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ORIGINAL ARTICLE

Heterogeneity of epithelial and stromal cells of head and neck squamous cell carcinomas in ex vivo chemoresponse

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Abstract
Background Valid prediction of the effectiveness of chemotherapeutic agents in individual head and neck squamous cell carcinoma (HNSCC) is desirable and might be achieved using ex vivo assays.
Methods Three biopsies from each of 15 HNSCC were taken, minced and digested by collagenase. The digested HNSCC was added to serial dilutions of either cisplatin (CIS) or docetaxel (DTX), which were prepared under flavin-protecting conditions in ECM-coated microtiter-plates. After 72-h incubation, cultures were methanol-fixed and Giemsa-stained. The cutoff concentration (COC; concentration completely suppressing colony formation) for epithelial cells (EC) and stromal cells (SC) was evaluated.
Results 12/15 HNSCC (80%) were evaluable. Despite significant correlation of COC of CIS in respect of colony formation of EC or SC, no significant differences in response of individual HNSCC specimens were found in the *t* test for paired samples ($p > 0.16$). The same applied to DTX. However, EC and SC showed heterogeneity in chemoresponses leading to COC variability of more than one titration step in 44.1% (CIS) and 20% of HNSCC (DTX). No significant correlation between the COC of both cell populations was found in HNSCC specimens.
Conclusions The ex vivo chemoresponse of EC and SC of HNSCC must be analyzed separately.

Keywords Heterogeneity · HNSCC · Ex vivo chemoresponse · Epithelial cells · Stromal cells

Introduction

During the last five decades, many in vitro chemoresponse assays were developed with the aim of achieving information on a tumor's sensibility or resistance to cytostatic drugs [1–3]. Especially in the 1980s and 1990s, predictive tests were quite popular and the subject of many studies [4–8]. But due to severe limitations of these test protocols [9–11], and corresponding increased skepticism, chemoresponse assays were never included in the clinical routine.

Over the same time period, chemotherapy in head and neck oncology has changed from an only palliative treatment to an important component in multimodal therapies. For example, in larynx organ preservation protocols, induction chemotherapy followed by irradiation has been proven to be sufficient with moderate late toxicity outcomes in nearly every second patient with head and neck squamous cell carcinoma (HNSCC) [12]. But chemotherapy (CT) and chemoradiation are not always curative since the responses to treatment of individual tumors differ widely. Moreover, the necessary doses required to have an effect on the individual tumor's growth are not known. Applying inadequate doses of chemotherapeutics probably also induces further increase of chemo-resistance in HNSCC to the applied agents and possibly additionally also to other potential

For the Head and Neck Cancer study group.

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therapeutics [13–15]. There is a high risk of ending up with inefficient, but nevertheless toxic, treatment of HNSCC patients.

Until the last 10 years, there was only limited need for predictive assays of HNSCC because of the small number of approved cytostatic agents and the limited number of first and second-line treatment protocols in use that conformed to guidelines. But today (and in particular, in the contradictory context of increasing number of new emerging cytostatic compounds and biologicals, allowing for chemotherapy or radiochemotherapy expanded by targeted therapy, and also the possibility to choose the false treatment modality), there is increasing pressure to select correctly the most appropriate pharmaceuticals and to apply individualized treatments adapted to the requirements of the particular HNSCC. This can be resolved only on the basis of knowledge of the individual tumor's reactivity to the eligible compounds in the context of maximum acceptable dosage. Thus, the need for an ex vivo chemoresponse assay in head and neck oncology to predict the success of individual types of multimodal therapy protocols has increased. However, conventional in vitro testing was judged to be useless as a predictive assay [10, 11]. Indeed, more efficient solutions to overcome critical limitations of conventional in vitro assays by utilizing flavin-protecting conditions [15] to eliminate flavin-mediated photochemical artifacts [16] are available. Advantageously, they are quality controlled, and allow also for earlier availability (4 days) of data with respect to dosages required to achieve the intended response of the individual HNSCC [17, 18].

To use such a predictive assay appears to be even more important, since only a small number of markers for chemoresponse to HNSCC has been known until now, partially with contrary interpretations of their meaning [19–22]. Also, all of the so far known markers do not accurately predict the individual sensibility or resistance of a given tumor. Furthermore, cell line-based chemosensitivity testing in highly selected tumors do not reflect the needs of average tumor behavior in individual patients.

We already used this predictive short-time ex vivo colony-forming assay to confirm that the chemoresponse of epithelial cells within a biopsy in principal is representative of a given HNSCC [17]. But within that study, the ex vivo chemoresponse of stromal cells was not analyzed. Moreover, it might be mistakenly concluded from the data shown in [17] that any biopsy of a tumor provides sufficient information about the chemoresponse of all cell types present within the tumor. Thus, the aim of this consecutive study was to analyze potential differences in the chemoresponse of epithelial and stromal cells in individual HNSCC. Therefore, three separate biopsies of an HNSCC ("fragments") were taken from spatially distant regions. Subsequently, the chemoresponse of epithelial and stromal cells was obtained using our ex vivo colony-forming assay [18] and analyzed regarding differences in the chemoresponse of epithelial and stromal cells, as well as representativeness of a single specimen for the other tissue samples and thus for the whole HNSCC.

Materials and methods

Patients and HNSCC specimens

Included in this study were 15 patients with histologically confirmed primary HNSCC. The stages following criteria of the American Joint Committee on Cancer (AJCC [23]) were as follows: 8.3% stage I, 16.7% stage II, 16.7% stage III and 58.3% stage IV (Table 1). After obtaining patient's informed consent, three biopsies (>60 mg) from spatially distant tumor areas were taken during surgical tumor resection under general anesthesia. The tissue samples were placed in tubes containing tumor medium (TM) and immediately transferred to the laboratory, where thse were weighed and processed under flavin-protecting conditions (see below).

