doi: 10.1111/j.1600-0714.2006.00503.x

J Oral Pathol Med (2007) 36: 177–83

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Effects of cisplatin, α-interferon, and 13-cis retinoic acid on the expression of Fas (CD95), intercellular adhesion molecule-1 (ICAM-1), and epidermal growth factor receptor (EGFR) in oral cancer cell lines

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BACKGROUND: Previous studies showed that many chemotherapeutic agents can induce immuno-suppression at therapeutic drug concentrations whereas low drug doses induce immuno-augmentation.

METHODS: The effect of low-dose cisplatin, interferon-alpha, and 13-cis retinoic acid on receptors involved in immune-mediated apoptosis (Fas/CD95), cell growth (epidermal growth factor receptor) and lymphocyte adhesion (intercellular adhesion molecule-1) was investigated in two oral cancer cell lines (UT-SCC-20A and UT-SCC-24A). Different methods for cell preparation were studied: mechanical and enzymatic detachment, and culture on chamber slides. Receptor expression was investigated using immunohistochemical staining. The amount of soluble and cell-bound Fas was determined with the ELISA technique, and the functional relevance of Fas expression, apoptosis induction, was analyzed.

RESULTS: Cisplatin enhanced cytoplasm and membrane staining for Fas in both cell lines. After cisplatin treatment, the amount of soluble Fas was increased in UT-SCC-20A cultures, but no effect was observed in the UT-SCC-24A cell line. Apoptosis, measured as enhanced caspase-3 activity, was induced by an agonistic Fas antibody (CH11) after cisplatin treatment in UT-SCC-24A cells.

CONCLUSIONS: Low-dose cisplatin treatment enhanced Fas expression in both cell lines and increased susceptibility to apoptosis in one of them.

J Oral Pathol Med (2007) **36**: 177–83

Keywords: apoptosis, 13-cis retinoic acid, cisplatin, EGFR, Fas (CD95), ICAM-1, α-interferon, oral cancer

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Accepted for publication September 13, 2006

Introduction

Curative treatment for squamous cell carcinoma of the head and neck (HNSCC) is based on surgery and/or radiotherapy. Chemotherapy alone has been less effective, and currently is mostly used in combination with radiation, or palliatively. However, increasing knowledge of the immunogenicity of HNSCC has created interest in immune therapy as an alternative treatment modality (1). In addition to proper presentation of tumor-associated antigens (2), the expression of several receptors on tumor cells is of importance for immune-mediated cancer control. ICAM-1 is an intercellular adhesion molecule which in addition to its pivotal role in initiation of an immune response also plays an important role in cell-to-cell interactions between lymphocytes and tumor cells (3). Non-functional apoptosis signaling in cancer cells may cause resistance to immune-mediated apoptosis. The Fas receptor is a transmembrane death receptor expressed in both normal and tumor tissue (4). Fas-Fas ligand interaction plays a critical role in cytotoxic T-cell killing and negative regulation of immune processes (5). Thus, in order to predict and optimize the outcome of cancer treatment based on immune therapy, it is important not only to obtain information on the reactivity of the immune system but also on the sensitivity of the tumor cells to this type of treatment. The epidermal growth factor receptor (EGFR) is a new target molecule in therapy of epithelial cancers (6). Treatment of loco-regionally advanced head and neck cancer (stage III or IV) with concomitant high-dose radiotherapy plus cetuximab, an anti-EGFR-antibody, was recently shown in a multinational study to significantly prolong progression-free survival (7). EGFR tyrosine kinase inhibitors have shown favorable results in phase II trials both as monotherapy and in combination with chemotherapy (8).

