; Malhotra et al., 1986; Oakes et al., 1990). For glioma tissue

Abbreviations: COT, CUSA obtained tissue; CUSA, cavitron ultrasonic surgical aspirator; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; f, female; FACS, fluorescence activated cell sorting; FITC, fluorescein-5-isothiocyanat; HBSS, Hanks' Balanced Salt Solution; HSC, human Schwann cells; IF, immunofluorescence; IgG, immunoglobulin G; ln, logarithmus naturalis; m, male; MIB, Molecular Immunology Borstel; n.d., not detectable; NHDF, normal human dermal fibroblasts; OD, optical density, PBS, phosphate buffered saline; td, doubling time; VS, vestibular schwannoma; XTT, 2,3-bis-(2-methoxy-4nitro-5-sulfophenyl)− 2 H-tetrazolium-5-carboxanilide.

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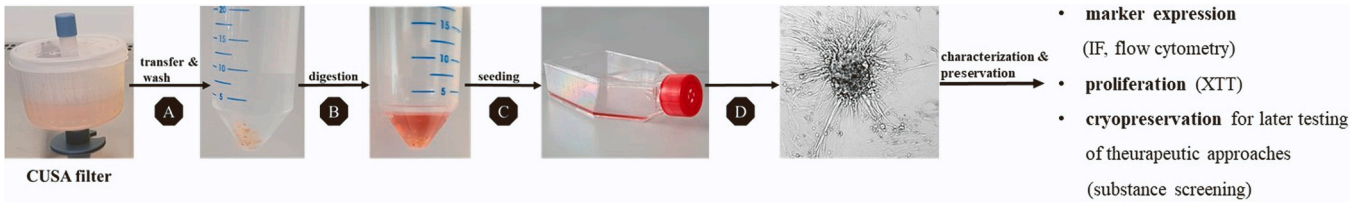

Fig. 1. Schematic workflow of VS cell isolation. CUSA obtained tissue was collected intraoperatively (A). After washing with PBS, tissue was digested with collagenase type I-S, hyaluronidase type I-S, trypsin and DNase (B) and seeded on precoated cell culture flasks. When the cells had reached at least 80% confluency, they were analyzed with S100 immunofluorescence staining and then cryopreserved for later testing of therapeutic approaches. IF, immunofluorescence; XTT, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)− 2 H-Tetrazolium-5-Carboxanilide; CUSA, cavitron ultrasonic surgical aspirator.

the usage of COT was shown to establish primary cultures, tumor spheroids and cancer stem cells (Beckner et al., 2007; Behnan et al., 2016; Jensen et al., 2013). In comparison to gliomas, VS are usually smaller and the tumor material is limited. Therefore, the aim of this study was to develop an effective method to culture primary VS cells from COT. In the last decades, some protocols for the isolation of primary human and murine Schwann cells from tissue samples have been established (Breun et al., 2020; Calderón-Martínez et al., 2002; Casella et al., 1996; Niapour et al., 2010; Weiss et al., 2018). Our method is based on a modified and simplified protocol by Dilwali et al. (2014). We characterized the CUSA obtained VS primary cells with immunofluorescence (IF) staining, viability and growth rate. Fibroblast contamination was analyzed. Because of limited tumor material, the COT opens up a new tissue resource for research accessibility of VS e.g., for drug screening, protein secretion and release of extracellular vesicles, which could contribute to a better understanding of tumor development.

2. Methods

2.1. Ethics statement

The collection of patient tissue was approved by the ethics committee of the Medical Faculty, Martin Luther University Halle-Wittenberg with process number 2020–122. Excluded were samples from patients under 18 years, with hereditary NF2-related Schwannomatosis, recurrences and preoperative irradiated tumors. After written informed consent was obtained from the patients, the samples were collected and analyzed pseudonymously. The patient data were taken from the medical record.

2.2. Tumor volume

The tumor volume was measured with Brainlab Origin Server 1.1 iPlan Net, version 3.7.0.64 (Brainlab AG, Munich, Germany) using thin-slice preoperative magnetic resonance images (T1- or T2-weightened). Images with a slide thickness under 2.5 mm were accepted.

2.3. Material collection

Sample material was obtained using the CUSA (CUSA Excel+, Integra Lifesciences, Tullamore, Ireland). The extracted material was collected via a disposable tissue collection filter (Medela Healthcare, Eching, Germany; hereafter named CUSA filter).

