Original Article
CD44\textsuperscript{high}/ESA\textsuperscript{low} squamous cell carcinoma cell-derived prostaglandin E\textsubscript{2} confers resistance to 5-fluorouracil-induced apoptosis in CD44\textsuperscript{high}/ESA\textsuperscript{high} cells

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Abstract: We previously found that CD44\textsuperscript{high}/ESA\textsuperscript{low} head and neck squamous cell carcinoma (HNSCC) cells harboring high dihydropyrimidine dehydrogenase (DPD) expression exhibited potent resistance to 5-fluorouracil (5-FU)-induced apoptosis. In addition, susceptibility of HNSCC cells to 5-FU was compromised in the presence of cyclooxygenase 2 (COX2)-derived prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). In this study, we examined 5-FU-induced apoptosis in sorted cell populations (i.e., CD44\textsuperscript{high}/ESA\textsuperscript{low}, CD44\textsuperscript{low}/ESA\textsuperscript{high}, and CD44\textsuperscript{low}/ESA\textsuperscript{low} cells from the HNSCC cell line A253) to clarify the anti-apoptotic effect of PGE\textsubscript{2} on CD44\textsuperscript{high} cells. Notably, CD44\textsuperscript{high}/ESA\textsuperscript{low} cells upregulated PGE\textsubscript{2} compared with other populations. To investigate the effect of CD44\textsuperscript{high}/ESA\textsuperscript{low} cell-derived PGE\textsubscript{2}, we co-cultured CD44\textsuperscript{low}/ESA\textsuperscript{low} cells to examine the percentage of apoptotic cells in a culture of CD44\textsuperscript{low}/ESA\textsuperscript{low} cells. Furthermore, 5-FU-induced apoptosis of CD44\textsuperscript{high}/ESA\textsuperscript{low} cells was significantly increased in the presence of an inhibitor of the PGE\textsubscript{2} receptors (EP\textsubscript{1}/EP\textsubscript{2}) when CD44\textsuperscript{high}/ESA\textsuperscript{low} cells were co-cultured with CD44\textsuperscript{high}/ESA\textsuperscript{low} cells. These results suggest that CD44\textsuperscript{high}/ESA\textsuperscript{low} cells may contribute to the inhibition of 5-FU-induced apoptosis in CD44\textsuperscript{high}/ESA\textsuperscript{low} cells. Additionally, NR4A2 knockdown enhances 5-FU-induced apoptosis in CD44\textsuperscript{high}/ESA\textsuperscript{low} cells, suggesting that PGE\textsubscript{2} may attenuate 5-FU-induced apoptosis in an NR4A2-dependent manner in CD44\textsuperscript{high}/ESA\textsuperscript{low} cells. In conclusion, CD44\textsuperscript{high}/ESA\textsuperscript{low} cells contribute to induction of resistance to 5-FU in CD44\textsuperscript{high}/ESA\textsuperscript{low} cells through the delivery of PGE\textsubscript{2}, which may be involved in enhancement of the cancer microenvironment [7]. We suspect that PGE\textsubscript{2} might be involved in enhancement of the anti-apoptotic effect of PGE\textsubscript{2} on CD44\textsuperscript{high} cells.

Keywords: Head and neck squamous cell carcinoma (HNSCC), apoptosis, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), CD44, NR4A2

Introduction

While the mortality rate of head and neck squamous cell carcinoma (HNSCC) has improved recently, survival rates remain lower than those of other malignant tumours, such as colorectal and breast cancers [1]. In particular, patients with advanced-stage HNSCC exhibit high rates of recurrence and metastasis [2, 3]. This worsened prognosis may be associated with the behaviour of a small population of cancer stem cells that may be less sensitive to conventional cancer therapy [4]. In HNSCC, populations of CD44\textsuperscript{high} cells exhibit cancer stem cell (CSC) properties, as well as phenotypic plasticity, such as mesenchymal (i.e., CD44\textsuperscript{high}/ESA\textsuperscript{low} cells) to epithelial transition (i.e., CD44\textsuperscript{high}/ESA\textsuperscript{high} cells) [5]. In addition, CD44\textsuperscript{high}/ESA\textsuperscript{low} cells exhibited strong resistance to 5-fluorouracil (5-FU)-induced apoptosis, along with high dihydropyrimidine dehydrogenase (DPD) expression [6]. Therefore, we hypothesized that surviving CD44\textsuperscript{high}/ESA\textsuperscript{low} cells may play an important role in the poor prognosis associated with HNSCC. Additionally, we have reported that susceptibility of HNSCC cells to 5-FU might be compromised following cyclooxygenase 2 (COX2)-derived prostaglandin E\textsubscript{2} delivery to the cancer microenvironment [7]. We suspect that PGE\textsubscript{2} might be involved in enhancement of the anti-apoptotic effect of PGE\textsubscript{2} on CD44\textsuperscript{high} cells.
anti-apoptotic effect in HNSCC cells. However, it remains unknown whether PGE$_2$ can block apoptosis in CD44$^\text{high}$ cells (i.e., HNSCC cells with CSC properties). In this study, we examined the effect of exogenous PGE$_2$ on 5-FU induced apoptosis in CD44$^\text{high}$ subpopulations in HNSCC cells. In addition, we examined 5-FU-induced apoptosis of CD44$^\text{high}$/ESA$^\text{high}$ cells that were co-cultured directly or indirectly with CD44$^\text{high}$/ESA$^\text{low}$ cells.