The large majority of chemotherapeutic agents exert their anti-tumor effects through anti-proliferative and cytotoxic mechanisms, and cytotoxic drugs are predominantly used at maximally tolerated doses (MTD). During the last few decades, evidence on the immune-modulating properties of these drugs has increased (9), and the augmentation of immune responses seems to be dose-dependent (10). Cisplatin (CDDP) is a DNA-interactive chemotherapeutic drug commonly used in the treatment of HNSCC. There is increasing evidence that low to moderate doses of cisplatin induce immuno-augmentation while high doses induce immuno-suppression (11–13). α-Interferon (IFN-α) is another potential immune response modifier in the treatment of HNSCC. It possesses anti-viral, anti-proliferative, and immuno-modulatory effects (14). Moreover, vitamin A derivates, retinoids, are essential for growth and cell differentiation (15). Hong et al. (16) reported that 13-cis retinoic acid (13-cisRA) suppressed the oral pre-malignant lesions and decreased the incidence of second primary tumors in head and neck cancer.

Given the poor efficacy of chemotherapy, immunotherapy, and molecular-targeted therapy of cancer when used as single modalities, it is clear that the efficacy of combination treatment should be investigated. The objective of the present investigation was to study the effects of low-dose cisplatin, IFN-α, and 13-cisRA on the expression of receptors crucial to lymphocyte adhesion (ICAM-1), cell growth (EGFR), and apoptosis induction (Fas) in two oral squamous cell carcinoma cell lines. To improve the validity of the results, the influence of different cell preparation techniques on receptor expression was also studied.

Materials and methods

Cell lines and culture conditions

Two recently established squamous cell carcinoma cell lines, UT-SCC-20A and UT-SCC-24A, were investigated (17). UT-SCC-20A has mutated p53 (18), and UT-SCC-24A has one normal p53 allele (19). The cell lines were established from primary squamous cell carcinomas of the mobile tongue (UT-SCC-24A) and the floor of the mouth (UT-SCC-20A), and passages 10–20 were used in the present study. The cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, non-essential amino acids, antibiotics (streptomycin 50 μg/ml and penicillin 100 IU/ml), and 10% foetal calf serum (all from Gibco, Paisley, UK). All cells were cultured in plastic flasks (NUNC, Rochester, NY, USA) and incubated at 37°C in a humidified air atmosphere with 5% CO_2.

Detachment procedure

The cells were harvested at sub-confluent growth in flasks, and trypsinization or mechanical cell detachment with a cell scraper was performed in parallel flasks. A solution with 0.01% trypsin (Sigma-Aldrich, Gillingham, UK) and 0.02% K-EDTA was used for enzymatic detachment. Both trypsinized and scraped cells were prepared on cytospin glasses (7 min at 8.5 *g*), or, alternatively, the cells were cultured on glass chamber slides for 72 h, then directly dried and stored at −70°C until staining.

Drug modulations

After trypsinization the cells were seeded onto glass chamber slides, UT-SCC-20A at 40 000 cells/ml and UT-SCC-24A at 50 000 cells/ml. After 24 h, the culture medium was changed and CDDP (0.5 μg/ml), IFN-α (1000 U/ml), and 13-cisRA (0.003 μg/ml) were added. The cells were cultured with or without drugs for 48 h and the slides were then gently washed with phosphate-buffered saline (PBS) solution, dried, and stored at −70°C.

Immunologic staining

The cell preparations were fixed with phosphate-buffered 4% paraformaldehyde (pH 7.4) for 5 min, and then blocked with 10% human AB serum for 15 min. The cells were incubated with primary mouse anti-human antibodies Fas 50 μg/ml (clone DX2; Dako Corp, Carpinteria, CA, USA), ICAM-1 8 μg/ml (clone 6.5B5; Dako, Glostrup, Denmark), EGFR 1 μg/ml (Labvision, Fremont, CA, USA), and IgG 50 μg/ml (IgG_1; Dako). The antibody dilutions used differed depending on the purpose of the staining; the antibodies used in staining after drug modulations were deliberately more diluted in order to make it easier to observe any receptor upregulation (Fas 25 μg/ml, ICAM-1 0.125 μg/ml, EGFR 0.125 μg/ml, and IgG 25 μg/ml). All primary antibodies were incubated for 30 min except anti-EGFR mAb and the negative control, which were incubated for 60 min. After incubation with the primary antibodies the slides were washed in BSS and Tris-buffered saline (TBS) and incubated with Envision+, Peroxidase, Mouse (Dako Corp) for 30 min. The slides were then washed in TBS and incubated with AEC+ substrate-chromogen (Dako Corp) for 20 min. The washing was repeated after AEC+ incubation, the slides were then counterstained in Mayer's hematoxylin for 1 min and mounted in Glycergel (Dakopatts, Stockholm, Sweden). The staining results, antigen distribution, staining intensity, and frequency of positive cells were separately evaluated by two people.