2.4. VS cell isolation and culturing

The protocol was modified from Dilwali et al. (2014). At first, 4 °C cold phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS; ThermoFisher Scientific, Waltham, USA) was added to the tissue collected by CUSA filter to prevent drying out. Then, the sample pieces were transferred with a sterile forceps into a 50 mL tube filled with 40 mL 4 °C cold PBS, spun down (10 min with 300 g at 4 °C) and washed again two times with 40 mL 4 °C cold PBS (Fig. 1A). Afterwards, the supernatant was removed completely and Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (1:1, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine (Biochrom AG, Merck, Darmstadt, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin (ThermoFisher Scientific, Waltham, MA, USA), 160 U/mL collagenase type I-S and 250 U/mL hyaluronidase type I-S (both enzymes Merck, Sigma-Aldrich, Darmstadt, Germany) was added to the tissue, that the sample was completely covered (0.5–2.0 mL), and incubated for approximately 14 h at 37 °C. Then, the digestion of tissue was stopped by diluting the enzyme mix through the addition of 10 mL supplemented DMEM/F12 (10 % FCS, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin). The sample was centrifuged down, the supernatant was discarded and the tissue pellet was incubated with 2.5 % trypsin and 250 µg/mL DNase diluted in Hanks' Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+}, ThermoFisher Scientific, Waltham, MA, USA) for 5 min at 37 °C (Fig. 1B). Then, the cells were resuspended with 1000 µL-pipette by carefully pipetting up and down. The tissue digestion was stopped with 10 mL complete DMEM/F12, spun down and cell pellet was resuspended in 10 mL supplemented media. The cell suspension was seated on poly-L-ornithine and poly-L-lysin (both Merck, Sigma-Aldrich, Darmstadt, Germany) pre-coated 75 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) and incubated at 37 °C with 5% CO_2 (Fig. 1C) under humidified atmosphere. The medium was renewed after 16–18 h and then every three to four days. Microscopic pictures and fluorescence images were made with Keyence BZ-800E microscope (Keyence, Neu-Isenburg, Germany, Fig. 1D). An overview of time-lapse schedule and total procedure time is shown in Supplementary Fig. 1.

Table 1
Used antibodies and antibody dilutions for IF analysis.

Antibody	Species	Dilution	Manufacturer
S100A10	mouse monoclonal	1:1000	Cell Signaling Technology
Ki-67	rabbit monoclonal	1:400	
Anti-Mouse IgG (H+L), F(ab')2 Fragment Alexa Fluor 488 Conjugate	goat	1:1000	
Anti-rabbit IgG (H+L), F(ab')2 Fragment Alexa Fluor 488 Conjugate	goat	1:1000	

2.5. Immunofluorescence staining

5×10^5 VS cells were seeded in 3 mL complete DMEM/F12 per well on poly-L-ornithine and poly-L-lysin (0.01 %, EMD Millipore Corporation, Burlington, VT, USA) precoated cover slips (2-well Tissue Culture Chambers, Sarstedt, Nümbrecht, Germany) and incubated for 24 h at 37 °C and 5 % CO_2 in humidified atmosphere. At first, cells were washed with 1.5 mL per well HBSS (with Ca^{2+} and Mg^{2+}) containing 3 mmol/L $MgCl_2$ (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). Then, the slides were two times washed with HBSS and fixed with − 20 °C-cold

Table 2
Patient data and corresponding primary cell characteristics.

Sample number	Patient characteristics					Cell characteristics		
	gender	diagnosis	Koos classification	tumor volume (cm^3)	MIB (%)	doubling time (hours)	immunofluorescence $S100^+$-cells (%)	$Ki\text{-}67^+$-cells (%)
1	f	VS	4	8.972	2	67.2	100.0	10.4
2	m	VS	2	0.409	1	n.d.	98.5	0.0
3	f	VS	4	7.918	1–2	n.d.	66.6	9.3
4	f	VS	2	0.248	5	52.5	58.1	0.3
5	w	VS	3	1.825	1	n.d.	82.4	6.2
6	m	VS	4	6.694	< 5	62.0	96.0	12.5
7	m	VS	2	0.264	5	67.1	98.3	5.4
8	f	VS	4	2.431	4	64.2	95.7	7.7
9	m	VS	4	3.055	4	92.3	81.9	8.3
10	f	VS	1	0.305	0	n.d.	87.0	1.0
11	f	VS	3	0.913	3	114.5	95.0	10.6
12	f	VS	3	2.806	1	69.6	56.4	1.8
13	m	VS	4	2.649	4	112.8	71.0	7.9
14	f	VS	2	0.689	< 3	73.9	64.5	0.3
15	f	VS	4	5.288	3	105.6	68.0	0.0
16	m	VS	2	0.532	n.d.	45.3	89.7	0.8
17	m	VS	4	11.677	4	173.0	97.2	4.5

