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Short Communication

Enhancement of docetaxel efficacy in head and neck cancer treatment by G0 cell stimulation

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ARTICLE INFO

Article history:
Received 22 August 2005
Accepted 2 September 2005
Available online 23 May 2007

Keywords:
Docetaxel
Chemotherapy
G0 cells
SCCHN

ABSTRACT

Objectives: Docetaxel has recently taken part in new chemotherapy regimens with promising activity especially in the first line therapy (induction chemotherapy) of head and neck cancer (SCCHN). Nevertheless a major problem concerning the response of SCCHN to chemotherapy is the high percentage of resting cells (G0-phase cells) being resistant to chemotherapy. To overcome this phenomenon we have investigated the capacity of several cytokines to switch on cells into division cycle and progress to the chemosensitive phases (S, M-phase).

Methods: Il-6, Serotonin, G-CSF and EGF were used to stimulate G0-phase squamous cell cancer cells (Detroit 562, A431, UM-SCC 10B) for reentry in the cell cycle to enhance the response to docetaxel. The proportion of G0-phase cells was detected through multicolor FACS analysis and Ki67 staining.

Results: Cell cycle reentering was most effective after combination treatment with Serotonin + EGF. The proportion of G0 phase cells was significantly reduced after stimulation with Serotonin + EGF ($p < 0.05$). Corresponding to cell cycle reentry the cytotoxic effect of docetaxel was significantly ($p < 0.04$) enhanced in the prestimulated cells compared to the control (docetaxel monotreatment).

Conclusions: Our investigations demonstrate for the first time that sensitizing G0 phase squamous cell carcinoma cells for docetaxel treatment is possible by prestimulation with target cytokines. Considering that up to 95% of tumor cells are in the resting (G0) phase of the cell cycle at the initiation of chemotherapy, prestimulation with EGF and serotonin could contribute to a synchronization of cancer cells. This would clearly enhance the cytotoxic effect.

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1. Introduction

Docetaxel has been assigned to the treatment of head and neck cancer in recent years.[1,2] Induction chemotherapy in the treatment of advanced head and neck cancer has been resumed since taxanes were added to cisplatin-based regimens. Several studies demonstrated high response rates of head and neck squamous cell carcinoma following treatment with docetaxel, cisplatin and 5-FU even during radiation.[3–17] However, despite complete remission rates after therapy, few patients suffer from recurrences during a follow up period of 2 years.[18]

Overexpression of the epidermal growth factor receptor (EGFR) is associated with poor prognosis.[19–22] This may be due either to the enhancement of cell proliferation or the

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doi:10.1016/j.ejca.2005.09.037

activation of resting (G0) cells. We assume that these cells were less vulnerable to injury by radiation and/or chemotherapy. It has recently been demonstrated that unresponsiveness of leukemic cells to both chemotherapy and immunotherapy could be due to residence in resting G0 phase of the cell cycle.[23] Recruitment of leukemic cells from dormant G0 phase into activated phases of the cycle by activation or induction of proliferation restored their sensitivity.[24]

Squamous cell carcinoma cells passing through the cell cycle depend on growth factor receptor signals (i.e. EGFR, GCSFR, etc.). Among others, IL-6, Serotonin, EGF and GCSF are proteins able to stimulate cytokine and growth receptors resulting in cell cycle progression. These proteins have been demonstrated to activate STAT3 dependent signal transduction pathways, which provoke enhanced transcription in the nucleus.[25–30] We therefore tested these proteins in vitro for their potential to recruit G0 cells into activated phases of the cell cycle in order to restore their vulnerability to chemotherapy. The most active combination (IL6 and EGF) was then used to stimulate squamous cell carcinoma cells before treatment with docetaxel.

2. Methods

2.1. Cell cultures

Three different SCCHN cell lines were used in this investigation. Detroit 562 (pharyngeal squamous cell carcinoma) and A431 (vulvar squamous cell carcinoma) were obtained from ATCC (American Type Culture collection). Thomas Carey, University of Michigan, has kindly provided UM-SCC 10B (laryngeal carcinoma). All cell lines were tested for overexpression of EGFR.

2.2. Fluorescence Activated Cell Sorter (FACS) analysis

A detergent and proteolytic enzyme-based technique was used for nuclear isolation and DNA content analysis of cells in different phases of cell cycle. Cells were harvested after treatment with serotonin, EGF, GM-CSF and IL-6 and processed for propidium iodide staining using a Cycle TEST™ Reagent Kit (Becton Dickinson, San Jose, CA). The cellular DNA content was analyzed by the Becton-Dickinson FACScan™ flow cytometer. At least 10,000 cells per sample were analyzed in the gated regions. The ranges for G0/G1, S, G2/M, and sub-G1 phase (apoptotic) cells were established based upon their corresponding DNA contents of the histograms. Results were analyzed and expressed as percentages of the total gated cells using the Modfit LT™ Software (Becton Dickinson).

