The Impact of Stromal Cell Contamination on Chemosensitivity Testing of Head and Neck Carcinoma

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Abstract. Background: Reliable chemosensitivity testing of head and neck squamous cell carcinoma (HNSCC) still faces methodical limitations. Since stromal cell contamination has been found to preclude reliable radiosensitivity testing of HNSCC as well as chemosensitivity testing of lung tumors, the present study investigates the impact of stromal cell contamination on chemosensitivity testing of HNSCC. Patients and Methods: Seventeen biopsies from HNSCC were analyzed. The specimens were investigated using an ex vivo colony formation assay which allows for the quantitative and separate determination of the overall, as well as the epithelial, and stromal response to carboplatin, 5-fluorouracil and docetaxel. Results: The overall chemoresponse was dominated by stromal cell multidrug resistance. However, by selective evaluation of the epithelial chemoresponse, individual chemosensitivity patterns could be identified. Conclusion: Multidrug-resistant stromal cells preclude the reliable assessment of the chemoresponse of HNSCC specimens. Careful correction for stromal cell effects is a prerequisite for the generation of therapeutically useful information.

In vitro chemosensitivity testing of human malignancies for the prediction of individual chemoresponse has been the focus of many studies over the past decades (1-3). Recently, the role of chemotherapy in the first-line treatment of head and neck squamous cell cancer (HNSCC) has changed due to the introduction of primary chemoradiation as an alternative to primary surgery (4-6). The underlying concept of functional organ preservation through primary chemoradiation prompted secondary research on predictors to identify individual responders (7-11). The attempt to predict the individual response to chemoradiation has also renewed the discussion on a possible role of in vitro chemosensitivity assays in head and neck cancer. However, in vitro chemosensitivity testing of solid tumors still faces crucial methodical limitations (1,3). One of the most challenging aspects is the architecture of solid tumors per se: malignant epithelial and accompanying non-malignant stromal cells in different ratios and patterns.

Stromal cell contamination has been shown to preclude reliable radiosensitivity determination in HNSCC specimens using the clonogenic assay (12). Furthermore, high levels of multidrug resistance were observed in stromal cell colonies present in primary cultures from bronchoscopic lung tumor explants (13). The impact of such stromal contamination on chemosensitivity testing of HNSCC has not been addressed so far.

The present study addresses the impact of stromal cell contamination on the quantitative determination of HNSCC biopsies ex vivo. Therefore, an ex vivo colony formation assay was established which allows for parallel determination of the overall drug response, as well as the specific drug response of epithelial and stromal elements from HNSCC biopsies. Using this assay, the present study investigates whether stromal cell contamination influences the ex vivo identification of individual cytostatic drug response profiles in HNSCC specimens.

Patients and Methods

Patients and HNSCC specimens. Thirteen patients with histologically confirmed diagnosis of primary head and neck squamous cell carcinoma (HNSCC) were enrolled in this study (for...
After obtaining individual informed consent, a total of 17 biopsies was taken from primary tumors (n=12), or from cervical lymph node metastases (n=5) during surgery at the Department of Otorhinolaryngology, Head and Neck Surgery, University of Heidelberg, Germany. The specimens were kept in ice-cold culture medium for a maximum of 8 hours prior to further processing in the cell culture laboratory.

**Drugs, enzymes and reagents.** Carboplatin (CP, Bristol, München, Germany), 5-fluorouracil (5-FU, Ribosepharm, München, Germany) and docetaxel (DTX, Rhone-Poulenc Rorer, Köln, Germany) were purchased as pharmaceutical preparations. Nystatin, G-penicillin-Na, gentamycin and streptomycin were purchased from Sigma (München, Germany). Trypsin and collagenase (EC 3.4.24.3, type IV) were obtained from Difco (Detroit, MICH, USA) and Sigma, respectively. All other reagents were of analytical grade.