Quantification of soluble and membrane-bound Fas

The cells were seeded onto 6-well plates (NUNC, Life Technologies); UT-SCC-20A at 9×10^4 cells/ml, and UT-SCC-24A at 11×10^4 cells/ml. After 24 h of plating, the culture medium was changed in all dishes and different modulating agents (CDDP, IFN-α, and 13-cisRA in the same concentrations as in earlier modulations) were added. After 48 h, the cells were gently harvested using a cell scraper. The cell suspension consisting of cells and culture medium was collected and centrifuged at 420 *g* for 5 min. The culture medium was collected separately and the cells were washed once in PBS. All samples were stored at −70°C until receptor quantification. The amount of Fas receptor in both membrane-bound and soluble form was determined

using Fas/APO-1 enzyme-linked immunosorbent assay (ELISA) (Oncogene; Calbiochem, Darmstadt, Germany). All assays were conducted according to the suppliers' recommendations. The optical density of all samples was determined using a microplate reader (Versa max; Molecular Devices Corp, Sunnyvale, CA, USA). Protein determination of samples was performed using a bicinchoninic acid protein (BCA) assay kit (Sigma, St Louis, MO, USA), following the detection protocols recommended by the manufacturers.

Apoptosis induction and detection

The cells were seeded into 6-well plates (Costar, Cambridge, MA, USA) at a density of 12 000/cm^2 (UT-SCC-20A) and 15 000/cm^2 (UT-SCC-24A). After 24 h, fresh medium was added either with or without cisplatin (0.5 μg/ml) and after another 40 h an apoptosis-inducing Fas antibody (clone CH11; Upstate, Lake Placid, NY, USA; 50 ng/ml) was added for 8 h. Cells were then trypsinized and together with detached cells collected in the culture medium and centrifuged (420 *g* for 5 min). The cell pellet was re-suspended in 100 μl PBS and subjected to cytospin centrifugation at 8.5 *g* for 5 min. The cells deposited on slides were fixed in 4% neutral buffered formalin for 20 min, rinsed in water, and mounted in Vectashield® mounting medium supplemented with the fluorescent dye DAPI (1.5 μg/ml; Vector Laboratories, Burlingame, CA, USA), which stains DNA. Nuclear morphology was examined in a Nikon microphot SA fluorescence microscope (Nikon, Tokyo, Japan) using UV exciting light and a blue barrier filter. Samples were blinded and the number of cells with nuclear apoptotic morphology was determined. When an apoptosis-inducing Fas antibody was added with cycloheximide (1 μg/ml) to cell cultures for the last 8 h, apoptotic nuclei were observed. Thereafter, caspase-3 activity was measured in the cell samples.

Measurement of caspase-3 activity

Caspase-3 activity was measured using the fluorescent substrate Ac-DEVD-7-amino-4-methyl coumarin (Ac-DEVD-AMC; Becton-Dickinson, Mountain View, CA, USA), which is cleaved by caspases with caspase-3-like activity. Measurements were performed according to the manufacturer's instructions. After incubation for 1 h at 37°C, the amount of released AMC was analyzed at λ_{ex} 380 nM and λ_{em} 435 in an RF-1501 Spectrofluorophotometer (Shimadzu, Kyoto, Japan). The amount of released AMC was calculated using a standard curve, and correlated with total protein determined with the method described by Lowry et al. (20). Caspase-3 activity was expressed in nmol AMC/mg protein/h.

