



Pre-Irradiated Fibroblasts Influence the Chemosensitivity of Co-Cultivated Squamous Cell Carcinoma Cells

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Abstract

Objective: Tumor stroma mainly consists of fibroblasts, which have a multitude of interactions with the cancer cells they surround. Since irradiation and chemotherapy are common therapeutic options for squamous cell carcinoma of the head and neck, the effects of irradiation on tumor stroma and their sensitivity to chemotherapeutic agents are of significant therapeutic interest.

Methods: FaDu head and neck squamous cell carcinoma cells (HNSCC) were cultivated with fibroblasts from pre-irradiated and non-irradiated human skin for 24 hours. Then the co-cultures were treated with either Cisplatin, Paclitaxel or 5-Fluorouracil for 48 hours. Analysis of tumor viability and apoptosis were conducted via the MTT assay and the Annexin V-propidium iodide test. Secretion of interleukin-8 (IL-8) was analyzed with an enzyme-linked immunosorbent assay.

Results: Co-cultures with pre-irradiated fibroblasts showed decreased viability, higher rates of apoptosis and necrosis, and lower levels of IL-8 as compared to co-cultures with non-irradiated fibroblasts in the presence of chemotherapeutic agents as well as in the control group.

Conclusion: We therefore postulate an influence of a previous irradiation of fibroblasts on the chemosensitivity of co-cultured tumor cells. To achieve a better understanding of the effects of cytostatic treatment in pre-irradiated head and neck cancer patients, further investigations are warranted.

Keywords

Fibroblast; Squamous cell carcinoma; Tumor stroma; Co-culture; Irradiation; Chemosensitivity; Cisplatin; Paclitaxel

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) have accounted for 4% of all cancer cases in the U.S. in the years 2005-2011 [1]. Five-year survival has only increased marginally for oral cavity and pharyngeal cancer to about 64% in 2011, while carcinoma of the larynx even showed a decline in survival in the same time [1]. With the human papillomavirus (HPV) as an emerging risk factor

for oropharyngeal cancer, the incidence in HNSCC is expected to increase in the coming years [2].

The stroma is a critical compartment in normal tissue and in tumor tissue, although both types of stroma have different properties [3]. More evidence is showing that normal stroma has distinct anti-cancer activity [4]. But when converted to tumor stroma, this effect is changed to promote cancer progression instead, as the tumor stroma is suggested to co-evolve with the tumor and adapt according to its needs [5,6]. Besides the extra-cellular matrix (ECM), the stroma consists of various stromal cell types; among these are mesenchymal cells like fibroblasts and adipocytes, cells of the vascular system and of the immune system [7]. Of these cell types, the fibroblasts are by far the most abundant, and have therefore been focused when targeting tumor-stromal interactions. When expressing smooth muscle actin, activated fibroblasts are referred to as myofibroblasts and constitute a major part of carcinoma-associated fibroblasts (CAFs) in tumors [8]. These CAFs have been demonstrated to stimulate cancer cell growth, angiogenesis and invasion [9,10]. Another way for CAFs to promote tumor growth is by enhancing its resistance to a variety of drugs, which has already been published for Cisplatin or Tamoxifen regarding breast and skin cancer [11,12]. Although radiation is a core component of most HNSCC cancer treatment regimes, information on its influence on tumor stroma is scarce. According to recent studies, CAF's themselves showed no significant changes in growth or proliferation when exposed to radiation *in vitro* [13,14]. The capability of fibroblasts to promote survival of co-cultured cancer cells seems to be enhanced after an application of low dose radiation (>20 Gy) *in vitro* [15]. However, the irradiation was delivered to cells *in vitro*. In a previous study, we could demonstrate that fibroblasts cultivated from pre-irradiated skin samples significantly reduced the viability of co-cultured HNSCC cells *in vitro* [16]. Moreover, we could demonstrate an increased secretion of Interleukin-6 and Interleukin-8 of HNSCC cells co-cultivated with pre-irradiated fibroblasts [16]. Both of these cytokines are major factors in the metastatic potential of different cancer entities [17,18]. They have also been shown to promote cancer resistance against different drugs, including cytostatic agents like Cisplatin [17].