**Cells and cell culture methods.** KB cells (ATCC, Bethesda, MD, USA) were propagated, without antibiotics, in flavin-free RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Integro, Zaandam, Holland), 1.134 g/l sodium bicarbonate and 1.072 g/l HEPES. KB cells were grown at 36.5°C under 2.5% CO2 in humidified air (standard conditions) and were routinely subcultivated at alternating intervals of 3 and 4 days, respectively. All experimental steps were carried out under exclusive illumination with sodium-discharge lamps, emitting monochromatically at λ=589 nm (Philips, Marburg, Germany). This specific illumination was used to avoid the known adverse flavin-mediated photooxidative effects in cell culture systems (14-16), especially in chemosensitivity testing (17,18).

**Evaluation of cytostatic drug response in KB cells.** Cytotoxicity tests with CP, 5-FU and DTX were performed in 24-well plates (Greiner, Frickhausen, Germany). After inoculation of freshly harvested and resuspended KB cells (3.5 × 105 /well), diluted gradients from frozen (-20°C) stock solution of either CP, 5-FU or DTX were added, except for the control wells. The test plates were

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**Table I. Patients and tumor characteristics.**

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age/ Gender</th>
<th>HNSCC localization</th>
<th>Stage (UICC 1997)</th>
<th>Biopsy site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70 / m</td>
<td>Larynx</td>
<td>T4N2cM0</td>
<td>P, LN</td>
</tr>
<tr>
<td>2</td>
<td>47 / m</td>
<td>Oropharynx</td>
<td>T2N2cM0</td>
<td>P, LN</td>
</tr>
<tr>
<td>3</td>
<td>61 / m</td>
<td>Hypopharynx</td>
<td>T3N0M0</td>
<td>P</td>
</tr>
<tr>
<td>4</td>
<td>75 / f</td>
<td>Hypopharynx</td>
<td>T4N2cM0</td>
<td>P</td>
</tr>
<tr>
<td>5</td>
<td>74 / m</td>
<td>Hypopharynx</td>
<td>T2N2cM0</td>
<td>P, LN</td>
</tr>
<tr>
<td>6</td>
<td>52 / m</td>
<td>Oropharynx</td>
<td>T3N2cM0</td>
<td>P</td>
</tr>
<tr>
<td>7</td>
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<td>Hypopharynx</td>
<td>T3N2cM0</td>
<td>P, LN</td>
</tr>
<tr>
<td>8</td>
<td>75 / f</td>
<td>Oropharynx</td>
<td>T3N2cM0</td>
<td>P</td>
</tr>
<tr>
<td>9</td>
<td>72 / m</td>
<td>Larynx</td>
<td>T3N0M0</td>
<td>P</td>
</tr>
<tr>
<td>10</td>
<td>53 / m</td>
<td>Larynx</td>
<td>T2N2cM0</td>
<td>P</td>
</tr>
<tr>
<td>11</td>
<td>42 / m</td>
<td>Hypopharynx</td>
<td>T4N3M0</td>
<td>LN</td>
</tr>
<tr>
<td>12</td>
<td>64 / m</td>
<td>Larynx</td>
<td>T3N0M0</td>
<td>P</td>
</tr>
<tr>
<td>13</td>
<td>59 / m</td>
<td>Oropharynx</td>
<td>T4N2cM0</td>
<td>P</td>
</tr>
</tbody>
</table>

* Biopsies were taken from the primary tumor (P) or from lymph node metastasis (LN).
The mean wet weight of the harvested specimens was 89.4±35.9 mg (range: 56.7-145.3 mg).