Results

Effects of different cell detachment procedures

The growth of epithelial cells is anchorage-dependent, therefore such cells have to be detached from the surface of the culture vessel in preparation for the analysis of cellular substances. However, detachment is often achieved by mild trypsinization, which might seriously influence the concentration and distribution of cellular substances. In order to examine the effect of trypsinization and mechanical cell detachment on protein expression and distribution, we compared mechanically and enzymatically detached cells with cells that were cultured on glass chamber slides.

Cells detached using trypsin became spheroid; they had a compact cytoplasm, and blebbing of the cell membrane occurred. Even though detachment of cells by scraping was made very gently, this procedure resulted in a large number of naked nuclei with only a few faintly staining cells. Compared with cells cultured on chamber slides, cytospin slides prepared from trypsinized cells showed more intense Fas staining of the cytoplasm, obscuring the discrete membrane staining, particularly in cell line UT-SCC-20A. After trypsinization, distribution of the cytoplasmic staining changed; a distinct peri-nuclear staining appeared in a large number of cells (Fig. 1). The effect of the different cell detachment procedures on the appearance of the other receptors was very similar. The cytoplasmic EGFR staining was more intense after enzyme treatment, again obscuring the membrane staining in UT-SCC-20A, whereas in UT-SCC-24A, an accentuation of this staining was seen despite an increased staining of the cytoplasm. The cytoplasmic staining for ICAM-1 was also markedly more pronounced in trypsinized cells, but

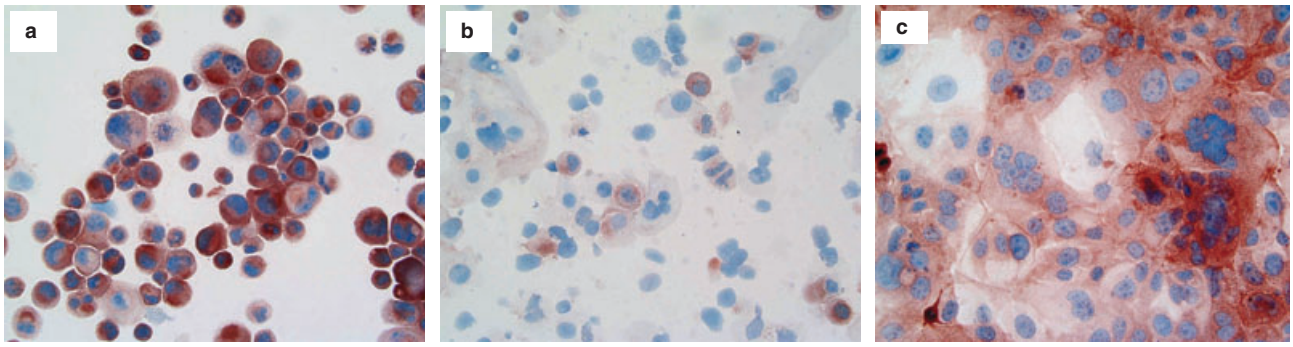

Figure 1 Immunohistochemical staining for Fas in UT-SCC-20A after trypsinization (a), detachment with a cell scraper (b), and culture on glass chamber slides (c).

the membrane staining in these preparations was clearly shown in both cell lines. Thus, the more intense staining of the cytoplasm was consistently found for all analyzed receptors in cell line UT-SCC-20A. Based on these observations, studies of the modulation of receptors were performed on glass chamber slides, where the cells could be studied while still attached to the surface of the culture vessel.

The growth pattern in cultures intended for immunochemical analyses and determination of apoptosis was very similar to the one seen in flasks. The UT-SCC-20A cells had varying sizes, with both small and large cytoplasm. Three distinct growth zones were identified in 72-h cultures; the center was dominated by confluently growing cells with small cytoplasm, the periphery contained a large number of cells with large cytoplasm, and the intermediate zone consisted of both cell types. The cells of the other cell line, UT-SCC-24A, were more homogenous in size, but there were still differences between the center and the periphery. Immunohistochemical analysis was therefore performed separately in three growth zones for UT-SCC-20A and two for UT-SCC-24A.