(n.d., not detectable; m, male; f, female; VS, vestibular schwannoma; MIB, Molecular Immunology Borstel; Koos classification according to Koos et al. (1998).

methanol (Merck, Sigma-Aldrich, Darmstadt, Germany) on ice for 10 min. Subsequently, cells were incubated with 1 mL per well blocking buffer containing PBS, 5 % normal goat serum (Cell Signaling Technology, Danvers, USA) and 0.3 % Triton X-100 (Carl Roth, Karlsruhe, Germany) for 1 h. Then, the respective primary antibody (Table 1), diluted in 900 µL PBS with 1 % bovine serum albumin (Carl Roth, Karlsruhe, Germany) and 0.3 % Triton X-100 (antibody diluting solution), was added to the cells for 16–18 h at 4 °C. Next day, cells were washed three times with 1 mL PBS followed by incubation with secondary antibody for 90 min at room temperature in the dark. After washing twice with PBS, the chamber was removed and the slide rinsed again with PBS. Then, the slides were covered with mounting medium containing DAPI (ImmunoSelect Antifading Mounting Medium DAPI, Dianova, Hamburg, Germany) and dried for 24 h. The imaging was done with Keyence BZ-800E microscope. Human Schwann cells (HSC) purchased from ScienCell Research Laboratories (Carlsbad, CA, USA), were used as a positive control for S100 staining. HSC were cultured with Schwann cell media (ScienCell Research Laboratories, Carlsbad, CA, USA) as recommended by manufacturer. The cover slips used for HSC were coated with poly-L-lysine solution (0.01 %, EMD Millipore Corporation, Burlington, VT, USA). Cells stained with DAPI, Ki-67 antibody and S100 antibody were counted using the IndentifyPrimaryObjects tool of the software CellProfiler (Version 4.2.4, Broad Institute, Cambridge, Massachusetts, United States). The percentage of Ki-67- and S100-positive cells in the total cell count was calculated. The number of DAPI-positive cell nuclei was taken as the total cell count.

2.6. Proliferation assay

The doubling time was determined using the Cell Proliferation Kit II (XTT, Merck, Sigma-Aldrich, Darmstadt, Germany) according to manufacturer's instructions. Briefly, 5×10^3 cells per well were cultured in 200 µL complete DMEM/F12 medium without phenol red in pentaplicates in 96-well plates (TPP, Trasadingen, Switzerland) and incubated for 96 h at 37 °C and 5 % CO_2. Every 24 h the mitochondrial dehydrogenase activity was measured by conversion of the tetrazolium salt XTT to formazan after incubation of the cells with the XTT substrate mixture for four hours. The color change was determined at a wavelength of 490 nm using Tecan F200Pro plate reader (Tecan, Männedorf, Switzerland). Representative growth curves are shown in Supplementary Fig. 2. The cell doubling time was calculated during the exponential phase of cell growth ($td = \frac{\ln(2)}{(\frac{OD2-OD1}{t2-t1})}$).

2.7. Flow cytometry

1×10^6 were washed twice with 2 mL PBS supplemented with 1% FCS and 0.1% NaN_3 (flow cytometry buffer). Then, 10 µL CD90-fluorescein-5-isothiocyanat (FITC) direct conjugated mouse monoclonal antibody (FITC Anti-CD90 / Thy1 antibody; Abcam, Cambridge, UK) was added to the cells resuspended in 100 µL flow cytometry buffer and incubated for 30 min in the dark. After washing cells twice with flow cytometry buffer, cell surface FITC signal was measured using BD LSRFortessa Cell Analyzer (Becton Dickinson, Heidelberg, Germany). Data analysis was done with BD FACSDiva Software (Becton Dickinson) and FCS Express Version 7 (De Novo Software, Pasadena, CA, USA). Normal human dermal fibroblasts (NHDF) served as positive control (Kisselbach et al., 2009) and A549 lung cancer cells as negative control (Yan et al., 2013), as previously described.

3. Results

Seventeen CUSA filter obtained cultures from VS of different volumes and Koos grades were prepared according to the method described above. Immunofluorescence staining for detection of Schwann cell marker S100 and proliferation marker Ki-67 was performed. All primary cultures were positive for S100 staining. Immunofluorescence of Ki-67 differed in the included primary cultures. Regardless of sex and Koos grade, the doubling time of schwannoma cells could be determined in 13 of the 17 primary cultures. The doubling time ranged between 45.3 and 173.0 h (Table 2).