Materials

TM was used as cell culture medium for chemoresponse testing of the HNSCC samples as well as of the KB cells for quality control. TM consisted of a phenol red- and flavin-free RPMI 1640 (Biochrom®, Berlin, Germany) supplemented by

Table 1 Age, gender, localization and stage of disease with respect to AJCC criteria of patients included in the study and TNM classification of the HNSCC, of which three tissue specimens were investigated in the ex vivo chemoresponse test

Patient ID	Age	Gender	Localization of HNSCC	Stage (AJCC)	TNM classification
1	58	Male	Oral cavity	I	$T_1 N_0 M_0$
2	62	Male	Oropharynx	IVa	$T_2 N_{2b} M_0$
3	57	Male	Oropharynx	IVa	$T_2 N_{2b} M_0$
4	52	Female	Oropharynx	IVa	$T_2 N_{2b} M_0$
5	42	Male	Larynx	IVb	$T_{4b} N_{2c} M_0$
6	45	Male	Oropharynx	IVa	$T_2 N_{2b} M_0$
7	65	Male	Oropharynx	IVa	$T_2 N_{2b} M_0$
8	50	Male	Oropharynx	III	$T_3 N_1 M_0$
9	53	Male	Oropharynx	IVa	$T_1 N_{2b}$ M0
10	71	Male	Hypopharynx	III	$T_3 N_0 M_0$
11	50	Male	Oral cavity	II	$T_2 N_0 M_0$
12	52	Male	Oropharynx	II	$T_2 N_0 M_0$
13	31	Male	Sphenoid bone	IVa	$T_{4a} N_0 M_0$
14	58	Male	Larynx	IVa	$T_{4a} N_{2c} M_0$

10% (v/v) fetal calf serum (FCS; Biochrom), amikacin, nystatin, penicillin and streptomycin (all from Sigma, Munich, Germany). After addition of FCS, the TM contained 20 nM of riboflavin. Consequently, all the following experimental steps were carried out utilizing flavin-protecting conditions (avoidance of adverse flavin-mediated photo-induced reactions by exclusive illumination using Philips SOX-E sodium-discharge lamps with monochromatic excitation at the wavelength of $\lambda = 589$ nm). Cisplatin was purchased from Sigma. Docetaxel (Taxotere®) was purchased as a pharmaceutical preparation from Sanofi-Aventis Deutschland GmbH (Berlin, Germany).

KB cell chemoresponse assay

For quality control purposes and examination of reproducible test conditions, along with each HNSCC colony-formation assay, a KB cell chemoresponse assay was carried out simultaneously as previously described [17]. Briefly, KB cells from stock cultures adapted to RPMI 1640 free of phenol red and containing only 20 nM of riboflavin were harvested and adjusted to give a final number of 10^4 KB cells/well. The KB cell suspension was added to wells of microtiter plates containing dilutions of either cisplatin or docetaxel derived from those dilutions created for the ex vivo test of the individual HNSCC. KB cells were harvested after 72 h of incubation (36.5°C, 3.5% CO_2, humidified air) and counted using a semi-automated cell counter (Casy TT®, Schärfe System, Reutlingen, Germany). The inhibitory concentrations (IC) of cytostatic drugs, leading to diminished proliferation or even cytotoxicity and causing a drop in cell counts to 50% of the cell numbers counted in control wells receiving only medium (IC_{50}), were calculated. These IC_{50} values were compared with the previously published IC_{50} values for KB cells [24, 25].

HNSCC colony-formation assay

During tumor resection of 15 HNSCC, biopsies within a mass range from 34 to 676 mg were obtained. The tissue samples of each HNSCC were handled separately. Each sample was minced into pieces of about 1 mm^3, transferred into pre-warmed TM and disintegrated using collagenase type IV (230 U/ml; Sigma). After 16 h of incubation, the digests were washed by centrifugation. The pellet of disintegrated HNSCC was resuspended and pipetted into wells of ECM-coated microtiter plates (Paesel & Lorey, Hanau, Germany) containing serial dilutions of cisplatin or docetaxel. In consequence of the sometimes limited sample size, and despite the aim of starting the chemoresponse test by adding 2 mg of collagenase digest, only 1.91 ± 0.13 mg (mean ± SEM) of HNSCC digests were seeded per well of the microtiter plate. The applied concentrations of the chemotherapeutics were chosen based on the data specifying the tolerable plasma level (TPL) in humans (cisplatin, 6.67 µM [26]; docetaxel, 0.55 µM [27]). After 72 h at 36.5°C and 3.5% CO_2 in humidified air, supernatants were discarded and the wells gently washed twice using phosphate-buffered saline. Adherent cells and cell colonies were fixed with 90% (v/v) methanol and air-dried. Giemsa staining allowing for differentiation of epithelial and stromal cell colonies was performed as described [17].

Cutoff analyses and statistical investigations

Colonies were counted independently by two well-trained investigators using a Zeiss Axiovert 200 M inverted microscope (Zeiss, Jena, Germany). Afterward, the results were compared, discussed and deviant findings reanalyzed together to achieve concordance in interpretation of microscopic observations and to accomplish decision-making by consensus.

A colony was defined as a cell group consisting of at least eight cells of the particular cell type. Endothelial colonies and those of fibrocytes were summarized as stromal colonies. Their numbers were compared with those of epithelial colonies. Cutoff analyses were performed only if sufficient colony formation (i.e., at least two epithelial and two stromal colonies of ≥8 cells each) was detected in all control wells without any chemotherapeutics.

Cutoff values for epithelial as well as stromal cell colonies of the individual HNSCC fragments were compared in the *t* test for paired samples regarding correlation and significance of differences using SPSS Statistics 15.0 for Windows version 15.0.1 (SPSS Inc., Chicago IL, USA). A p value less than 0.05 was regarded to be significant.