**Table II. Pharmacological indices used for calibration of cytostatic drug concentration gradients in the ex vivo colony formation assay.**

<table>
<thead>
<tr>
<th>CP</th>
<th>5-FU</th>
<th>DTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (KB cells)$\dagger$</td>
<td>2.08 μM (±0.07)</td>
<td>1.23 μM (±0.16)</td>
</tr>
<tr>
<td>Clinically tolerated drug plasma levels (Cp)$#$</td>
<td>24.0 μM (19)</td>
<td>1.5 μM (20)</td>
</tr>
</tbody>
</table>

Cytostatic drug concentration gradient 2.0-200.0 μM 1.2-307.2 μM 0.28-71.68 nM

$\dagger$ Mean IC50 values (±S.D.) resulted from five independent cytotoxicity tests (n=5) each done in duplicate.
$\#$ References are given in parenthesis.

Figure 1. Results of control experiments with KB cells. The growth inhibitory effect of the IC50 of carboplatin (CP), 5-fluorouracil (5-FU) and docetaxel (DTX) is shown (open circles). Filled circles with error bars: Mean±S.D. (n=12).
incubated for 72 hours under standard conditions. At harvest, trypsinized KB cells were resuspended in culture medium. Cell densities were determined using a Casy I cell analyzer equipped with Casystat software (Scharfe System, Reutlingen, Germany). Growth inhibition values (N) were expressed as a percentage as follows:

\[
N(\%) = 100 \frac{(t-i)}{c-i}
\]

where t, c, and i represent test, control and inoculum KB cell densities, respectively. The fifty percent growth inhibitory concentration (IC50) was determined from semilogarithmic drug concentration vs. growth inhibition plots.

Control experiments with KB cells. Control experiments with KB cells were performed as part of each colony formation assay. Using the same drug solutions and culture media as in the colony formation assays, the control experiments were necessary to verify the cytostatic potency of each drug in the colony formation assay. Control experiments with KB cells were performed as described for cytotoxicity tests in KB cells, although only one concentration (IC50 value) was tested per drug. The percent growth inhibition value (N) was determined according to equation (1) and should give a fifty percent growth inhibition (+/- 10%) to ensure adequate pharmacological assay conditions. 

Figure 2. Microscopic aspects of Giemsa-stained epithelial and stromal cell colonies from HNSCC specimen#7. Figures A,B,E and I: control wells showing epithelial as well as stromal cell colonies. Figures C and D: epithelial colonies grown in the presence of CP. Figures F, G and H: epithelial and stromal (*) colonies grown in the presence of 5-FU. Figures J, K and L: stromal (*) and epithelial colonies grown in the presence of DTX. The drug concentrations are indicated. Magnification: approx. 100x, scale bar: 100 μM.
Handling of HNSCC specimens. The handling of the specimens as well as the colony formation assay are adaptations to HNSCC of procedures developed for lung tumor explants (13). After harvesting, specimens were minced generating tumor fragments of about 3 mm³ in size. After dissection, the fragments were transferred into 30 ml of prewarmed, flavin-free RPMI 1640 medium containing 10% (v/v) FBS, nystatin, G-penicillin, gentamycin and streptomycin. For enzymatic disintegration, 300 IU/ml collagenase type IV were added and the suspension was incubated for 24 hours under standard conditions. After centrifugation (100 x g, 5 min), the pellets were carefully resuspended in 30 ml culture medium supplemented as described above (w/o collagenase).

Colony formation assay. Three hundred μl aliquots of the suspension were transferred to microwells coated with extracellular matrix (Pessael & Lorey, Hanau, Germany). After a short sedimentation period, diluted cytostatic drug solutions (3 μl/well) were added to establish drug concentration gradients (see below) for CP, 5-FU and DTX. Each test plate included a minimum of eight drug-free control wells. After 72 hours incubation time under standard conditions, the cells were washed twice (phosphate-buffered saline, pH 7.8) and the adherent cells and cell colonies were fixed with methanol before Giemsa staining.

Drug concentration gradients. The cytostatic drug concentration gradients in the colony formation assay were calibrated using two pharmacological indices: i) the respective IC₅₀ values for KB cells (see Figure 1), and ii) the clinically achievable plasma concentration of the cytostatic drugs (19-21). Accordingly, for each drug a six-step concentration gradient was defined starting with the IC₅₀ for KB cells. The gradients covered, as well as exceeded, the clinically achievable plasma drug levels. The exact concentration gradients are given in Table II.

