Slice cultures from head and neck squamous cell carcinoma: a novel test system for drug susceptibility and mechanisms of resistance

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Background: Human head and neck squamous cell carcinoma (HNSCC) fundamentally vary in their susceptibility to different cytotoxic drugs and treatment modalities. There is at present no clinically accepted test system to predict the most effective therapy for an individual patient.

Methods: Therefore, we established tumour-derived slice cultures which can be kept in vitro for at least 6 days. Upon treatment with cisplatin, docetaxel and cetuximab, slices were fixed and paraffin sections were cut for histopathological analysis.

Results: Apoptotic fragmentation, activation of caspase 3, and cell loss were observed in treated tumour slices. Counts of nuclei per field in untreated compared with treated slices deriving from the same tumour allowed estimation of the anti-neoplastic activity of individual drugs on an individual tumour.

Conclusion: HNSCC-derived slice cultures survive well in vitro and may serve not only to improve personalised therapies but also to detect mechanisms of tumour resistance by harvesting surviving tumour cells after treatment.

To bypass this conflict, an ongoing trial (DeLOSII) uses the tumour response after short induction chemotherapy as assumed indication of susceptibility for the planned therapeutic protocol. In addition, we have exposed tumour-derived cells cultured under flavin-free conditions (FLAVINO assay) to cytotoxic drugs in an attempt to test their efficiency ex vivo (Dollner et al, 2004; Dietz et al, 2010; Wichmann and Dietz, 2011; Wichmann et al, 2011; Schaefer et al, 2012). Using this system, we demonstrated differences in the response of epithelial vs stromal cells in HNSCC (Horn et al, 2010). As extracellular matrix (ECM) epitopes, which are lost in cell cultures, provide important signals for cell differentiation and survival (Schaefer et al, 2005; Babelova et al, 2009), we now established a protocol to culture organotypic slices from HNSCC and tested their response to established cytotoxic...
drugs (cisplatin, docetaxel and cetuximab) currently used in standardised treatment concepts for HNSCC.

Tumour-derived organotypic slice cultures provide a unique tool to study tissue responses to various stimuli, including drugs and irradiation (Nitsch et al, 2000; Merz et al, 2010; Rajendran et al, 2010; Vaira et al, 2010; Merz and Bechmann, 2011; Holliday et al, 2013). The open system allows direct access and the option to collect samples from media over time. At the end of the experiment, slices can be embedded in paraffin for histological analysis using for example, immune cytochemistry for the detection of DNA damage after irradiation (γH2AX), apoptosis (activated caspase 3), or proliferation (Ki-67) (Merz et al, 2013). Here, we show that HNSCC-derived slices survive well in culture for up to at least 6 days and exhibit the expected cell loss upon treatment with cytotoxic drugs rendering them a promising tool to predict tumour responses and to study mechanisms of tumour resistance.

**MATERIALS AND METHODS**

**Tumour slice preparation.** Informed consent was obtained from all patients, and the investigation was approved by the ethics committee of the Medical Faculty of the University Leipzig (votes No. 201-10-12072010 and No. 202-10-1207210). Tissue was obtained from patients during resection of HNSCC (tonsils, base
of tongue, larynx, pharynx or metastases in cervical lymph nodes) at the Clinic of Otorhinolaryngology, University Hospital Leipzig, Leipzig, Germany. The specimens for experiments were kept in tumour medium (TM, please see below) and transported to the lab, where they were put into an incubator until further preparation. In parallel, the tumour and its histology was classified by a board certified pathologist (CW). Tissue slices of 350 μm were prepared under sterile conditions either using a vibratome (Leica VT 1000, Leica Microsystems GmbH, Wetzlar, Germany) or a tissue chopper (McIlwain TC 752; Campden Instruments, Lafayette, IL, USA) between 3 and 48 h after surgery. Slices were then placed on the membrane culture inserts (Millipore Corporation, Billerica, MA, USA) in six-well-plates containing TM. TM is a custom-designed phenol red and riboflavin-free cultivation medium (RPMI 1640; **080611**). Figure 1. See caption for next page
Bio & Sell, Feucht, Germany) consisting of 1.134 g l⁻¹ NaHCO₃, 14.5 mM HEPES and 20 mg l⁻¹ l-cystine. TM was supplemented before use with 10% (v/v) fetal calf serum (PCS; Invitrogen, Darmstadt, Germany), amikacin, nystatin, penicillin and streptomycin (all from Sigma, Munich, Germany) and underwent sterile filtration (0.22 μm). Following the addition of PCS, TM contained 20 nM of riboflavin. Later, slices were cultivated in a humidified incubator at 5% CO₂ and 37 °C. Medium was changed at least twice a week.