Immunohistochemical investigation of receptor expression modulation

Modulation of receptor expression was studied using cultures on glass chamber slides with sub-toxic concentrations of cisplatin, IFN-α, and 13-cisRA, that is, concentrations which were found to produce minimal growth retardation, cell death, and intercellular dissociation between attached cells. Compared with untreated controls, cisplatin enhanced the membrane and cytoplasmic staining for Fas in the central and intermediate growth zones of UT-SCC-20A and in both growth zones of UT-SCC-24A (Fig. 2). Cisplatin had no effect on the expression of EGFR or ICAM-1 staining in UT-SCC-20A, but enhanced the cytoplasmic staining for ICAM-1 in the central growth zone of UT-SCC-24A. IFN-α induced a change in growth pattern in cultures of UT-SCC-20A – the zone of cells with small cytoplasm in the middle of the confluent areas was reduced and the number of cells with large cytoplasm was increased in this area – but receptor expression was unaffected. In the other cell line, UT-SCC-24A, the cytoplasmic staining intensity for EGFR and ICAM-1 was enhanced in the central growth zone but no effect was found on Fas expression. 13-cisRA had no effect on EGFR staining in UT-SCC-20A, but both cytoplasmic and membrane staining for ICAM-1 was enhanced in the central and peripheral growth zones. The effect of 13-cisRA on Fas expression was variable in cell line UT-SCC-20A. 13-cisRA also increased the cytoplasmic and membrane staining for Fas and EGFR in the central growth zone of UT-SCC-24A, but no staining intensity changes were observed in peripheral growth zones. ICAM-1 membrane staining was not affected by any of the modulating agents in UT-SCC-24A cultures.

Quantification of soluble and cell-bound Fas after drug addition

The total amount of soluble and cell-bound Fas receptor was further quantified using the ELISA technique in three parallel cultures (Fig. 3). Both cell-bound Fas and Fas secreted into the medium were normalized to the total amount of protein in the culture. In accordance with the staining results, the three modulating drugs showed almost no toxicity at the concentrations used; the amount of protein in cultures with modulating drugs was only slightly reduced compared with control cultures (data not shown). The amount of soluble Fas in the conditioned medium of UT-SCC-20A was increased by all three modulating drugs, whereas the amount of cell-bound receptor was unaffected. Drug modulations had a different effect on UT-SCC-24A; no increase in the two receptor forms was observed.

Apoptosis induction

The biological significance of Fas expression was further analyzed by incubating the two cell lines with an agonistic Fas monoclonal antibody (CH11) to induce apoptosis. Particular attention was paid to the effect of cisplatin, the immunohistochemistry of which had shown to enhance the expression of cell-bound and membrane-bound Fas in both cell lines. In order to optimize the experimental conditions, apoptosis was

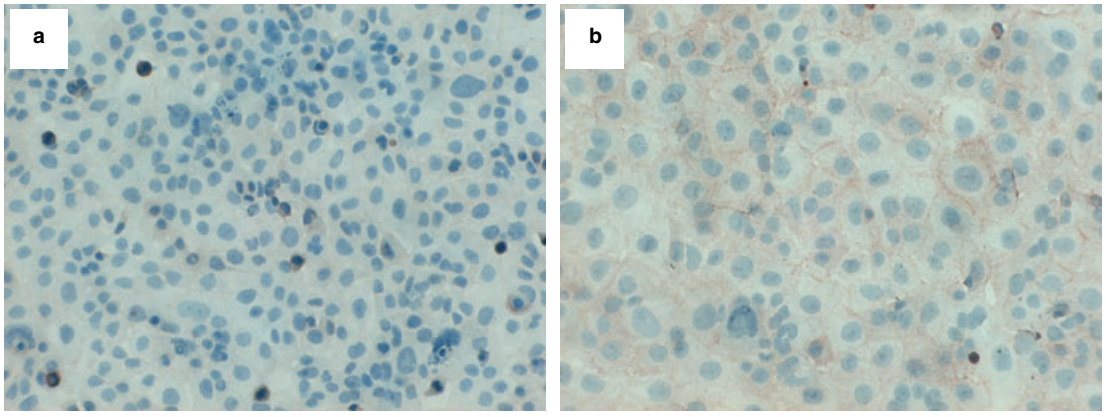

Figure 2 Immunohistochemical staining for Fas in UT-SCC-24A cells with no (a) or 48-h exposure to cisplatin (b).