Materials and methods

Cell culture and treatment

A HNSCC cell line, A-253 (American Type Culture Collection (ATCC), Manassas, VA, USA), was used in this study. Cells were cultured in a high-supplemented epithelial growth medium (Dulbecco’s Modified Eagle’s Medium: DMEM), with 10% FBS, under 5% CO$_2$ in air at 37°C [8]. For re-plating and for assays, cells were released into suspension using Accutase (Nakalai Tesque, Kyoto, Japan). To investigate the effect of CD44$^\text{high}$/ESA$^\text{low}$ cells on CD44$^\text{high}$/ESA$^\text{high}$ cells, direct co-culture assays were performed by culturing the two cellular populations together. An indirect co-culture system, using Transwell cell culture inserts with micro-porous membranes (pore size: 1.0 $\mu$m) (BD Falcon, Milian, Italy) was also employed to prevent direct cell-to-cell communication. PGE$_2$ (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO). DMSO was used as control for PGE$_2$. PGE$_2$ was added after cells had been cultured without FBS for 48 h. SC-51089 (Cayman Chemical, Ann Arbor, MI, USA) and AH-6809 (Cayman) were used as EP1 receptor and EP1/EP2 receptor antagonist, respectively. H-89 (Enzo Life Sciences, Farmingdale, NY, USA) was used as a cAMP-dependent protein kinase (PKA) inhibitor.

Fluorescence-activated cell sorting

Anti-CD44-PE-conjugated antibody (BD Pharmingen, San Diego, CA, USA) and anti-ESA-APC-conjugated antibody (BD Pharmingen) were used for fluorescence-activated cell sorting (FACS). 7-AAD (BD Pharmingen) was used to exclude dead cells during FACS analysis. According to our previously published methodology [9], samples were assayed on a Becton Dickenson FACSCalibur$^\text{TM}$ (BD Biosciences, San Jose, CA, USA); CD44$^\text{high}$/ESA$^\text{low}$, CD44$^\text{high}$/ESA$^\text{high}$, and CD44$^\text{low}$ cells were sorted using Becton Dickenson FACSArria equipment.

Apoptosis analyses

We examined apoptosis induced by 5-FU (Wako, Osaka, Japan) in A-253 cells. $1.0 \times 10^4$ cells were seeded in 6-well plates and grown for 24 h, then exposed to 25 $\mu$g/mL 5-FU for 48 h. Cells were collected and stained with 7-AAD and AnnexinV-Cy5 (BD Pharmingen), then AnnexinV-positive apoptotic fractions were analysed by FACS. Results are expressed as the mean $\pm$ SD for three independent experiments.

Introduction of green fluorescent protein (GFP) through viral infection

Introduction of GFP into the cells was performed according to our previously published method [10]. Briefly, virus host cells (HEK293FT cells) were cotransfected with a viral vector plasmid encoding EGFP (pLenti 6.3) (Invitrogen) and packaging plasmid Virapower mix (Invitrogen), using the EGF X-treme GENE HP reagent (Roche, Basel, Switzerland). After 48 h, the viral supernatant was mixed with 8 $\mu$g/mL polybrene (Sigma-Aldrich), and then used to infect the target cells by spinoculation.