This raises the question whether pre-irradiation of fibroblasts has an influence on the Chemosensitivity of co-cultured HNSCC. The objective of the present study was to examine the *in vitro* effects of co-cultured fibroblasts from pre-irradiated tissue on the Chemosensitivity of HNSCC cell lines compared to non-irradiated fibroblasts with respect to viability, apoptosis and the secretion of IL-8.

Material and Methods

Acquisition and culture of fibroblasts

Fibroblasts were obtained from skin samples from voluntary patients undergoing neck surgery at the Department of Otolaryngology, Head and Neck Surgery, at the University Hospital Würzburg, Germany, between October 2012 and September 2013. 10 of 20 patients had been treated with intensity-modulated irradiation with 60-70 Gy for 6 weeks during head and neck cancer therapy during the previous 6-18 months. Approval was obtained from the Ethics Committee of the Medical Faculty, University of Würzburg

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(Approval nr. 12/06), as well as informed consent from all individuals included in the study. Tissue preparation was done as described in our previous study [16] in a modification of the protocol described by Vangipuram et al. [19]. In summary, the skin samples were cleared of fat and cut into small pieces of 2-3 mm, which were then seeded on 6-well plates. After 60 minutes of culture without medium at 37°C and 5% CO₂, the tissue pieces adhered to the bottom of the plates well enough so that Dulbecco's Modified Eagle Medium (DMEM) (Gibco Invitrogen) with 10% FCS (Biochrom AG, Berlin, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (DMEM-expansion medium [DMEM-EM]) could be added without the pieces being washed away. From these tissue pieces the fibroblasts grew out into the periphery. Every other day medium was replaced and passaging was performed after reaching 70%-80% confluence by trypsinization (0.25% trypsin, Gibco Invitrogen), followed by washing and seeding in new flasks or treatment wells.

Culture of FaDu cell line

The HNSCC cell line FaDu was established from a human hypopharyngeal squamous cell carcinoma [20]. FaDu cells (American Type Culture Collection, LGC Standards, Wesel, Germany) were cultivated in RPMI-1640 medium (Biochrom AG, Berlin, Germany) with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, 100 U/ml penicillin, 1% sodium pyruvate (100 mM, Biochrom AG) and 1% non-essential amino acids (100-fold concentration) (RPMI-expansion medium [RPMI-EM]). Culture conditions included temperature at 37°C with 5% CO₂ in culture flasks. Every other day medium was replaced and passaging was performed after reaching 70%-80% confluence by trypsinization (0.25% trypsin, Gibco Invitrogen, Karlsruhe, Germany), followed by washing and seeding in new flasks or treatment wells. For performing experiments, cells in the exponential growth phase were used.

Cytostatic agents

For our experiments, we used the chemotherapeutic agents Cisplatin, Paclitaxel and 5-Fluorouracil. Cisplatin is a platinum-based molecule whose antitumor properties were first described by Rosenberg et al. in 1969 [21]. It is a common therapeutic agent for HNSCC, used for primary chemoradiation as well as in combination with other cytostatic agents as a palliative chemotherapy. Paclitaxel was isolated from *Taxus brevifolia* in the late 1960s, with its antitumor properties being discovered in 1971 [22]. 5-Fluorouracil is a fluorinated pyrimidine whose antitumor properties were first described by Heidelberger et al. in 1957 [23]. Both Paclitaxel and 5-Fluorouracil are most commonly used either in combination with Cisplatin for induction chemotherapy prior to irradiation, or in palliative chemotherapeutic regimes.

Group composition

Altogether 3 main groups with 4 subgroups each were formed. Every group consisted of 10 transwells, one for each of the different patient samples. The 3 main groups consisted of a mono-culture of FaDu cells without fibroblasts, a co-culture of FaDu cells with non-irradiated fibroblasts and a co-culture of FaDu cells with pre-irradiated fibroblasts. Each of these 3 main groups had 4 subgroups, one for each of the three cytostatic agents used and a control group without any cytostatic substance (Figure 1).