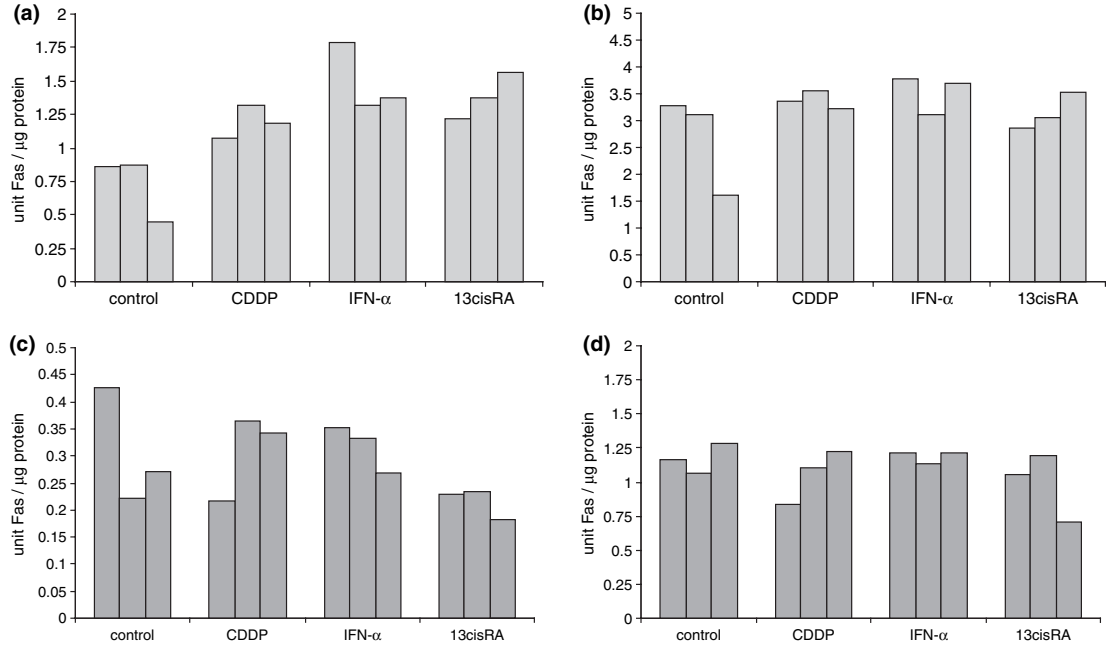

Figure 3 The amount of soluble (a, c) and cell-bound (b, d) Fas in three parallel cultures after incubation with low-dose modulating drugs. The three adjacent bars show results of parallel culture experiments. UT-SCC-20A (a, b), UT-SCC-24A (c, d).

first studied as nuclear condensation in DAPI-stained preparations, but no enhanced apoptosis induction was observed in either cell line (data not shown). Addition of cycloheximide (CHX) to all antibody concentrations resulted in apoptosis after 8 h. Induction of apoptosis, under these experimental conditions, was then quantified by determination of caspase-3 activity measured as release of AMC, and all values obtained in caspase-3 activity analyses were normalized to the total protein amount in each cell sample. The diagrams in Fig. 4 show the results of one of two representative experiments. A low spontaneous caspase-3 activity was found in both cell lines; 0.9 nmol AMC/mg protein in UT-SCC-20A and 0.253 nmol AMC/mg protein in UT-SCC-24A. Addition of CHX had a slight upregulating effect on this activity – 1.459 and 0.497 nmol AMC/mg protein, respectively. Stimulation with cisplatin alone or in combination with CHX showed similar results with no increase in activity. Addition of the apoptosis-inducing antibody CH11 with CHX resulted in over 10-fold enhancement of the caspase-3 activity in both cell lines (10.903 and 3.257 nmol AMC/mg protein, respectively), and in cell line UT-SCC-24A, cisplatin further increased the apoptotic activity (5.22 nmol AMC/mg protein).