Results

Types of ex vivo formed colonies derived from HNSCC specimen

After Giemsa staining, differentiation of epithelial and stromal colonies was carried out in each well of the microtiter plates by microscopic examination as described before [17]. In general, epithelial cells are represented by big cuboid cells with wide cytoplasm margin and a big round cell core. Fibroblasts, fibrocytes and endothelial cells were summarized as stromal cells. Fibrocytes and fibroblasts grew fusiform. They had a small cytoplasm margin and small oval cell cores. The fibroblast colonies showed fish-bone-like growth patterns. The endothelial cells, in contrast, grew triangle like and had a wider cytoplasm margin, but usually also a bigger round cell core than the fibroblasts. Examples of the different colony types consisting of epithelial and stromal cells are shown in Fig. 1.

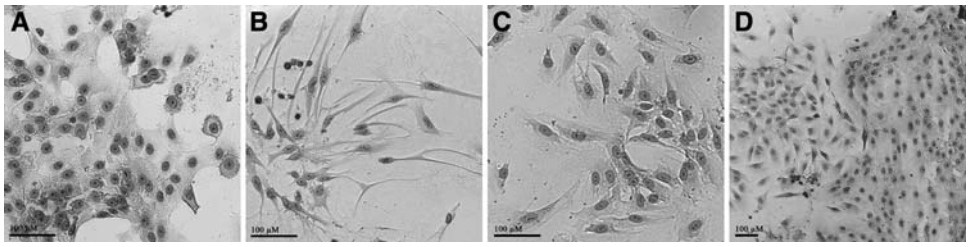

Fig. 1 Giemsa-stained colonies of an HNSCC cultured 3 days in the flavin-protecting ex vivo colony-formation assay. **a** Epithelial cell colonies. **b** and **c** stromal cell colonies consisting of either fibrocytes (**b**) or endothelial cells (**c**). **d** Mixed colonies (simultaneous appearance of epithelial and stromal colonies in close proximity) *Bar* 100 μM

Evaluability of chemoresponse tests

A colony-formation assay was regarded as evaluable, only if at least two of the three fragments showed sufficient colony formation of epithelial, as well as stromal, cells in control wells, and, in addition, the KB cell chemoresponse assays confirmed the correct efficiency of the cytostatic drugs in the determination of the corresponding IC_{50} value (±30%). Of the 15 colony-formation assays, 12 with cisplatin as well as docetaxel met these inclusion criteria for cutoff determination. This corresponds with a success rate of 80%. In some tumors, only two samples were evaluable. Three assays showed no colony formation in all of the three fragment samples, but this was not caused by technical limitations or contaminations. Neither of the tests had to be excluded because of bacterial or fungal contamination. All 14 concomitant KB cell experiments confirmed the predetermined IC_{50} values of 200 nM cisplatin [24] (220 ± 8 nM corresponding to 110 ± 4%) and 280 pM docetaxel [25] (280 ± 8.1 pM corresponding to 100 ± 2.9%). These data show that all experiments were performed under reproducible conditions and therefore allow for reliable quantification of the effective chemotherapeutic dosage in the colony-formation assay.

Cutoff determination with respect to formation of epithelial cell colonies

Since cutoff determination was carried out separately for epithelial and stromal cell colonies for both cytostatic drugs, the outcome of cutoff determination is shown separately for these major types in Table 2.

Following cisplatin treatment, the range of cutoff concentrations for colony formation of epithelial cells varied essentially between the different tumors. For individual HNSCC, cutoff concentration of 3.3 up to >50 μM was observed. In contrast, the cutoff for the three fragments of a given tumor was reached at almost identical concentration. In 8 of the 12 tumors, the cutoff was identical in all fragments. Regarding the remaining four HNSCC, the cutoff variation between the fragments was only one titration step. Cutoff was reached in all assays below the highest tested cytostatic concentration of 213 μM (corresponding to 32-fold TPL). For epithelial cell colony formation, there was a significant correlation ($R^2 > 0.78$) between the HNSCC fragments with respect to their chemoresponse to cisplatin (Fig. 2). However, the *t* test for paired samples revealed that there were no significant differences ($p > 0.34$).

Cutoff concentrations of docetaxel in colony formation by epithelial cells were in the range of 0.5 to >17.60 μM. Also, the intratumoral cutoff variation was only marginal (Table 2). Cutoffs were identical for all HNSCC fragments in eight cases and varied in only one titration step in the other four assays. Colony formation at the highest tested docetaxel concentration (17.60 μM) was detectable in all assayed fragments of six HNSCC. Two HNSCC showed high resistance to docetaxel in one and two of the three tested fragments, respectively. A significant correlation ($R^2 > 0.71$) was found between the separate tumor fragments for epithelial cell colony formation following docetaxel treatment (Fig. 2), but significant differences could not be shown in the *t* test for paired samples ($p > 0.18$).

Cutoff determination respective to formation of stromal cell colonies

Intertumoral cutoff concentrations of cisplatin for stromal cell colonies were reached in all tests below the highest tested cisplatin concentration of 213 μM. They ranged from 1.67 to >106 μM. In contrast to this high intertumoral variability, the intratumoral cutoff concentrations varied only marginally (Table 2; Fig. 3). This corresponds to the observations in the analyses of colony formation by epithelial cells. Cutoff concentrations were identical for all fragments in eight assays and differed in four assays only by one titration step (Table 2). There was a significant correlation ($R^2 > 0.98$) regarding the stromal colony formation between the HNSCC fragments without significant differences in the *t* test for paired samples ($p > 0.17$).