Transwell culture

A transwell system (Corning Incorporating Costar, Wiesbaden,

Germany) was used to analyze the effects of fibroblasts on the HNSCC cell line FaDu. First a co-culture was generated: 5 x 10⁴ FaDu cells were coated with 1 ml RPMI-EM on the bottom of a 12-well plate. 5 x 10⁴ fibroblasts with 0.5 ml RPMI-EM were coated in transwells and then transferred to the wells containing FaDu cells. After one day of co-culture, the 3 different chemotherapeutic agents were added, except for the controls. Cisplatin was added in a molar concentration of 10 µmol/l to one group with a FaDu mono-culture, one group with a pre-irradiated fibroblast co-culture and one group with a non-irradiated fibroblast co-culture. The same was done with Paclitaxel (10 nmol/l) and 5-Fluorouracil (50 µmol/l). The concentrations needed were evaluated by measuring the half maximal inhibitory concentration (IC₅₀) with FaDu cells in advance (data not shown). After 2 days of further incubation, the analytical assays were performed.

MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) (Sigma-Aldrich) colorimetric staining method according to Mosmann (24) was used to study the viability of cells. All wells were incubated with 1 ml of MTT (1mg/ml) for 5 hours at 37°C with 5% CO₂. MTT was then removed and 1ml of isopropanol was added, followed by another incubation period of 1 hour at 37°C with 5% CO₂. Measurement of the color conversion of the blue formazan dye was done using a multi-plate reader (Titertek Multiskan PLUS MK II, Labsystems, Helsinki, Finland) at a wavelength of 570 nm.

Annexin V-propidium iodide test

The Annexin V-APC kit from BD Pharmingen (BD Bioscience, Heidelberg, Germany) was used to evaluate apoptosis, following a modification of the method described by Span et al. [25]. Cells in suspension and adherent cells were harvested and washed twice with PBS, followed by resuspension in 1:10 binding buffer (0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM CaCl₂) at a concentration of 1 x 10⁶ cells/ml. 100 µl aliquots of this cell suspension (1 x 10⁵ cells) were then transferred to a 5 ml culture tube. 5 µl of propidium iodide and 5 µl of Annexin V-APC were added to each aliquot. After 15 minutes of incubation at room temperature in the dark, cells were resuspended with 400 µl 1:10 binding buffer. A FACScanto flow cytometer (Becton Dickinson, Heidelberg, Germany) was used to analyze the samples. Only cells with damaged membranes were stained by propidium iodide.

IL-8 enzyme-linked immunosorbent assay (ELISA)

The evaluation of IL-8 secretion was conducted in a modification of a method described by DeForge et al. [26]. For measurement of the secretion of IL-8, the supernatants were collected and stored at -20°C in sterile tubes until further use. DMEM-EM served as control. Human IL-8 kit (Diacclone SAS, Besancon, France) was used and experiments were performed in duplicate. The ELISA plate was read at a wavelength of 450 nm (Titertek Multiskan PLUS MK II, Thermo Labsystems, Thermo Fisher Scientific, Inc.). The concentration of IL-8 was determined by constructing a standard curve using recombinant IL-8. Since FaDu cells alone showed negligible amounts of IL-8 secretion in a previous study [16], IL-8 was only tested in the two co-culture settings.

Statistical analysis. The data collected was transferred to standard spreadsheets and statistically analyzed using Graph Pad Prism Software (version 6.0; Graph Pad Software, Inc., San Diego, CA, USA). Analytical assays were performed in triplicates, if not otherwise stated. The Gaussian distribution was tested via first column analysis. In case of a Gaussian distribution, two-way analysis of variance followed by