Discussion

Cell surface receptors such as Fas, EGFR, and ICAM-1 are involved in control or progression of malignant tumors. Therapeutic drugs can potentially modulate the expression of these receptors and thus modulate the

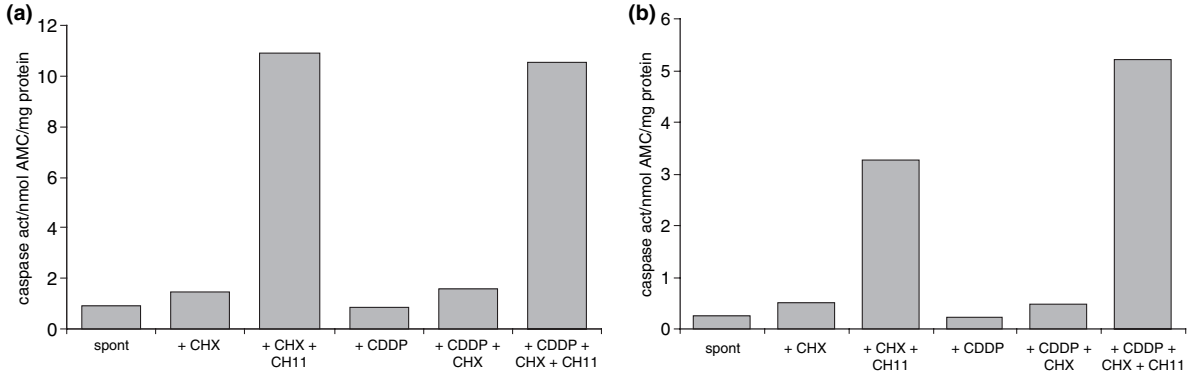

Figure 4 Caspase-3 activity in UT-SCC-20A (a) and UT-SCC-24A (b) cells spontaneously and after stimulation with different agents for 8 h. Results of one of two representative experiments are shown. Each bar represents a mean value of three samples. spont, spontaneous caspase-3 activity; CHX, cycloheximide (1 µg/ml); CH11, agonistic Fas-antibody (50 ng/ml); CDDP, cisplatin (0.5 µg/ml).

therapeutic efficacy of targeting drugs. The aim of this study was therefore to investigate the effect of three potentially modulating therapeutic drugs – cisplatin, IFN-α, and 13-cisRA – on the expression of Fas, EGFR, and ICAM-1 on two squamous cell carcinoma cell lines from the oral cavity.

Cultured adherent cells intended for various types of analyses are usually detached from the surface of the culture vessel by mild trypsinization. However, as we have shown, this procedure can profoundly change the cellular appearance and could possibly also result in proteolytic release of cell surface receptors. Epithelial cells, which are enzymatically detached from the culture vessel surface, round up and minimize their cell membrane area compared with cells attached to and stretching out on the surface of the culture vessel. This can give the impression of higher membrane and cytoplasmic staining intensity in trypsinized cells, as the concentration of both membrane-bound and cytoplasmic epitopes per area will increase. This is a reasonable explanation for the weaker staining intensity of cells cultured on glass chamber slides. In addition, enzymatic unmasking of epitopes might play a role in modulating the intensity of staining in immunohistochemical studies. Thus, analysis of various cell constituents after enzymatic treatment should be performed with great care even several days after sub-cultivation, as it is difficult to determine when a full recovery has taken place.