With regard to docetaxel, the intertumoral range of cutoff concentrations for stromal cell colonies was 1.10–17.60 μM, whereas the variability of intratumoral cutoff

Table 2 Cutoff concentrations (concentrations of either cisplatin or docetaxel required to suppress formation of colonies) for three tissue samples of individual HNSCC with respect to colonies of epithelial and stromal origin

Patient ID	Fragment	Cutoff concentration for epithelial colonies (μM)		Cutoff concentration for stromal colonies (μM)	
		Cisplatin	Docetaxel	Cisplatin	Docetaxel
01	01-1	53.33	0.55	13.33	8.80
	01-2	53.33	1.10	13.33	8.80
	01-3	53.33	0.55	13.33	8.80
02	02-1	26.67	8.80	1.67	8.80
	02-2	26.67	8.80	1.67	8.80
	02-3	53.33	8.80	1.67	8.80
03	03-1	3.33	4.40	13.33	17.60
	03-2	3.33	4.40	13.33	8.80
	03-3	6.67	4.40	13.33	8.80
04	04-1	13.33	17.60	13.33	17.60
	04-2	13.33	17.60	13.33	17.60
	04-3	13.33	17.60	6.67	17.60
06	06-1	13.33	17.60	106.67	17.60
	06-2	13.33	17.60	106.67	17.60
	06-3	13.33	17.60	106.67	17.60
07	07-1	13.33	17.60	26.67	17.60
	07-2	6.67	17.60	26.67	17.60
	07-3	6.67	17.60	26.67	17.60
09	09-1	13.33	17.60	26.67	17.60
	09-2	13.33	17.60	26.67	17.60
	09-3	6.67	17.60	26.67	17.60
10	10-1	6.67	17.60	6.67	8.80
	10-2	6.67	17.60	13.33	8.80
	10-3	6.67	17.60	13.33	8.80
11	11-1	13.33	8.80	13.33	1.10
	11-2	13.33	8.80	6.67	1.10
	11-3	13.33	8.80	6.67	1.10
12	12-1	13.33	8.80	13.33	8.80
	12-2	13.33	8.80	13.33	8.80
	12-3	13.33	17.60	6.67	8.80
13	13-1	3.33	17.60	n.e.	17.60
	13-2	3.33	8.80	3.33	8.80
	13-3	3.33	17.60	3.33	17.60
14	14-1	26.67	17.60	13.33	17.60
	14-2	26.67	17.60	13.33	17.60
	14-3	n.e.	n.e.	13.33	8.80

concentrations was small. In nine cases, all fragments of a given tumor had identical cutoffs, while this parameter varied only by one titration step in the three divergent cases. In 7 out of 12 chemoresponse tests, stromal cell colony formation was detectable up to the highest tested docetaxel concentration (17.60 μM). Despite the significant correlation ($R^2 > 0.55$), the *t* test for paired samples revealed that following docetaxel treatment, there were no significant differences among the cutoff values of the three fragments ($p > 0.17$).

Comparison of the cutoff concentrations in formation of epithelial and stromal cell colonies

Chemoresponse assays with absence of either epithelial or stromal colonies in control wells were not included in this comparison. Consequently, 69 of 90 single fragments (76.7%) from the total 15 tested HNSCC specimens were evaluable for differences with regard to cutoff concentrations of both chemotherapeutics in the formation of epithelial and stromal cell colonies.

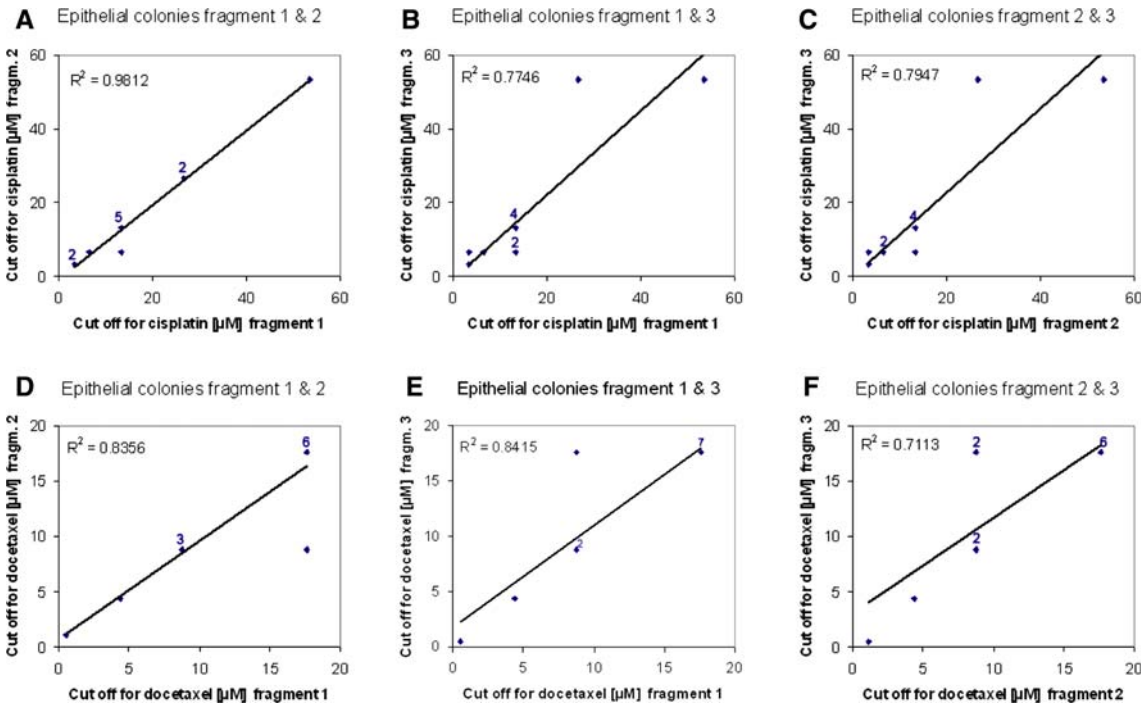

Fig. 2 Correlation of cutoff concentrations in the formation of epithelial cell colonies of the three fragments of the 12 HNSCC, fulfilling inclusion criteria for cutoff determination. **a–c** Cutoff concentrations of cisplatin. **d–f** Cutoff concentrations of docetaxel. As shown by the coefficient of determination (R^2), a significant correlation between cutoff concentrations of the three fragments is generally found. If identical cutoff concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates was inserted in *Arabic numbers*