Brightfield and immunofluorescence images were obtained to analyze the VS primary cells (Fig. 2). The typical clusters in which schwannoma cells grow are visible, and these clusters are known to originate from a single cluster of cells. For better visualization of the cell clusters, staining with S100 was performed using immunofluorescence. Both, HSC and VS primary cultures showed strong positive staining with S100 antibody. However, the normal human Schwann cells (HSC) showed no clusters but only two-dimensional cells, the arrangement in the VS primary culture differed. The cells showed a distinct three-dimensional clustering with spindle-shaped palisade-like cell clusters, which are characteristically found for schwannoma cells. An investigation of the growth marker Ki-67 detected only a few positive cells in the most VS primary cultures (Table 1, Fig. 2) as expected.

To exclude fibroblast contamination, flow cytometric analysis of four primary cultures of different Koos grades was performed. The percentages of fibroblasts in the VS primary cultures are shown (Table 3). Only

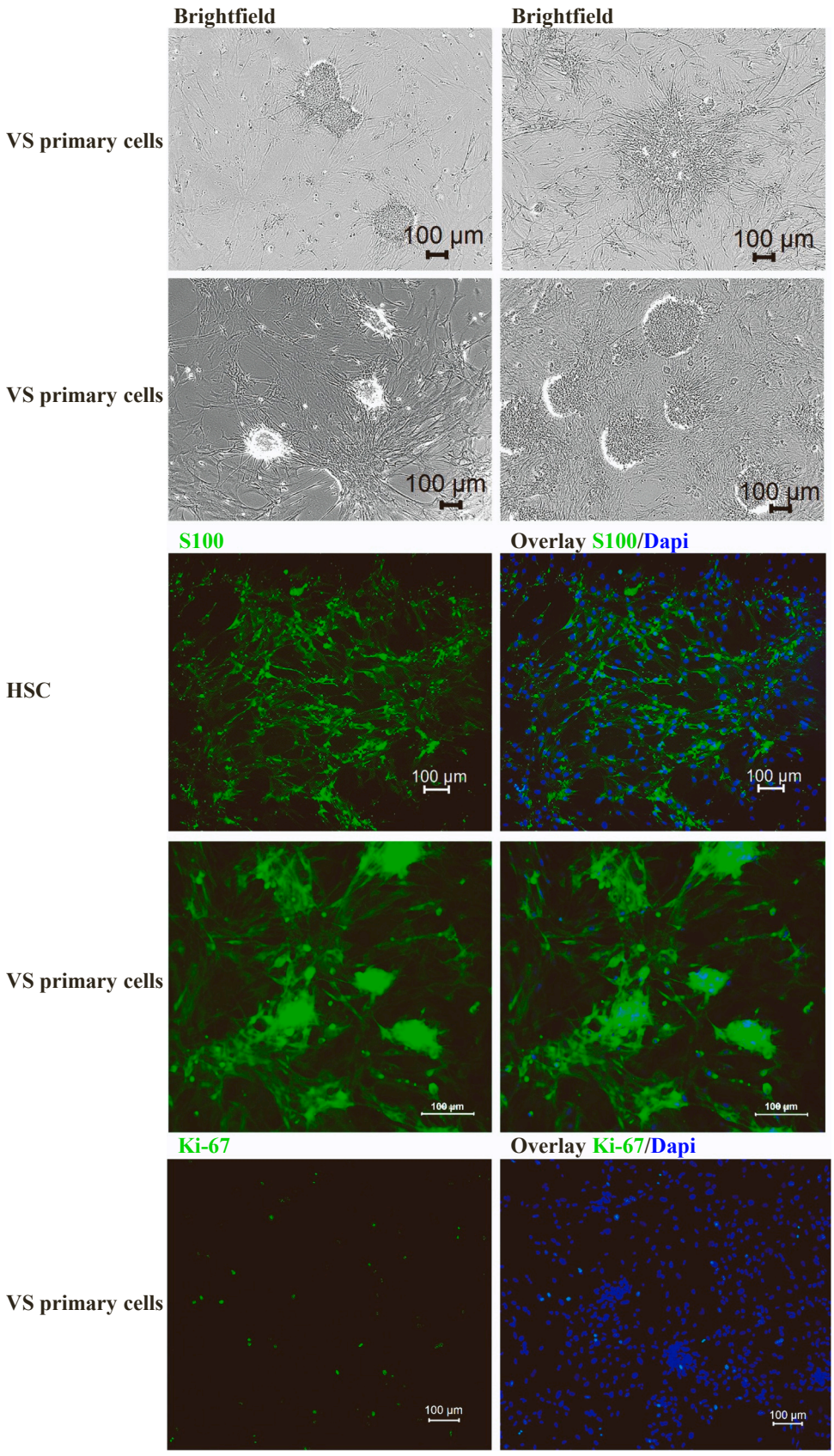

Fig. 2. Exemplary brightfield images and immunofluorescence staining of S100 and Ki-67 in VS primary cultures from CUSA obtained tissue. In the microscopic images, typical schwannoma-derived cell clusters were visible. Comparative immunofluorescence of human Schwann cells and schwannoma cells in the VS primary culture using S100 showed spindle-shaped palisade-like cell clusters exclusively in the VS primary culture. Immunofluorescence staining with Ki-67 showed few positive cells in the VS primary culture.