Cytotoxic treatment of slice cultures. For treatment of slices, cisplatin ((SP-4-2)-Diamindichloridoplatin(II); DDP; Sigma), doxetaxel (Taxotere; 20 mg ml⁻¹; Sanofi-Aventis Deutschland GmbH, Berlin, Germany) and cetuximab (Erbitux; 5 mg ml⁻¹; Merck Serono, Munich, Germany) were used. Cisplatin was dissolved in sterile H₂O and diluted in culture medium to final concentrations of 0.66 and 3.33 μM. Before testing doxetaxel in its final concentrations of 0.55 and 0.275 μM, it was dissolved in ethanol and diluted in culture medium. Cetuximab was tested in a concentration of 66 μg ml⁻¹ and diluted in TM. The incubation period was up to 7 days, and medium was changed at least two times with newly prepared dilutions of drugs. After the treatment, slices were fixed and processed as described below.

Staining procedure for morphological analysis. After a minimum of 5 h and a maximum of 7 days, the tumour slices were fixed in paraformaldehyde (4%), washed with phosphate-buffered saline (PBS) and embedded in paraffin. Before staining with hematoxylin/eosin (HE) for histology, sections were cut on a microtome (4–7 μm). In order to perform immunohistochemical stainings, paraffin sections were dewaxed in xylene, rehydrated in graded alcohol and consecutively retrieved in citrate buffer at 96 °C for 10 min in a microwave. Sections were then rinsed in PBS/Triton (0.3%) and blocked with 5% normal goat serum in 0.3% PBS/Triton for 1 h. The primary antibodies against Ki-67 (Becton Dickinson, Franklin Lakes, NJ, USA; mouse, 1:200), cleaved caspase 3 (Cell Signaling Technology, Inc., Danvers, MA, USA; rabbit, 1:400), IBA1 (Wako Chemicals GmbH, Neuss, Germany; rabbit, 1:400), cytokertatin (Dako Denmark A/S, Glostrup, Denmark; Clone D5/16 B4, mouse, 1:150) and γH2AX (Millipore, mouse, 1:100) were allowed to incubate overnight at 4 °C. Sections were then rinsed again with 0.3% PBS/Triton and incubated with appropriate fluorescent-labelled secondary antibodies (goat-anti-mouse, goat-anti-rabbit, Alexa Fluor 568 and 488) and Hoechst 33342 for visualisation of nuclei. Images were taken and analysed using an Olympus BX51 fluorescence microscope (Olympus Europa Holding GmbH, Hamburg, Germany) or a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) for fluorescent staining and a Zeiss Axioplan 2 (Carl Zeiss AG) for HE staining.

Quantification of nuclei and analysis of cell death. Total cell numbers of sections were determined by counting nuclei in HE-stained slices with the Image J plug-in Cell Counter (NIH, Bethesda, MD, USA). Apoptosis was also determined using Cell Counter by identifying caspase-3-positive cells in relation to total cell number (Hoechst positive). Pictures taken at a magnification of ×400 were analysed to this end. Statistics were calculated with Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) (t-test for two or one-way ANOVA for more groups; P<0.05 was considered significant).

RESULTS

Slice culture of HNSCC. Tumour tissues obtained from patients after surgical resection were cut using a vibratome (Leica VT1000) or a tissue chopper (Mcllwain TC 752) at a thickness of 350 μm and transferred onto membrane inserts (Millipore) within six-well plates for further cultivation (Figure 1Aa). Owing to the texture of the tissue, some tumours could not be cut properly with a vibratome or consecutive sections revealed different thicknesses, whereas cutting with a tissue chopper provided better and more precise results.