2.3. Western Blot Analysis

Whole cell extracts (50 μg/lane) were electrophoresed through 8% sodium dodecyl sulfate (SDS)–polyacrylamide gel and were transferred onto a Hybond-C-super nitrocellulose membrane (Amersham, Buckinghamshire, UK). Prestained molecular weight markers (Life Technologies, Inc. Gaithersburg, MD) were included. Membranes were blocked for 30 min in Tris-buffered saline (TBS, pH 7.5) with 0.5% Tween-20 (TBST) and 5% nonfat dry milk. After blocking, membranes were incubated for 60 min with a Stat-3 and AKT as well as with phosphorylation specific p-Stat-3 and p-AKT mouse monoclonal antibody (Cell Signaling). After incubation with horseradish peroxidase–conjugated secondary antibody, the membranes were scored by using the enhanced chemiluminescence (ECL) detection system (Amersham).

2.4. Ki 67 staining

Cells from each treatment group have been transferred from culture medium to glass slides and prepared for staining with Ki-67 monoclonal antibody (1:20 dilution; Dako). The slides were subsequently incubated with biotinylated goat antirabbit/antimouse IgG for 20 min (Dako LSAB2 System) and then with streptavidin horseradish peroxidase for 20 min. For immunostaining, the slides were incubated in 3,3′-diaminobenzidine (Dako) solution containing 0.06 mmol/L 3,3′-diaminobenzidine and 2 mmol/L hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips with Permount mounting medium (Proscitech, Kirwan, Australia). Ki 67 negative cells, which represent the G0 phase cells, were counted three times on three different vertical lanes of the glass slide.

2.5. Controls

The control group of each cell line (Detroit 562, A431, UM-SCC 10B) was not treated during the observation period, during which the cell culture medium (Dulbeccos modified eagle medium, DMEM + 10% FCS) was changed daily. For estimating the effect of cytokine stimulation the prestimulated docetaxel group was compared with the docetaxel monotreatment group after stimulation (serotonin + EGF).

3. Results

3.1. Stimulation of SCCHN cells with cytokines

Detroit 562 cells were incubated with combinations of IL-6, Serotonin, EGF and GCSF (2 cytokines per group). As displayed in Fig. 1, the combination of 1μg/μl EGF and 1 mM/ml Serotonin yielded the highest transition rate of cells from the G0 into the G2/M phase. Compared to the control, cells of the G0 and G1 phase were reduced up to 10% transferring them mainly in the G2-M phase (+10%). Similar results were obtained with other cell lines (data not shown).

3.2. Cell cycle transition of SCCHN cells through EGF/serotonin incubation

Detroit 562 cells were incubated with increasing concentrations of Serotonin and EGF. As revealed by FACS analysis, coincubation of cancer cells with EGF and Serotonin increased the percentage of cells in the G2/M phase by corresponding reduction in the percentage of G0/G1 phase cells at the same

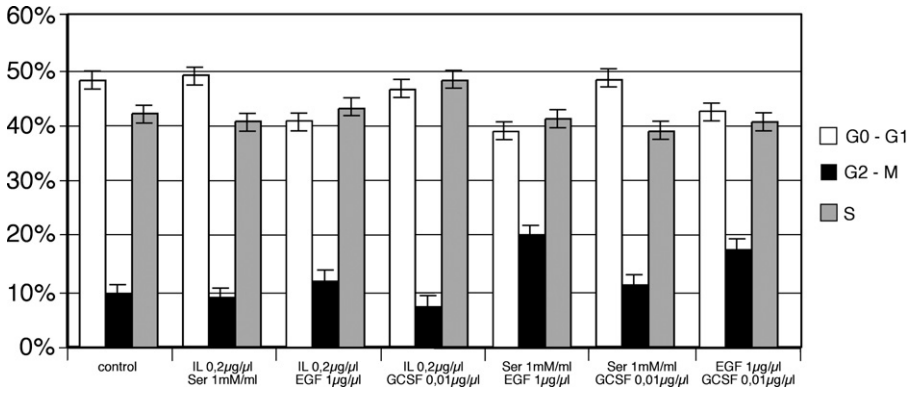

Fig. 1 – Detroit 562 cells were incubated with Interleukin 6, Epidermal growth factor, granulocyte colony stimulating factor and serotonin (concentrations displayed) for 24 h. After that, cells were analyzed for cell cycle distribution using flow cytometry. The combination of 1 µg/µl EGF and 1 mM/ml Serotonin revealed to be the treatment with highest efficacy concerning cell cycle transition. Compared to the control, cells of the G0 and G1 phase were reduced up to 10% and transferred to the G2-M phase (+10%) [$p < 0.05$]. The figure displays the mean value of proportions ±S.E.

time (Fig. 2). The number of cells in S-phase did not change. As displayed in Fig. 2, this effect seems to be dose dependent. Similar results were obtained other cell lines (data not shown).