Microscopic evaluation of the drug response. Giemsa staining allowed for microscopical identification of stromal and epithelial cell colonies (≥ 16 cells) in all specimens tested. To determine the overall drug response (OR) of a specimen to a certain drug, the drug concentration which caused a complete suppression of any ex vivo colony formation (C₁₀₀) was determined. Considering the clinically achievable drug concentration (Cp; Table II), the specimens were classified as sensitive, if the C₁₀₀ was below or equal to the respective Cp and as resistant, if the C₁₀₀ exceeded the respective Cp (Table II).

Analogously to the microscopic identification of the C₁₀₀, those drug concentrations were identified which caused a complete suppression of epithelial (Cₑ₁₀₀), or stromal (Cₛ₁₀₀) colony formation ex vivo. The specimens’ epithelial drug response (ER) and the stromal drug response (SR) were classified in the same manner as described for the overall drug response (OR).

Results

Evaluable rate. To consider a HNSCC specimen evaluable in the colony formation assay, the following criteria were applied: i) all control wells contained epithelial, as well as stromal colonies, and ii) the IC₅₀ values for the tested cytostatic drugs were reproduced (+/- 10%) in the control experiments with KB cells. Accordingly, 16 out of 17 specimens were evaluable (evaluability rate: 94.1%). One specimen (patient #13, Table I) was excluded due to fungal contamination of the ex vivo culture. The results from control experiments are shown in Figure 1.

Types of colonies formed from HNSCC specimens. Giemsa staining of the ex vivo formed colonies allowed for microscopical identification of epithelial as well as stromal colony formation (≥ 16 cells) in each well of the microtiter plate. In control wells, colonies of either epithelial or of stromal cells (Figures 2A, 2E and 2I) were found in explants from primary tumors and from lymph node metastases. The proportion of epithelial and stromal colonies in the control wells varied markedly between the specimens. A microscopical comparison of the stromal proportion in the ex vivo cultures with hematoxylin/eosin-stained histology from the same tumors was done independently by three investigators. No correlations between the original tumor (histology) and the ex vivo cultures were found (data not shown). Examples of epithelial and stromal cell colonies grown from a HNSCC specimen are shown in Figure 2.

Overall drug response of HNSCC specimens. The complete suppression of any ex vivo colony formation was investigated in 16 HNSCC specimens by graded exposure to CP, 5-FU and DTX. The overall response (OR) to each single drug is shown in Table III. Only specimen #13 (Table III) showed a sensitive OR to DTX. Apart from this selective sensitivity, the overall response of the remaining HNSCC specimens revealed resistant response patterns to DTX. All specimens were resistant to CP and 5-FU.

Cell type-specific drug response in HNSCC specimens. To identify the cell type-specific drug response, the suppression of epithelial as well as stromal ex vivo colony formation were determined separately. The results are shown in Table III.

Epithelial cytostatic drug response (ER). One specimen showed a sensitive ER to carboplatin (specimen #9, Table III). The remaining 15 specimens were found to be resistant according to the ER definition. For 5-FU, none of the 16 investigated specimens showed a sensitive ER. Four specimens (specimen #4, #9, #13 and #14) were found to be sensitive to DTX. In specimen #9 the sensitivity to DTX was combined with a sensitivity to carboplatin.