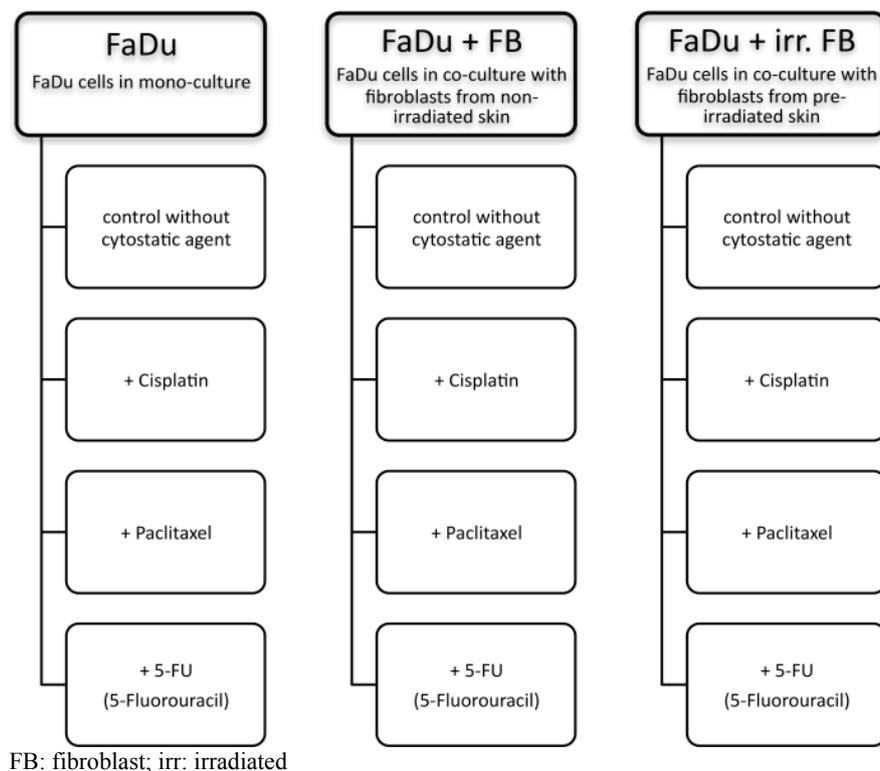


Figure 1: Schematic of group composition and abbreviations.

Turkey's multiple comparison tests was used. Data were presented in mean +/- standard deviation, if not otherwise stated. P-values were adjusted for multiplicity testing. Statistical significance was assumed at $p < 0.05$.

Results

MTT assay

A co-culture with fibroblasts from pre-irradiated skin samples showed a significant decrease in viability of FaDu cells compared to a FaDu mono-culture in the control group ($p = 0.0001$). Additionally, there were significant reductions in viability of FaDu cells in co-culture with pre-irradiated fibroblasts compared to a co-culture with non-irradiated fibroblasts in the control group ($p = 0.0001$), after addition of 5-FU ($p = 0.0001$) and Paclitaxel ($p = 0.0029$). The only statistically significant increase in FaDu cell viability was found for a co-culture with non-irradiated fibroblasts compared to a FaDu mono-culture after addition of 5-FU ($p = 0.0001$). In the Cisplatin group, no statistically significant differences between the 3 groups could be found ($p = 0.9990$, $p = 0.4531$ and $p = 0.4281$, respectively (Figure 2).

Annexin V-propidium iodide test

Annexin V-propidium iodide analysis revealed significant differences between the 3 groups (Figures 3 and 4). Increased rates of necrosis could be identified for co-cultures with pre-irradiated fibroblasts when compared to co-cultures with non-irradiated fibroblasts in the control group ($p = 0.0001$), as well as in the groups containing 5-FU ($p = 0.0051$) and Paclitaxel ($p = 0.0001$). In the Cisplatin group ($p = 0.0019$) and Paclitaxel group ($p = 0.0101$), there was also a statistically significant increase in necrosis when comparing

the pre-irradiated co-cultures with FaDu cells alone. Moreover, in the Cisplatin group ($p = 0.0038$) and Paclitaxel group ($p = 0.0331$), there was also a statistically significant decrease in necrosis when comparing the non-irradiated co-cultures with FaDu cells alone (Figure 4). A significantly higher percentage of viable cells could be found for co-cultures with non-irradiated fibroblasts compared to FaDu cells alone in the groups containing Cisplatin ($p = 0.0003$) and Paclitaxel ($p = 0.0070$). Accordingly, lower percentages of viable cells were found for co-cultures with pre-irradiated fibroblasts compared to non-irradiated fibroblasts in the control group ($p = 0.0001$), Paclitaxel ($p = 0.0001$) and 5-FU group ($p = 0.0007$), as well as compared to FaDu cells alone in the cisplatin ($p = 0.0282$) and Paclitaxel group ($p = 0.0016$) (Figure 4). Significantly higher levels of apoptosis were identified for pre-irradiated fibroblasts versus non-irradiated fibroblast co-cultures in the control ($p = 0.0123$), Cisplatin ($p = 0.0003$) and Paclitaxel group ($p = 0.0001$), as well as versus FaDu cells alone in the Paclitaxel group ($p = 0.0017$). Lower levels of apoptosis could be shown accordingly for non-irradiated fibroblast co-cultures versus FaDu cells alone in the Cisplatin ($p = 0.0001$) and Paclitaxel group ($p = 0.0202$) (Figure 4).