3.3. *Serotonin/EGF stimulation enhances treatment efficacy of docetaxel*

10,000 cells from A431, Detroit 562 and UMSCC 10B cell cultures were treated with 100 nM/l docetaxel with or without prestimulation by 10 mmol/ml Serotonin and 1 µg/µl EGF. After 7 days this treatment was repeated. The total amount of A431, Detroit 562 and UM-SCC 10B cells were significantly reduced in the prestimuled group compared to the control and CTX (docetaxel without stimulation) group ($p < 0.04$). While this effect has been comparable over all investigated cell lines, enhancement of proliferation in the stimulation group did vary among the cell type (Fig. 3a–c).

4. Discussion

Local recurrence of disease is the major problem in patients with head and neck cancer. Around 60% of stage III and IV patients suffer from recurrent tumors during 36 months after primary treatment.[31] Overall prognosis depends strictly on disease free survival. The goal of modern cancer treatment should therefore be the prolongation of disease free survival. Docetaxel has been assigned to the treatment of head and neck cancer in recent years.[1,2] Induction chemotherapy in the treatment of advanced head and neck cancer has been resumed since taxanes were added to cisplatin-based regimens. Several studies demonstrated high response rates of

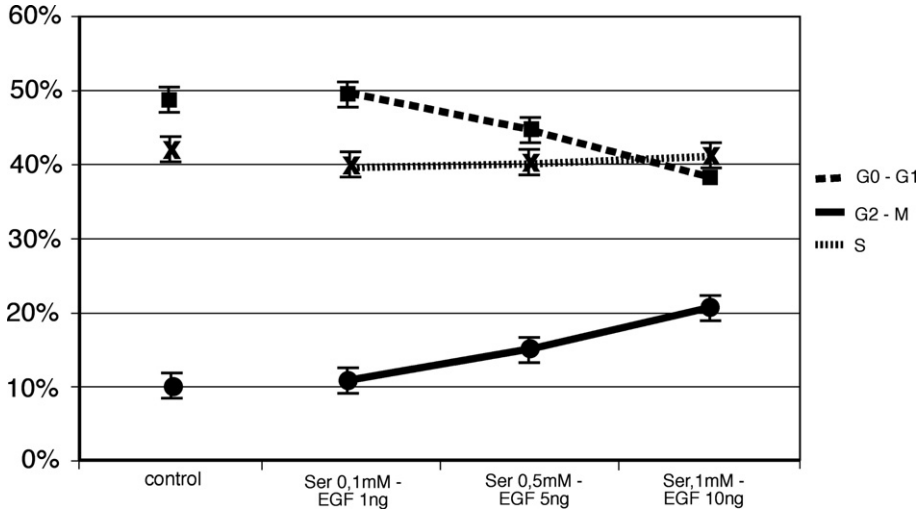

Fig. 2 – Detroit 562 cells have been incubated with epidermal growth factor and serotonin at different concentrations (see graph) for 24 h. After that, cells were analyzed for cell cycle distribution using flow cytometry. The stimulation increased the proportion of cells in the G2/M phase of the cell cycle corresponding with a decrease of G0/G1 phase cells. The number of cells in S-phase did not change. The effect was concentration dependent ($p < 0.05$). The figure displays the mean value of proportions ±S.E.