Stromal cytostatic drug response (SR). The drug response patterns of the stromal elements were different from those of the epithelial elements from HNSCC specimens. In all specimens stromal cells were found to be resistant to CP. Moreover, the comparison of the corresponding Cₛ₁₀₀ and Cₑ₁₀₀ for CP showed in 13 out of 16 specimens (81%) that
complete suppression of stromal colonies required similar or higher drug concentrations than required for suppression of epithelial colony formation (Table III). As for CP, the stromal response to 5-FU revealed resistance to 5-FU for all specimens. Again, the comparison of the corresponding Cs100 and Ce 100 for 5-FU showed in 87.5 percent of the tested specimens similar, or higher resistance levels in stromal colonies compared to the epithelial cell colonies. Distinct stromal sensitivities were found only for docetaxel. In 5 HNSCC specimens, stromal colony formation was suppressed by docetaxel in lower concentrations than the clinically achievable plasma concentration (specimen #7, #12, #13, #15 and #16, Table III). In the remaining eleven specimens, colonies from the tumor stroma were resistant to docetaxel.

**Discussion**

Stromal cell contamination is an extensively discussed methodical limitation of most *ex vivo* procedures for chemosensitivity testing of solid tumors (1-3). For radiosensitivity testing using the clonogenic assay, this contamination has been described to preclude reliable test results for HNSCC specimens (12). Up to now, the impact of stromal cell contamination to chemosensitivity testing of HNSCC specimens has not been systematically investigated.

The present study was designed to investigate whether stromal cell contamination has an impact on chemosensitivity testing of HNSCC specimens. Furthermore, we tried to estimate the relevance of a distinction between epithelial and stromal elements in such test procedures.

The *ex vivo* colony formation assay for HNSCC specimens used in this study aims to determine the quantitative chemosensitivity of a given specimen corrected for stromal cell contamination. This was achieved by unselected *ex vivo* cultivation and chemosensitivity testing of all cellular elements from each specimen. The later fixation and staining of the colonies allows for a morphological identification of epithelial and stromal colonies, and thereby the determination of the quantitative epithelial and stromal *ex vivo* drug response.

Since cellular cytostatic drug resistance in HNSCC has been commonly attributed to malignant cells (22), one would expect that the formation of non-epithelial colonies would
be suppressed by lower drug concentrations than epithelial colonies. In parallel to the findings concerning the radiosensitivity of tumor fibroblasts (12), our results indicate that stromal HNSCC tumor cells are also highly resistant to cytostatic drugs. Consistently, we found surprisingly low chemosensitivities of non-epithelial cellular elements in the tested HNSCC specimens. In fact, stromal ex vivo colony formation was suppressed in only 10 out of 48 tests by a lower cytostatic drug concentration than that necessary to suppress the formation of epithelial colonies (Table III).

It is essential for in vitro chemosensitivity tests to attribute the results to the malignant cell population from a given tumor. Our results indicate that the unselective determination of the “overall response” leads to crucial misinterpretations of test results. In the tests reported here, the overall drug response of the examined HNSCC specimens showed for 15 out of 16 specimens resistant drug response patterns to all drugs investigated. If the overall drug response were to be taken as the individual drug response, 4 out of 5 (80%) sensitive ER patterns would not have been identified due to stromal cell drug contamination (specimen #4, #9, #13 and #14; Table III). These findings are in agreement with the previously reported influence of stromal cell contamination in radiosensitivity testing of HNSCC specimens using the clonogenic assay (12).

The present study has focused on the impact of stromal cell contamination on chemosensitivity detection in HNSCC. The underlying mechanisms of the surprisingly high chemoresistance in stromal tumor cells was not investigated in detail. However, the finding was somewhat surprising since normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23). Nevertheless, several authors have described P-glycoprotein expression in non-malignant normal dividing cells of tumor patients are generally considered chemosensitive (23).

In conclusion, we want to emphasize that methods to detect individual drug response profiles in HNSCC should allow for stroma cell correction. Besides the colony formation assay reported here, the histoculture drug response assay (HDRA) seems to fulfil this requirement (1,31,32). Using this assay, Singh and coworkers have recently shown that survival in head and neck cancer patients is predictable by chemosensitivity determined by the HDRA (33).

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References
Dollner et al: Resistance to Cytostatic Drugs of Stromal Cells in Head and Neck Tumors


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