Quantitative analysis of IL-8 expression

The examination of the secretion of IL-8 revealed a general trend towards higher levels of IL-8 in the co-cultures with non-irradiated fibroblasts compared to co-cultures with pre-irradiated fibroblasts. However, this was only statistically significant in the group containing Paclitaxel ($p = 0.0247$) (Figure 5).

Discussion

We focused this study on the effects of a previous irradiation of fibroblasts on the Chemosensitivity of co-cultured HNSCC *in vitro*.

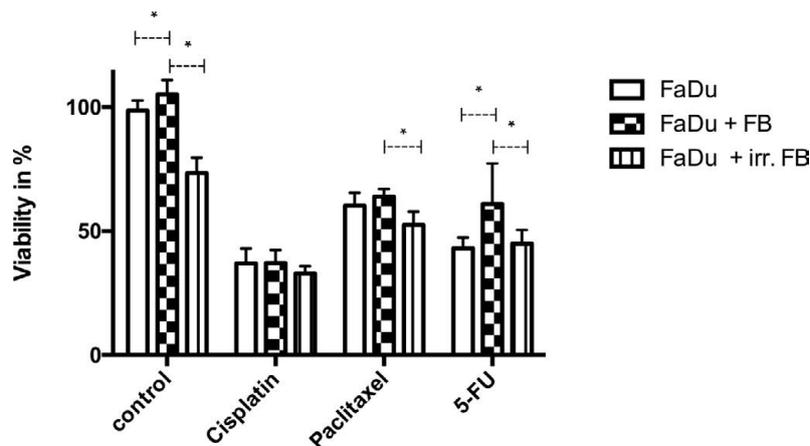


Figure 2: MTT assay of FaDu cells in mono-culture and co-culture with and without the addition of various cytostatic agents. Significant reduction in cell viability was observed in co-cultures with irr. FB compared to FaDu cells alone in the control group. Significant decreases in viability were also found in co-culture with irr. FB compared to co-cultures with FB in the control group, 5-FU group and Paclitaxel group. A significant increase was only found in the 5-FU group when comparing a co-culture with FB with FaDu cells alone. * $p < 0.05$ (FB: fibroblast; irr.: irradiated).