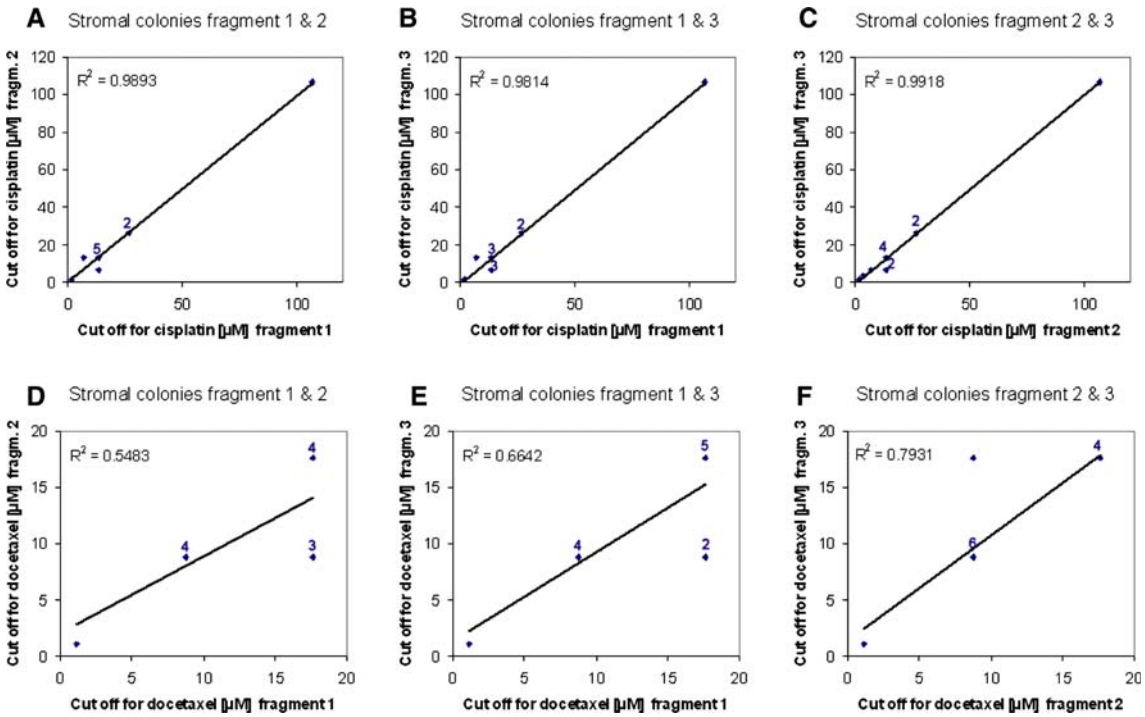

Fig. 3 Correlation of cutoff concentrations in the formation of stromal cell colonies of the three fragments of the 12 HNSCC, fulfilling inclusion criteria for cutoff determination. **a–c** Cutoff concentrations of cisplatin. **d–f** Cutoff concentrations of docetaxel. As shown by the coefficient of determination (R^2), a significant correlation among the cutoff concentrations of the three fragments is generally found. If identical cutoff concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates was inserted in *Arabic numbers*