For morphological analysis, HE stainings were performed on paraffin sections of tissue slices after a cultivation period of 5 h to 7 days. Slice cultures were viable and maintained their typical morphological features for up to 6 days in vitro (Figure 1Ad) as compared to the original diagnostic histopathology (Figures 1Ab and 1Ac). After 7 days, tissue quality was found to suffer in some tumour slices (not shown). Quantification of Hoechst-positive nuclei (Figure 1Ca) and morphological analysis of slices fixed at different time points within 6 days (Figures 1Cb and 1Cc) did not reveal severe tissue alterations within this period of time. Non-tumour cells, for example, endothelia (Figures 1Ae and 1Ah), stratified muscle cells (Figure 1Af) and glands (Figure 1Ag), revealed excellent preservation even after treatment with cytotoxic drugs (Figure 1Ah). In addition to morphological analysis by HE staining (Figures 1Ba, Bc Bb and Cc; Figures 2Ab and Ac; Figure 3;
Figure 4), evaluation of cytokeratin- or IBA1-positive cells (Figures 1Al and 1Aq)) was performed in order to investigate the cellular composition of the tissue. Cytokeratin-positive cells showed increased expression of activated caspase 3 after treatment compared with cytokeratin-negative cells and untreated controls (Figures 1Aq and 1Aq). Proliferation was detected by Ki-67 antibodies and nuclear counterstaining with Hoechst 33342 (Figures 1Bb and 1Bd; Figures 2Ad and 2Ae; Figure 2B). After 5 (Figure 1Bb) and 6 (Figure 1Bd) days in vitro, untreated slices maintained a high proliferative activity. With the help of γH2AX antibodies, double-strand breaks were visualised in slices before cultivation as well as in slices after 3 and 6 days of cultivation (Figures 1Cd–f). γH2AX foci were spread evenly in nuclei of all areas of the slice, and no distinct change was found at any of the analysed time points.

The membrane-supported cultivation technique produces two surfaces of the slice, one connected to the membrane and the medium and the other with contact to the air. Thus, vertical gradients in cell proliferation or apoptotic activity must be considered. Although it is not possible to distinguish between the upper and lower surface once fixation and automated embedding are done, transverse sections of the slices provide no evidence for proliferative or apoptotic gradients but detect evenly activated caspase 3 throughout the treated slices rather than showing predominant appearance at the surfaces (Figure 2B).

As the aim of this study was to evaluate effects of cytotoxic drugs in an attempt to establish a susceptibility assay, we restricted our further experiments to the period of 3–6 days in vitro.

An overview of the tissue samples used in this study and the experimental setup is given in Table 1.

**Treatment of HNSCC slices with cytotoxic drugs.** Slices were treated with cisplatin (3.33 and 6.66 μM; Desoize et al, 1996), cetuximab (66 μg ml⁻¹) and docetaxel (0.275 and 0.55 μM; Bisset et al, 1993) throughout the cultivation period of 3–6 days. Control slices of the same tumour were not exposed to cytotoxic drugs but cultured and fixed simultaneously. Potential cytotoxic effects of ethanol as diluent of docetaxel could not be detected, neither by HE-staining and count of nuclei (Figure 2Aa–c) nor by antibody staining against Ki-67 or caspase 3 (Figures 2Ad and 2Ae). All slices were then embedded in paraffin for histological analysis. In HE-stained sections of samples treated for 6 days, a higher number of fragmented nuclei was present compared with untreated controls; moreover, pyknotic alterations of nuclei, increased cellular polymorphisms and fragmented nuclei (Figures 3B–F) were prominent after cytotoxic treatment.

Next, we tested whether chemotherapy-induced changes could also be observed after reducing the period of treatment to 2 days. Slice cultures derived from a lymph node metastasis of a supraglottic squamous cell carcinoma were kept for 1 day without treatment and then exposed to chemotherapy for 2 days. Cell loss was evident in sections from all treated slices but not in controls (Figures 4B–G). Using the Image J plugin Cell Counter, we then quantified the numbers of nuclei. This analysis also revealed highly significant, dose-dependent cell loss induced by cytotoxic
be due to the lack of translational research efforts from bedside to bench and back to bedside in most of the technologies. More importantly, a potential source of artifacts relies in the singularisation of cells using trypsinisation with the resulting isolation from their normal environment, which significantly impacts on tumour growth (Pietras and Ostman, 2010; Hanahan and Weinberg, 2011). For example, ECM epitopes provide important differentiation cues, for example, by signalling via Toll-like receptors (Schaef er et al, 2005; Senner et al, 2008; Moreth et al, 2010; Merline et al, 2011) and integrins (Abdollahi et al, 2005; Alghisi et al, 2009). Aiming at partial preservation of such signalling pathways, we previously used collagenase IV, which causes particle separation rather than complete cellular isolation (Wichmann et al, 2009; Dietz et al, 2010; Wichmann and Dietz, 2011). Moreover, we used ECM proteins for coating of polystyrene surfaces allowing for specific interaction of integrins and other receptors with ECM, to further mimic in vivo conditions in the FLAVINO assay (Wichmann et al, 2009; Dietz et al, 2010, 2012; Horn et al, 2010; Schrader et al, 2012).