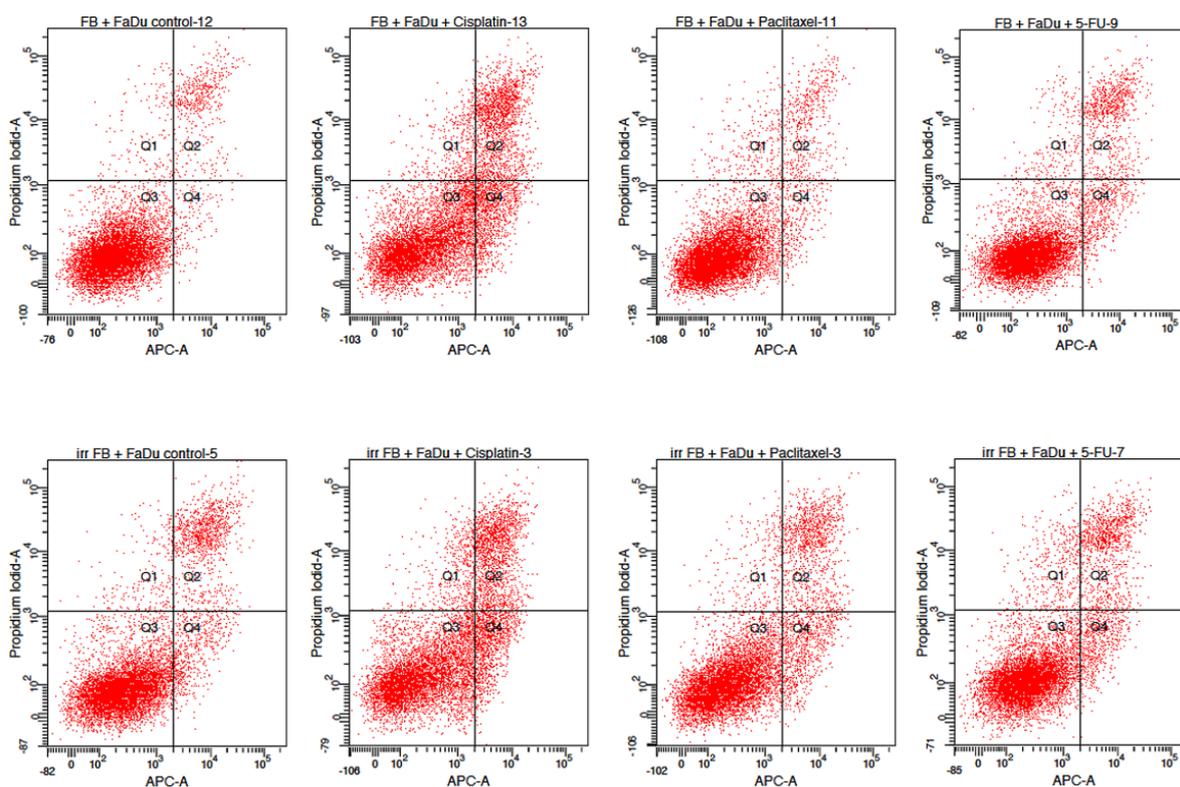


Figure 3: Representative flow cytogramme of Annexin V binding (abscissa) versus propidium iodide uptake (ordinate) in FaDu cells incubated with non-irradiated or pre-irradiated fibroblasts with or without addition of cytostatic agents. Upper row from left to right: non-irradiated co-culture in the control, Cisplatin, Paclitaxel and 5-FU group. Lower row from left to right: pre-irradiated co-culture in the control, Cisplatin, Paclitaxel and 5-FU group. For quantitative statistics, see Figure 4. Q1: % of damaged cells; Q2: % of necrotic cells; Q3: % of viable cells; Q4: % of apoptotic cells. APC-A: allophycocynin-A (FB: fibroblast; irr.: irradiated).

Another possible setup would be to apply conditioned fibroblast medium to tumor cells. Thereby it is possible to examine especially the influence of soluble factors produced by the fibroblasts on tumor cells, which has already been shown to induce EMT-like phenomena and to increase EGFR signaling as well as cell proliferation in HNSCC cells [27]. However, we chose a Trans well co-culture model because it allows the two cell types to mutually influence each other.

In a previous study, an influence of a pre-irradiation of fibroblasts on the proliferation, apoptosis and secretion of Interleukin-6 and Interleukin-8 of co-cultivated HNSCC [16] could also be demonstrated. These results could be confirmed in the present study, as one of the groups had no addition of a cytostatic agent. This is also consistent with data available from the literature for various other cell types, for example prostate cancer cells [28] and breast cancer cells [29].