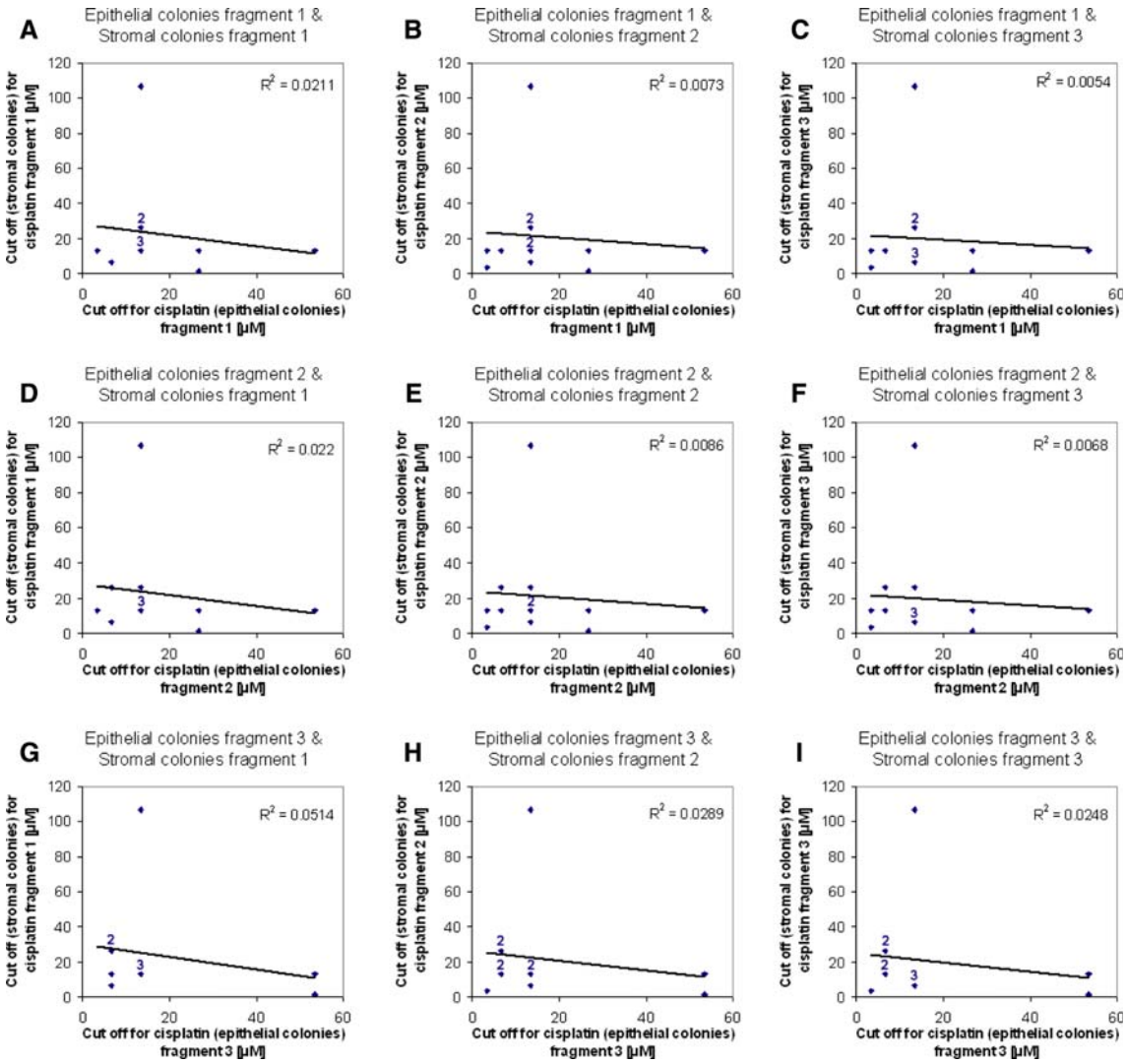

Fig. 4 Correlation of cutoff concentrations of cisplatin in the formation of epithelial (abscissa) and stromal cell colonies (ordinate) of the three fragments of the 12 HNSCC, fulfilling inclusion criteria for cutoff determination. **a–c** Cutoff concentrations for epithelial cells of fragment one and stromal cells in the three fragments. **d–f** Cutoff concentrations for epithelial cells of fragment two and stromal cells in the three fragments. **g–i** Cutoff concentrations for epithelial cells of fragment three and stromal cells in the three fragments. As shown by the coefficient of determination (R^2), a significant correlation among the cutoff concentrations of the three fragments is absent. If identical cutoff concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates was inserted in *Arabic numbers*

For cisplatin, suppressive activity in colony formation of epithelial and stromal cells was different, in general. Thus, no significant correlation was found in the colony-formation ability of the epithelial and stromal cells (Fig. 4).

The cutoff concentrations of docetaxel in colony formation of either epithelial or stromal cells were found to be more often identical within one individual HNSCC fragment. On the contrary, the cutoff values showed a much higher variability following cisplatin treatment (Table 2; Fig. 4) and also varied between the fragments of the same HNSCC in more than one titration step. This outcome was more frequently observed in response to cisplatin (44.1%) than to docetaxel treatment (only 20% of the cases; Table 3). Furthermore, it could be demonstrated that altogether 18.9% of the HNSCC showed an elevated chemoresistance of formation of stromal colonies in comparison to those of epithelial colonies. Noticeable also, the share of high-resistant stroma cells was higher in the treatment by cisplatin than following administration of docetaxel (26.4 vs. 11.4%). Thus, the chemoresponse of epithelial and stromal cells was found to be more homogeneous with docetaxel treatment than treatment with cisplatin (Tables 2, 3; Figs. 4, 5). However, a significant correlation of cutoff concentrations of docetaxel in the colony formation of epithelial and stromal cells of HNSCC was not found (Fig. 5).

Table 3 Comparative overview on the cutoff concentrations of cisplatin and docetaxel in the suppression of the formation of epithelial and stromal cell colonies

Chemotherapeutic agent	Number of tissue samples[a]	Cutoff concentration, C					
		$C_{epithelial} = C_{stromal}$		$C_{epithelial} = C_{stromal}$		$C_{epithelial} = C_{stromal}$	
		N	(%)	N	(%)	N	(%)
Cisplatin	34	06	17.7	19	55.9	09	26.4
Docetaxel	35	03	08.6	28	80.0	04	11.4
Sum	69	09	13.0	47	68.1	13	18.9

[a] The number given represents the number of tissue specimens, fulfilling inclusion criteria regarding formation of at least two colonies of both types of cells differentiated