Table 3
Percentage of fibroblasts in VS primary cultures.

Sample number	Percentage of fibroblasts (%)
9	13.5
10	0.1
11	1.3
14	0.0

in the primary culture of a Koos 4 VS increased percentage (13.5 %) of fibroblasts were detected. There were no CD90-positive fibroblasts detectable in the analyzed smaller VS cultures.

4. Discussion

So far, there are only few protocols to culture schwannoma cells (Dilwali et al., 2014; Landegger et al., 2017). Moreover, these cell cultures are primarily limited to NF2-associated schwannomatosis (Rosenbaum et al., 1998). Xue et al. established a immortalized human schwannoma cell line, which was obtained from a 41-year-old sporadic vestibular schwannoma patient (Xue et al., 2021). However, there are no established immortalized human vestibular schwannoma cell cultures purchasable for substance testing or in vitro testing of therapeutic approaches. This is in contrast to the fact that no pharmacological treatment has yet been established that prevents the development or progression of benign VS. Considering the risks involved in removing the tumor, the potential for research into a drug target for the treatment of VS would be of major importance. Preliminary studies on the implementation of an isolation technique of VS primary cells are provided only by Dilwali et al. (2015a,b,c); (2013); (2015d), whose protocol we adapted partially. However, for small VS it could be difficult to gain enough tissue material with conventional tissue removal. Since the recovery of primary cells from tissue obtained by CUSA has already been tested and established for a long time in other tumor entities such as gliomas, our derivation of primary cells was performed from the CUSA filter (Day et al., 2013; Malhotra et al., 1986). Because vestibular schwannoma does not infiltrate the surrounding tissue compared to glioma cells, the risk of contamination with non-tumor cells is even lower. S100 is the most commonly used schwannoma cell marker (Liu et al., 2015). Therefore, we used likewise the staining of S100 to characterize our primary cultures. The strong positive staining of cell clusters suggests that the cultured cells are schwannoma cells as described from previous authors (Kisselbach et al., 2009). We were able to demonstrate that the described method is suitable for the cultivation of schwannoma cells after microsurgical VS removal using the COT. The analyzed cell cultures showed a high variance in doubling time and expression of the proliferation marker Ki-67. Quantification of fibroblasts by flow cytometry revealed a minor percentage of fibroblasts in the cultures. Nevertheless, the cultivation of fibroblasts should be excluded before using the cultures for experiments (Kisselbach et al., 2009). In addition, the purity and identity of the culture should be verified after multiple passaging and cryopreservation. A transformation of the culture over time or an overgrowth by more proliferating fibroblast-like cells cannot be excluded generally. Nor indeed can it be guaranteed that the genotype or phenotype of the cells will change as the number of passages increases. This has already been shown for a number of other cultures (Hughes et al., 2007; Kim et al., 2017; Mouriaux et al., 2016; Oh et al., 2013). Dilwali and co-authors demonstrated that schwannoma cells retained 80 % purity for the majority of 12 weeks (Dilwali et al., 2014), which is in line with our observations. However, the cultures from CUSA obtained VS tissue have two advantages i) The material is already disintegrated by the ultrasound ii) The material is not suitable for histological examination and is normally discarded. In our view, this is an option, especially for smaller VS when less material can be obtained for research purposes. To summarize, the VS primary cells obtained from CUSA filters are suitable for various analyses. In addition, it could offer the opportunity to establish immortalized schwannoma cell lines from the primary cultures. In the long term, this method allows testing of pharmacological agents on the schwannoma cells to investigate a targeted drug therapy for VS.

5. Conclusion

The postoperative processing of a CUSA filter allows the culturing of schwannoma cells. These primary cultures are suitable for more precise analyses of the schwannoma cells.

CRediT authorship contribution statement

Sandra Leisz: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Clara Helene Klause:** Writing - original draft, Investigation, Formal analysis. **Anna-Louisa Becker:** Formal analysis, Writing – review & editing. **Maximilian Scheer:** Resources, Writing - review & editing. **Sebastian Simmermacher:** Writing – review & editing, Data curation. **Christian Strauss:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Christian Scheller:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2023.109955.

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