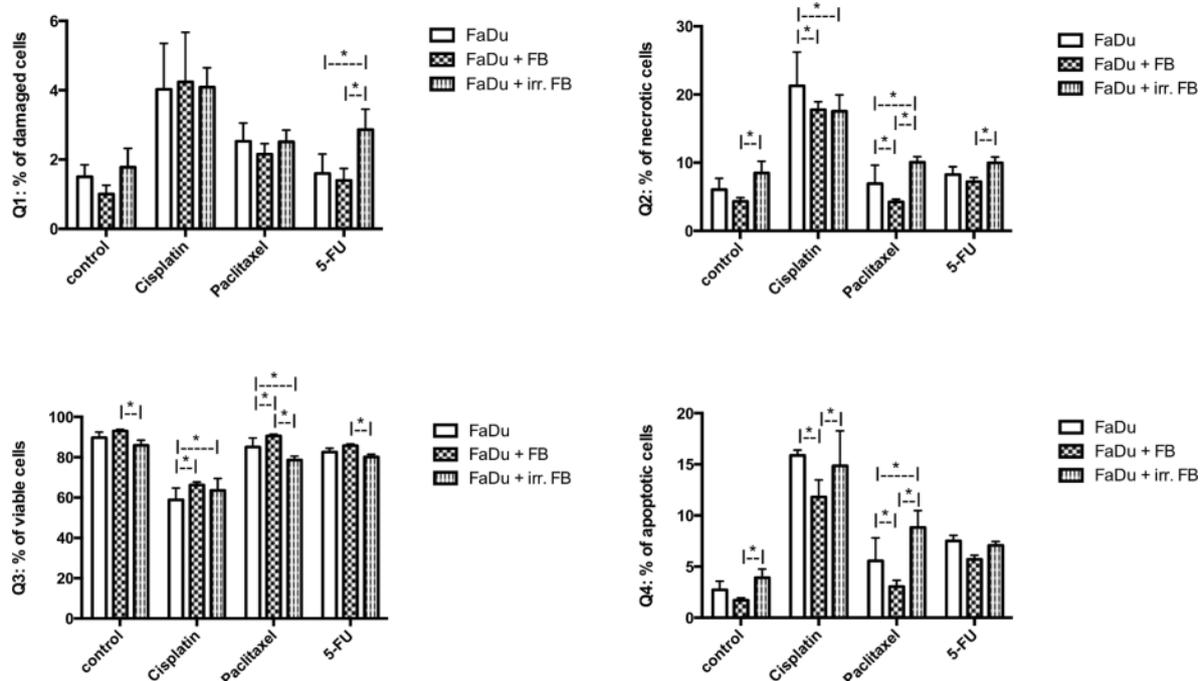


Figure 4: Quantitative assessment of the Annexin V-propidium iodide test. For co-cultures with pre-irradiated fibroblasts versus co-cultures with non-irradiated fibroblasts, significantly higher rates of apoptosis could be identified in the control, Cisplatin and Paclitaxel group; compared to FaDu cells alone, there was a significant increase in apoptosis in the Paclitaxel group. A decrease in apoptosis could be shown for non-irradiated co-cultures versus FaDu cells alone in the Cisplatin and Paclitaxel group. There were also higher rates of necrosis for pre-irradiated fibroblasts versus non-irradiated co-cultures in the control, 5-FU and Paclitaxel group; compared to FaDu cells alone, higher rates of necrosis were significant for the Cisplatin and Paclitaxel group. The rates of viable cells showed significant differences accordingly. * $p < 0.05$ (FB: fibroblasts; irr.: irradiated).

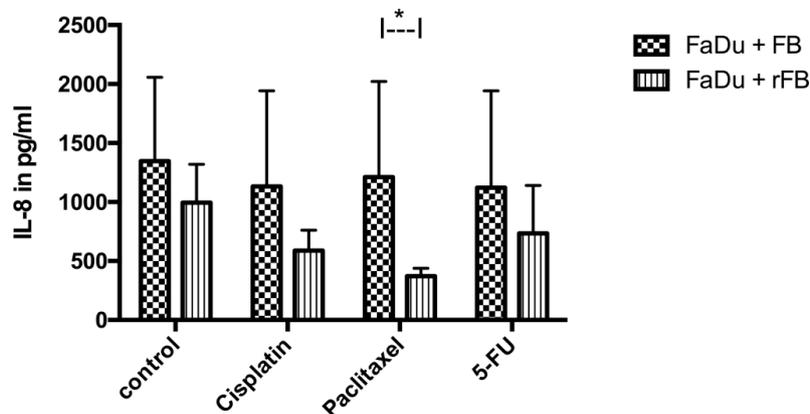


Figure 5: Concentration of interleukin-8 in both co-cultures. A general trend could be seen towards lower levels of secretion of IL-8 in pre-irradiated versus non-irradiated co-cultures. Statistical significance, however, was only observed in the group containing Paclitaxel ($p = 0.0247$). * $p < 0.05$ (FB: fibroblasts; irr.: irradiated).