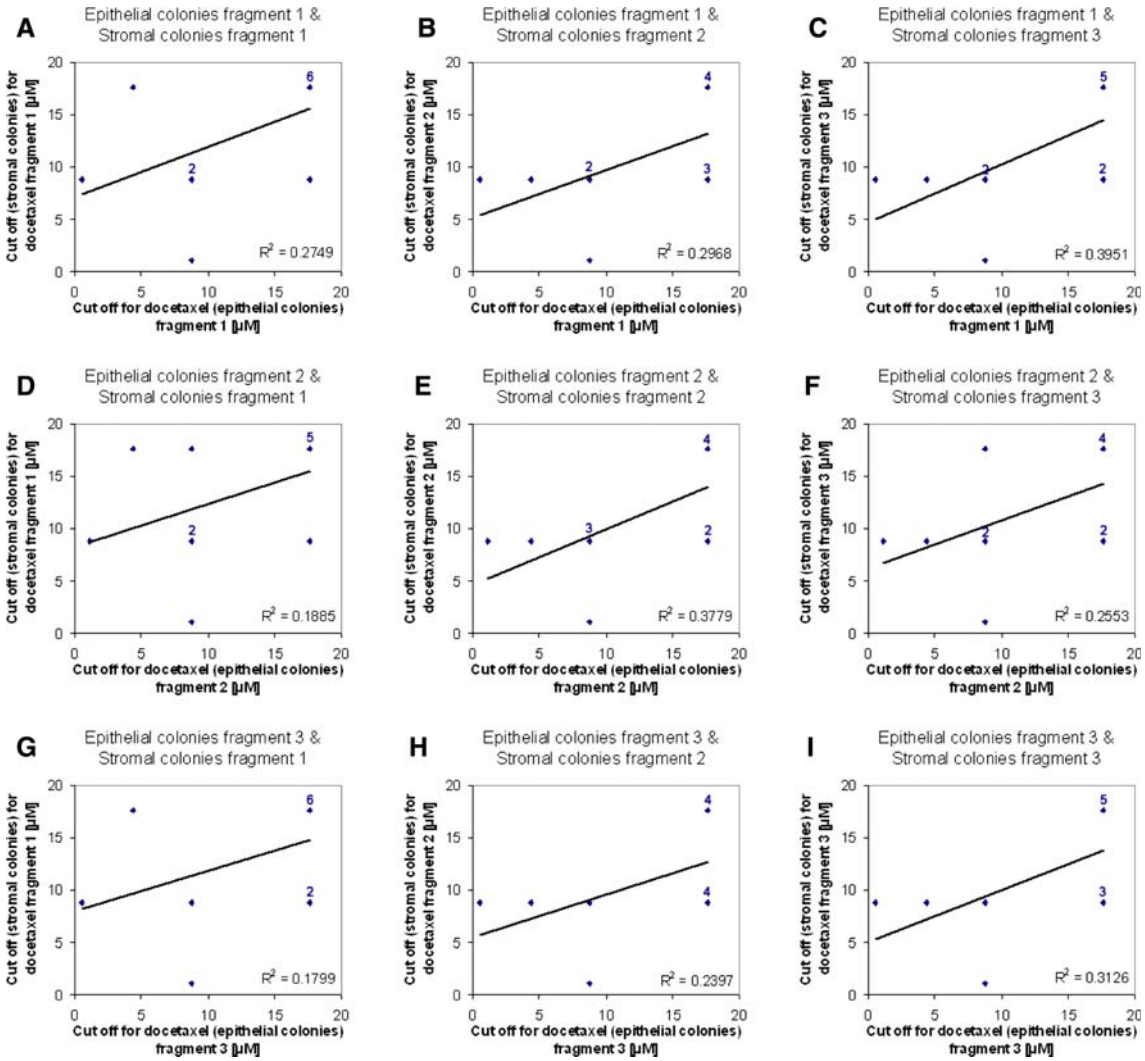

Fig. 5 Correlation of cutoff concentrations of docetaxel in the formation of epithelial (abscissa) and stromal cell colonies (ordinate) of the three fragments of the 12 HNSCC, fulfilling inclusion criteria for cutoff determination. **a–c** Cutoff concentrations for epithelial cells of fragment one and stromal cells in the three fragments. **d–f** Cutoff concentrations for epithelial cells of fragment two and stromal cells in the three fragments. **g–i** Cutoff concentrations for epithelial cells of fragment three and stromal cells in the three fragments. As shown by the coefficient of determination (R^2), no significant correlation among cutoff concentrations of the three fragments is found. If identical cutoff concentrations of two or more HNSCC were detected, the quantity of data points on the same coordinates was inserted in *Arabic numbers*

Discussion

In this study, the existence of highly chemoresistant stromal cell colonies in a high number of HNSCC and, in particular, a different chemoresistance of epithelial and stromal cell colonies to chemotherapeutics was demonstrated. This was found for docetaxel and cisplatin and was statistically significant in the data presented for colony formation of HNSCC ex vivo.

HNSCC consist in vivo of epithelial and non-epithelial (i.e., stromal) cells in different quantities. Since both cell types show differences in proliferation and growth, depending on various factors (growth factors, cytokines, etc.) present in their environment [28], there is not only a great intertumoral, but also a high intratumoral morphological heterogeneity. This morphological heterogeneity of a tumor in vivo is found also in the tumor growth in vitro [29].

In the study of Wichmann et al. [17], only with regard to epithelial cells the representativeness of the chemoresponse of a single biopsy for the whole given HNSCC was emphazised. Since it is desirable that not only epithelial, but all types of cells (in particular, the major cell types, the epithelial and stromal cells) proliferate and grow in the ex vivo chemoresponse assay in an in vivo like manner, it is also important to examine all cell populations separately regarding their response to chemotherapeutical treatments. This is necessary to avoid misinterpretation, due to limited data, and to reach a conclusion on whether or not the chemoresponse of one cell type allows inferences on the chemoresponse of all other cell types. Only such a complete approach allows for the translation of data gained ex vivo into the situation in vivo.

The used ex vivo chemoresponse tests as well as the concomitant KB cell experiment for verification of the test's reproducibility were carried out under flavin-protecting conditions to avoid flavin-mediated photoreactions [15, 16]. As demonstrated by the 14 KB cell tests, which in general confirmed the predetermined IC_{50} values of cisplatin and docetaxel [24, 25], flawless reproducibility has been proven. Hence, the execution of all assays was valid. Thus, it might be concluded that this is true also for the culturing of the major cell populations present in HNSCC within the ex vivo assay. Therefore, the tests allow for a detailed analysis of colony formation by epithelial and stromal cell under exposure to either cisplatin or docetaxel. Comparison of this test method with two well-known chemoresponse assays (the EDR and ATP-TCA assays) exposes some flaws in both of the established test systems. The EDR assay indicates only the chemoresistance of a malignant tumor; it does not indicate the chemotherapeutic agent that is best suited for sufficient treatment of the tumor's malignancy and, thus, has only limited value in determining a suitable treatment. Against a background of an increasing number of new cytostatic agents, including small molecules and biologicals, determination of the chemosensitivity of a cancer and so about a probable effective treatment option is preferable for decision-making in head and neck oncology than the declaration of the high chemoresistance of tumors. Besides, due to the cooling of the tumor material during the shipping before testing, and in particular the usage of medium with high riboflavin concentrations and the handling under normal illumination, phototoxic reactions may occur and influence the test results leading to chaotropism and unpredictability of chemoresponses of the malignancy to tolerable doses in vivo. Besides the limitations mentioned above, the ATP-TCA uses trypsination for digestion, which results in a suspension of singularized cells. Thus, the interaction between epithelial and stromal cells, as well as their different chemoresponse, is not reflected accurately. This results in basically and essentially in vitro deviations from those in vivo, since solid tumors in no way represent a singularized cell suspension. However, the measurement of sum signals reflecting colony formation, proliferation or cytotoxicity, and use of such measurements cannot be suitable for evaluation of the chemoresponse of an HNSCC. In particular, and as shown by our data, definite findings are not deducible about the chemoreactivity of a malignancy, and congruence in chemoresponse in vitro and in vivo will be hardly achieved whenever only sum signals are measured.