Here, we established organotypic slice cultures, which we have previously used to study mechanisms of neuroinflammation and neurodegeneration in rodent (Klüge et al, 1998; Hailer et al, 2001; Dehghani et al, 2003; Eryü poğlu et al, 2003; Prodinger et al, 2011) and human neural tissues (Nitsch et al, 2000; Merz et al, 2013). In the latter (deriving from epilepsy surgery), we have shown that human tumour-necrosis-factor-related-apoptosis-inducing ligand, which is not toxic in mice (Walczak et al, 1999), induced lysis of non-transformed brain cells (Nitsch et al, 2000). Such highly relevant species differences gained world-wide attention after the ‘London tragedy’ in 2006, when six volunteers developed toxic shocks and cytokine storms after administration of TGN1412, an anti-CD28 antibody, which was tested before in animals (Kenter and Cohen, 2006; Dowsett and Kendall, 2007; Merz and Bechmann, 2011; Seok et al, 2013), further arguing for the development of human test systems for drug toxicity. The London incidence may explain the current renaissance of this idea (van der Kuip et al, 2006; Merz et al, 2010; Vaira et al, 2010; Merz and Bechmann, 2011) despite the ASCO’s decision to abolish chemosensitivity testing in 2004 due to a lack of reliability or clinical practicability of available assays at that time (Schinköethe et al, 2007). This is where we see a potential advantage of the human tissue slice culture technology: they can quickly be set up while the complex cross-talk between matrix and cells as well as the individual cellular composition and susceptibility to drugs is preserved. In addition, healthy surrounding tissues such as stratified muscle cells and glands also exhibited excellent preservation (Figures 1Af and 1Ag). Vivid proliferation was still visible after 6 days in vitro using Ki-67 staining (Figures 1B, 2A and 2B). In untreated slices, it was possible to distinguish between the different components of the tissues and to evaluate the composition of the slice, even if the slices are certainly heterogeneous.

Following treatment with cytotoxic drugs, nuclear fragmentation was abundantly present in HE sections, and apoptosis induction was confirmed using antibodies to activated caspase 3 (Figures 3 and 5). Quantification of nuclei revealed significant cell loss after treatment (Figure 4). Whereas we could show late effects of the tested drugs by the visualisation of lower cell density through cell loss, earlier stages of apoptosis could be quantified by the cleavage of caspase 3. As the individual tumours react at different rates, it is important to consider both early and late stages of cell death, which is why we quantified both features in the samples.

We could show that activated caspase 3 is spread evenly throughout the slice rather than showing predominant appearance at the surfaces (Figure 2B) proving that drug penetration into the slices takes place. As for potential side effects of the drugs used here, we cannot make judgment on systemic effects, but we can
state that damage to the surrounding tissue would be visible in our experiment if it was consistently caused by an individual drug. As systematical follow-up of every aspect in each of the tumours tested was not possible due to the limited sample volume not needed for proper diagnosis, different aspects (e.g., viability over time or reaction to chemotherapeutics) therefore had to be addressed with different tumours.

The next step will be to test in a prospective study whether and which parameters of susceptibility to treatments of an individual tumour correspond to the respective patient’s response and clinical outcome. Only if this is the case, cultivation conditions can be regarded as mimicking the in vivo situation faithfully.