In literature, there are few studies examining the role of non-irradiated fibroblasts on the Chemosensitivity of co-cultured tumor cells, although even there data is scarce. Fujiwara et al. showed pancreatic cancer cells to be more resistant to Gemcitabine when in co-culture with fibroblasts [30]. Li et al. could demonstrate higher resistance to the cytotoxic agent YC-1 of breast cancer cells in co-culture with fibroblasts in a 3D-model [31]. This could be at least partially mirrored by the present study as well, where in some sub-groups with cytostatic agent's statistically significant increases in viability and lower rates of apoptosis and necrosis could be seen for non-irradiated co-cultures versus FaDu cells alone. In the case of

pancreatic cancer cells, this was seen as a consequence of the tumor cell EMT caused by fibroblasts, which led to a decrease in E-Cadherin and thereby increased invasiveness [30], or due to increased levels of fibroblast growth factor (FGF) and transforming growth factor- β (TGF- β) [32]. Yet, no such studies have been done investigating the chemo resistance of HNSCC with co-cultured fibroblasts, to which our studies may contribute important preliminary results.

To our knowledge, this is the first study to describe the effects of irradiated fibroblasts on the Chemosensitivity of co-cultured tumor cells. Pre-irradiated co-cultures generally showed less viability and correspondingly higher levels of necrosis and apoptosis of tumor

cells than non-irradiated co-cultures. These findings were especially significant for Paclitaxel-treated HNSCC, although in some subgroups of the experiment significant differences could also be identified for Cisplatin and 5-FU. Since there is also a corresponding difference in the control group, it remains unclear whether the effects seen in the cytostatic groups are independent signs of Chemosensitivity, or just a consequence of the anti-proliferative effects of irradiated fibroblasts on FaDu cells. Nonetheless, the addition of cytostatic agents did not equalize the effects of the co-culture, showing the anti-tumor properties of the co-culture are intact even during cytostatic treatment.

IL-8 was chosen to be analyzed in the present study because of its known tumor-proliferative effects [33], mainly through an increase in angiogenesis. IL-8 levels are significantly higher in patients with HNSCC than in healthy controls [34]. Bae et al. could demonstrate that Interleukin-1 produced by oral squamous cell carcinoma cells increased the secretion of IL-8 by co-cultured fibroblasts *in vitro* and *in vivo* [35]. In the present study, the interleukin-8 levels of co-cultures with pre-irradiated fibroblasts were generally lower than in co-cultures with non-irradiated fibroblasts, while this was only statistically significant for Paclitaxel. This is in accordance to the results of a former study without the addition of cytostatic agents [16], which showed higher levels in co-cultures with non-irradiated fibroblasts. For Paclitaxel, higher levels of IL-8 have already been described as a potential mechanism for chemo resistance in ovarian cancer cells [36]. In addition, inhibition of IL-8 has proven to enhance chemo sensitization for Paclitaxel and Cisplatin in multidrug-resistant breast cancer cells [37]. This is the first study, however, to examine HNSCC and cytostatic agents in this regard. For further studies, it will be crucial to examine the exact changes in the fibroblasts themselves through irradiation, so that further analysis of the anti-tumor effects of these cells can be investigated for specific targets. A reduction of interleukin-secretion, namely IL-6 and IL-8, may play an important role therein. This focus will be part of future experiments at our institution.

There are certain drawbacks to this study design, however. First, the fibroblasts used in this study were not CAF's, as they were not cultivated from the primary tumor site. In the present study, primary human fibroblasts from irradiated or non-irradiated skin and immortalized cancer cell lines were used. For an optimized physiological setting, however, both cancer cells and CAFs should be primary human cells and from the same patient. This is quite a challenge regarding the acquisition of a relevant number of patients, though. While CAFs are relatively easy to grow from specimens taken from HNSCC tumors, the culture of primary human cancer cells remains challenging. Nevertheless, since the present data was acquired in a non-physiological setting, the results cannot be transferred to clinical situations, and thus need to be affirmed in amore clinical significant setting. However, our data are an important first step towards understanding the complex changes irradiation implies on tumor stroma.

In conclusion, pre-irradiation of fibroblasts affects the growth of co-cultured HNSCCs, changing their secretory capacities with respect to IL-8 and thus the effect that cytostatic treatment has on them. What exact changes in the fibroblasts via irradiation are responsible for their effects on co-cultured tumor cells should be investigated further. Furthermore, a study with cells from irradiated cancer and the surrounding tissue together should be conducted to evaluate these findings in a more clinical setting.

Acknowledgement

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