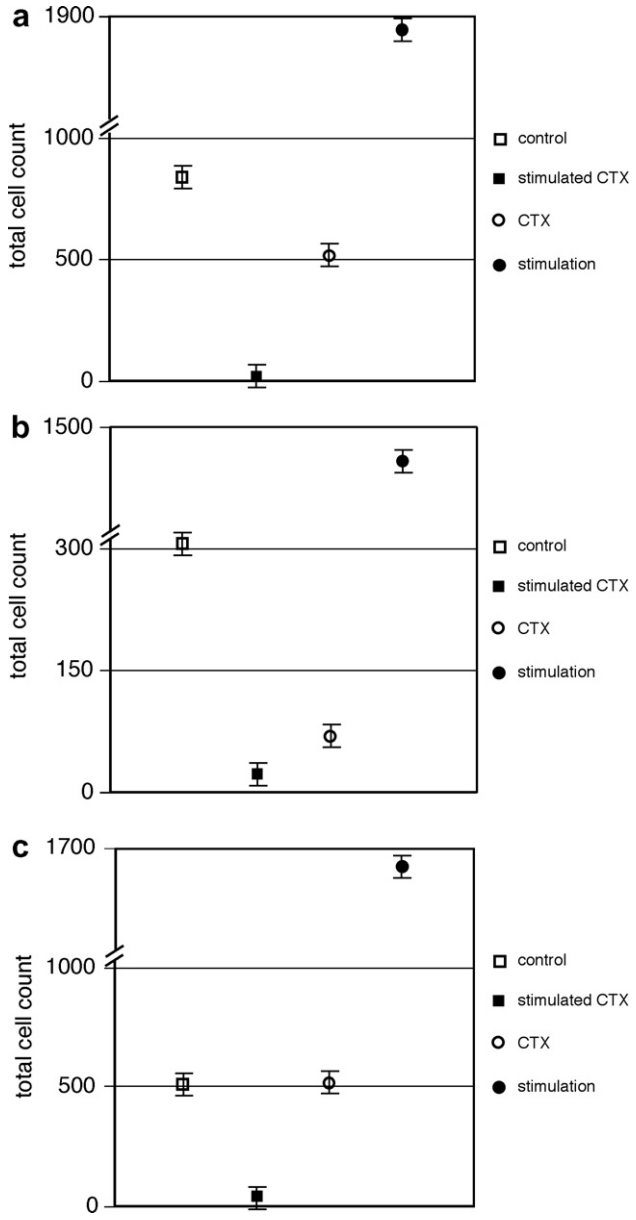

Fig. 3 – (a–c) The figure shows the total cell amount of the untreated control group (control), serotonin + EGF-prestimulated docetaxel group (stimulated ctx), docetaxel monotreatment group (ctx) and stimulation group (stimulation). 10 mmol/ml Serotonin and 1 μg/μl EGF were coincubated with 10000 Detroit 562 (Fig. 3a; A431: Fig. 3b; UM-SCC 10 B: Fig. 3c) cells for 24h and 100 nM/l docetaxel (ctx), respectively. After 7 days this treatment has been repeated. After treatment with 100 nM/l docetaxel the prestimulation group (stimulated ctx) showed a significant reduced amount of total cell count in all cell lines (Fig. 3 a–c) ($p < 0.04$). The amount of stimulated (without ctx) cancer cells compared to the control was higher in UM-SCC 10B and A431 cells than in Detroit 562 cells. The figure displays the mean value of total cell count ±S.E.

head and neck squamous cell carcinoma following treatment with docetaxel, cisplatin and 5-FU even during radiation.[3–17] However, despite complete remission rates after therapy, few patients suffer from recurrences during a follow up period of 2 years.[18]

In general, recurrence of disease may be due to either the generation of new cancer cells or activation of resting cells. Activation of resting cells indicates insufficient primary treatment. We assume that these cells were not vulnerable to radiation and/or chemotherapy.

It has recently been demonstrated that unresponsiveness of leukemic cells to both chemotherapy and immunotherapy could be due to residence in the resting G0 phase of the cell cycle.[23,24] Recruitment of leukemic cells from resting G0 phase into activated phases of the cycle by activation or induction of proliferation restored their sensitivity.

A few studies deal with arresting tumor cells in the G0 phase.[32–41] However, those cells remain in the tissue and may be activated after a while. It has been described, that the proportion of G0-phase cells during induction chemotherapy of acute myeloid leukemia predicts response to treatment.[42] Therefore reduction of the G0 fraction of a tumor may enhance treatment efficacy. Reentry of G0 cells into the cell cycle is associated with the expression of proliferation-associated antigens[43] It is known that squamous cell carcinoma cells need signals from growth factor receptors to proliferate.[20] We therefore investigated, whether growth factor receptor stimulating proteins were able to provoke a cell cycle transition of squamous cell carcinoma cells (Fig. 1). The combination of highest efficacy has been Serotonin + EGF. This combination has then been used to prestimulate cancer cells for chemotherapy with docetaxel. As shown in Fig. 2, G0 phase cells have been significantly reduced after Serotonin/EGF coincubation. Correspondent to this observation the cytotoxic effect of docetaxel against Detroit 562, A431 and UM-SCC 10B cells was enhanced affecting prestimulation with serotonin/EGF.

Our investigations demonstrate for the first time that sensitizing G0 phase squamous cell carcinoma cells for chemotherapy is possible by prestimulation with target cytokines. Considering that up to 95% of tumor cells are in the resting (G0) phase of the cell cycle at the initiation of chemotherapy, prestimulation with EGF and serotonin could contribute to a synchronization of cancer cells. This would clearly enhance the cytotoxic effect of docetaxel.

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