In the ex vivo chemorespones assay used, at least the major cell types present in HNSCC could be differentiated using Giemsa staining. Hence, colonies of fibrocytes, and endothelial and epithelial cells were clearly distinguishable (Fig. 1), based on the described morphology and the efficiency of the chemotherapeutics. To minimize possible mistakes in interpretation of microscopic observations, and in particular to achieve proper differentiation between colonies of epithelial and stromal cells that were formed during ex vivo treatment, and especially taking into account their somewhat irregular morphological appearance that may lead to misinterpretation by students and even pathologists without experience, the counting was carried out by two well-trained investigators independently. To allow in the future for an easier differentiation of all cell types and an analysis of the exact composition of the stromal colonies, new studies should include marker analyses using fluorescent-labeled antibodies. Despite the possible advantages of using sufficient markers and their labeling by fluorescent dyes, allowing also differentiation of cell populations by the untrained, a correct evaluation of the outcome in ex vivo testing is achievable by Giemsa staining (Fig. 1).

As shown here, the dose of cytostatics needed to achieve suppression of colony formation varies depending on the cytostatics used and the cell type examined. Regarding the total spectrum of the 15 HNSCC tested in this study, the

spectra of cutoff concentration for cisplatin were 3.3–53.3 μM for epithelial and 1.7–106.7 μM for stromal cell colonies. For docetaxel, the cutoff was between 0.5 and 17.6 μM for epithelial and 1.1 and 17.6 μM for stromal cell colonies.

In contrast to the great pharmacological heterogeneity of the 15 HNSCC tested, the chemoresponse of the three specimens of a given tumor was quite homogeneous. This homogeneity in general is the case in comparisons made for colonies of the same major cell population, either epithelial or stromal cells. It was found that the cutoff concentration of three fragments of an HNSCC varied for each of the particular cell population only marginally (about one titration step at most). This was regarded to be an assay-related fluctuation due to statistical reasons. Hence, there was a significant correlation among the three specimens of the individual HNSCC. Moreover, no statistical difference was found on applying the *t* test for paired samples, in the treatment with cisplatin (epithelial colonies, $p > 0.34$; stromal colonies, $p > 0.17$) or docetaxel ($p > 0.18$ and $p > 0.17$, respectively).

On comparing the cutoff concentrations of the epithelial and stromal colonies within a fragment, it was noticed that in 18.9% of all assays (and accordingly 26.4% of the assays with cisplatin and 11.4% with docetaxel), the cutoff concentration for the stromal colony formation within a fragment was considerably higher (>2 titration steps) than the cutoff for epithelial cell colonies (Table 3). Vice versa, higher resistance of epithelial cells was found only in 13% of all assays (accordingly 17.7% with cisplatin and 8.6% with docetaxel). This more often found resistance of stromal cells in comparison to epithelial cells is reflected by the graphs shown in Fig. 3. Such a higher resistance of stromal cells was also described as an outcome in ex vivo chemoresponse assays for HNSCC [25, 30] and lung cancer [31]. The chemoreactivity of epithelial and stromal cell was more homogeneous regarding treatment with docetaxel than with cisplatin. Thus, there was no significant correlation of colony-formation ability of epithelial and stromal cells for either cisplatin or docetaxel. These results demonstrate that the ex vivo chemoresponse assay is able to discover a distinct pharmacological behavior or, in other words, heterogeneity in colony-formation ability of different cell types treated with cytostatics. Moreover, it underlines that errors are unavoidable if the evaluation of chemoresponses based on cutoff concentrations does not differentiate between the major cell populations. In addition, there is a much higher homogeneity within the HNSCC specimens in the outcome of chemoresponse testing in separate analysis of each cell population. This, as already stated by Wichmann et al. [17] for epithelial cell colonies, allows for estimation of the chemoresponse of the total HNSCC with respect to a particular (but as shown here, only to the particular one) cell population analyzed.

Therefore, an analysis of the sum responses for the evaluation of the chemoresponse of HNSCC, like that commonly used in most of the publications [e.g., 32, 33], appears to be obsolete, because misinterpretation of the results might be possible due to the occurrence of high resistant stromal cells and a predominant lack of correlation between colony formation of epithelial and stromal cells.

To date, the cause of the more often observed increase in chemoresistance of stromal cells has not been resolved conclusively. It is known that fibroblast and fibrocytes affect tumor growth, progression, invasion and resistance to chemotherapeutics in the autocrine and paracrine signal pathways [34–37]. An efficient method to better predict the response of an individual HNSCC to a treatment in vivo is by analyzing the various cell types in HNSCC regarding their potentially different chemoresponse ex vivo and including also the knowledge about the interaction of these cells in the interpretation of the ex vivo data.

Conclusion

The existence of highly chemoresistant stromal cell colonies in some HNSCC and, in particular, a difference in the chemoresponse of epithelial and stromal cells within a given HNSCC to either cisplatin or docetaxel were demonstrated in this study. Consequently, the separate analysis of epithelial and stromal cell colony formation is necessary for sufficient evaluation of chemoresponses of HNSCC in an ex vivo assay to allow adequate predictive statements of the tumor's chemoreactivity in vivo.

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