The major effect of cisplatin was found on the expression of Fas, which was generally upregulated in all growth zones of both cell lines. The only other effect of this drug was upregulation of the cytoplasmic expression of ICAM-1 in one of the two cell lines. IFN-α only enhanced the cytoplasmic expression of EGFR and ICAM-1 in one of the cell lines. Cytoplasmic and membrane staining of Fas and EGFR were enhanced by 13cisRA in UT-SCC-24A, and ICAM-1 staining in UT-SCC-20A. These results clearly show that Fas, EGFR, and ICAM-1 expressions were modulated differently by the tested drugs in the two cell lines, which is in agreement with previous results (e.g. 21). Based on these observations it was decided to further explore the effect of cisplatin on Fas expression using quantitative methods.

As cisplatin is a cytotoxic drug, it is important to choose sub-toxic drug concentrations in studies of its modulatory effect on the expression of Fas, to avoid possible toxic effects. We therefore used a concentration of cisplatin, 0.5 μg/ml, that was found to have no or only minimal cytotoxic activity. This is in good agreement with earlier studies, in which cisplatin treatment with < 0.5 μg/ml showed less than 10% cytotoxicity in HNSCC cells (22) whereas treatment of HNSCC cells by higher cisplatin concentration (1–5 μg/ml) showed 20–50% cytotoxicity (22, 23). The results of some studies are difficult to interpret, as they used a high concentration (24) or reported high toxicity (25). Previously only one study of HNSCC cells used cisplatin concentration comparable to the one used in the present investigation (26); in line with our results, the expression of Fas was found to be increased. However, in the previous study Fas expression was determined by flow cytometry. Based on our results, it can thus be anticipated that the expression of Fas would have been seriously influenced by the cell preparation procedure.

There is an apparent discrepancy between the results regarding Fas expression analyzed by immunohistochemical staining and by quantitative determination on cell lysates using the ELISA technique. Fas expression was enhanced in the cytoplasm and on the cell membranes after immunohistochemical staining but the total amount of cell-bound receptor as determined by ELISA was not increased. This might be due to some heterogeneity in the expression of Fas, the different sensitivity of the two methods used, and also redistribution of Fas between cytoplasm and cell membrane.

Both cell lines were sensitive to apoptosis induction by an agonistic antibody (CH11) presently, irrespective of p53 status. However, when apoptosis was induced after cisplatin treatment, only one of the cell lines, UT-SCC-24A, showed an additionally increased caspase-3 activity. Interestingly, low-dose cisplatin treatment has been shown to induce apoptosis after Fas stimulation only in hepatic cancer cells that had wild-type p53 (27). The importance of wild-type p53 for Fas-mediated apoptosis is also supported by studies of lung and prostate cancer cell lines (28). The Fas-mediated cell death cannot be solely dependent on wild-type p53 as shown presently but a cisplatin-induced, further enhanced apoptotic cell death may be p53-dependent. p53 is a transcriptional regulator of the *cd95*(Fas) gene (29). In addition, the activated p53 can increase surface Fas expression by transport from the Golgi complex and thereby promote enhanced apoptosis (30). Other mechanisms, such as inhibition of FLIPs (death domain-like interleukin-1β-converting enzyme-inhibitory protein) by cisplatin, may be involved in regulation of Fas-mediated apoptosis (31). An enhanced secretion of death receptors by chemotherapeutic drugs can also contribute to resistance to cancer treatment. The secretion of Fas can theoretically protect cells from apoptosis if soluble receptors neutralize the Fas ligand in the extracellular space before they reach membrane-bound receptors; this might explain why apoptosis was not increased in our UT-SCC-20A cultures.

As molecular-targeted cancer therapy often achieves only disease stabilization, combination treatment with various drugs is currently being developed. It is therefore becoming more and more important to understand the interaction between different drugs in order to enable their optimal use. The present article demonstrates not only the usefulness of cell lines in this type of investigation but also the complexity of the procedure and the need to use a combination of immunohistochemical, quantitative ELISA, and functional methods. The two HNSCC cell lines studied showed different response patterns to drug modulation, which emphasizes the individual behavior of cancer and the need for individualized treatment when new and costly drugs are used in clinical practice.

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Acknowledgements

This study was supported in part by grants from the Ansgarius Foundation and the Research Council of South Eastern Sweden (FORSS).