The combined analysis of cell death (caspase 3 and cell density) is certainly time consuming and relies on an experienced, un-biased investigator. Instead of counting cells by hand or measuring cell density, we believe that measurement of biochemical markers in the supernatant or in homogenates of the slices will provide a more feasible readout of such an assay. Histology performed in our study was primarily designed to establish the tissue quality and longevity as well as detecting the effects of drugs in principle. For the general use in laboratory routine, the readout should be made as standardised and least time consuming as possible. Furthermore, kinetics are not uniform in all tumours, and care must be taken not to oversee drug effects when using cell density as paradigm. Therefore, we are testing alternate approaches to quantify cell death, for example, using soluble markers (LDH, M30, CK18) present in the TM, which may also allow for monitoring treatment-induced degeneration over time, or tissue homogenates for the analysis of RNA and/or proteins.
### Table 1. Detailed information about tissue samples used in this study

| Sample     | Tissue type/origin                  | Duration of cultivation | Slices acquired with | Fixation time |
|------------|------------------------------------|-------------------------|----------------------|---------------|
| **200111** | Carcinoma of the larynx           | 5h, 6d                  | Method establishment | Vibratome     |
| **190411** | Carcinoma of the trachea          | 1d, 2d, 6d, 9d, 12d    | Method establishment | Vibratome     |
| **270611** | Carcinoma of the tonsil           | 6d                      | See data (Figures 1A and 1B) | Vibratome |
| **080611** | Metastasis of an oropharyngeal carcinoma | 5d, 7d                  | See data (Figure 1B)  | Vibratome |
| **120911** | Metastasis of a carcinoma of the tonsil | 6d                      | See data (Figure 3)   | Tissue chopper |
| **071111** | Carcinoma of the base of tongue   | 3d, 6d                  | See data (Figures 1C and 5) | Tissue chopper |
| **070512** | Carcinoma of the larynx           | 4d                      | Method/analysis establishment | Tissue chopper |
| **160512** | Carcinoma of the base of tongue   | 4d                      | Method/analysis establishment | Tissue chopper |
| **240512** | Carcinoma of the base of tongue   | 4d                      | Method/analysis establishment | Tissue chopper |
| **020712** | Metastasis of a supraglottic carcinoma | 3d, 5d                  | See data (Figure 4)   | Tissue chopper |
| **040713** | Carcinoma of the pharynx          | 3d, 5d                  | See data (Figures 1A and 1C) | Tissue chopper |
| **040713** | Metastasis of a carcinoma of the pharynx | 3d, 5d                  | See data (Figures 1A, 2A and 2B) | Tissue chopper |

This table presents the type of the tumour tissues used in this study, the duration of cultivation, the cutting method, the use of each tumour and where the data are shown as well as the fixation time. The approach in our study was the following: We first tested vibratome sections (samples **200111, **190411, **270611 and **080611) of which only three could be kept in culture with good preservation until day 6 (**200111, **270611, **080611). As we found evident deterioration at day 7 in sample **080611, we did not focus any longer on establishing a maximal survival time for individual tumours. Our reasoning was that for the purpose of the method, it is much more important to establish a minimum survival time suitable to test drug effects. We found that slices cut with a tissue chopper were faster to obtain and more uniform in thickness and exhibited very good tissue preservation until day 6. Therefore, we looked at the effects of treatments on day 4 (samples **070512, **160512 and **240512) or – if there was enough tissue – on days 3 and 5 (samples **020712, **040713TM and **040713Mtx).

**Figure 5.** Activation of caspase 3 after treatment. (A) The number of Hoechst-positive nuclei in paraffin sections of a carcinoma of the base of the tongue did not reveal significant effects of treatments. (B–F) However, in sections co-stained with antibodies against activated caspase 3 (green), increased apoptosis was evident in all the treated samples compared with controls, but only docetaxel (DTX) treatment was considered significant (21.3% relating to total number of Hoechst-stained nuclei vs 2.5% in control). After treatment with cisplatin, 6.2% of all nuclei showed activated caspase 3, and after treatment with cetuximab, 5.4% of the cells were positive. ***P < 0.0001.
However, our current data demonstrate that HNSCC-derived slices can be kept in culture, may serve as prediction assay and can be used to better understand the mechanisms of tumour resistance by harvesting surviving tumour cells after treatment. Decision making between highly crucial different treatment options such as mutilating laryngectomy or organ-preserving primary chemoradiation (with potentially severe late complications) would highly benefit from a reliable predictive test system.

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