Measurement of PGE$_2$ concentration

Enzyme-linked immunosorbent assay (ELISA) was performed to measure PGE$_2$ concentrations via the Prostaglandin E$_2$ Monoclonal Kit (Oxford Biomedical Research, Oxford, UK), according to the manufacturer’s instructions. Briefly, after 24 h incubation of the cells in culture medium with or without FBS, culture media were harvested to determine the PGE$_2$ concentration. The absorbance values were measured using a microplate reader with a 450-nm filter. Results are expressed as the mean $\pm$ SD for three independent experiments.

Quantitative RT-PCR analysis

The RNAeasy Micro Kit (Qiagen, Hilden, Germany) was used for RNA extraction and the resulting total RNA was subjected to reverse transcription using the ReverTra Ace$^\text{TM}$ qPCR RT Kit (TOYOBO, Osaka, Japan). Quantification of mRNA levels was performed using the CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and SYBR Green PCR
Anti-apoptotic effect of PGE$_2$ on CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells

The reaction mixture contained 1.0 µg of cDNA, 10.0 µL of SYBR Green Mix, and 10 µmol of each pair of oligonucleotide primers. GAPDH was used as a reference control. The primer sequences were as follows: Cox2, 5'-TTGCTGG-AACATGGAATATTAC-3' (sense), 5'-TGCTGTCTGCTGTACTG-3' (antisense); NR4A2, 5'-GCTT- CACGTCTCGGACG-3' (sense), 5'-TTTTGACT-GTGTCCTTTAAA-3' (antisense); Bcl-2, 5'-CCTGTGGATGACTGTA-3' (sense), 5'-GCCAC- GTGCGTGCTCAAAT-3' (antisense); and G3PDH, 5'-GAACCATGAGAAGTATGACA-3' (sense), 5'-ATGAGTCCT- TCCAGTAC-3' (antisense). The PCR program was as follows: initial melting at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, 57°C for 30 sec and 72°C for 40 sec. Results are expressed as the mean ± SD for three independent experiments.

Western blotting

Protein samples were solubilized in sample buffer by boiling, then separated in a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Western blot bands were detected using an enhanced chemiluminescence Western blotting reagent (GE Healthcare, Piscataway, NJ, USA). Antibodies (all diluted at 1:1000) consisted of an anti-human EP-1 mouse monoclonal antibody (Abcam, Cambridge, UK), an anti-human EP-2 mouse monoclonal antibody (Abcam), and an anti-human GAPDH mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

siRNA knockdown

Stealth$^\text{TM}$ siRNAs were used for NR4A2 knockdown (Life Technologies, Gaithersburg, MD, USA) and a Stealth siRNA negative control (Life Technologies) served as the control for knockdown. Cells were transfected with siRNA using
HiPerFect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer’s recommendations.

**Statistical methods**

Statistical analysis was performed using Student’s t-test. P values < 0.05 were regarded as statistically significant.

**Results**

**PGE<sub>2</sub> inhibit 5-FU-induced apoptosis of CD44<sup>high</sup>/ESA<sup>high</sup> cells via EP1/2 receptor**

All cell fractions expressed PGE<sub>2</sub> receptor, EP1, and EP2 proteins, as shown by Western blotting (Figure 1A). Thus, we analysed the effect of exogenous PGE<sub>2</sub> on 5-FU-induced apoptosis in CD44<sup>high</sup>/ESA<sup>low</sup>, CD44<sup>high</sup>/ESA<sup>high</sup>, and CD44<sup>low</sup> cells. CD44<sup>high</sup>/ESA<sup>low</sup> cells exhibited the most potent resistance to 5-FU-induced apoptosis. Notably, PGE<sub>2</sub> significantly reduced the percentage of 5-FU-induced apoptotic cells in CD44<sup>high</sup>/ESA<sup>high</sup> cells at concentrations of 10 and 20 ng/mL (Figure 1B). However, PGE<sub>2</sub> did not significantly reduce 5-FU-induced apoptosis in CD44<sup>high</sup>/ESA<sup>low</sup> and CD44<sup>low</sup> cells (Figure 1B). 5-FU-induced apoptosis was examined in the presence of PGE<sub>2</sub> and either EP1 or EP1/EP2 receptor inhibitor. PGE<sub>2</sub>-inhibited apoptosis was significantly increased after the addition of either EP1 or EP1/EP2 receptor antagonist (Figure 1C).
Anti-apoptotic effect of PGE$_2$ on CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells

CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells produce an increased amount of PGE$_2$ compared than CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells

Next, we examined the PGE$_2$ production ability of sorted cells. After 24 h incubation of the cells in culture medium without FBS, culture media were collected to determine the PGE$_2$ concentration. CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells exhibited significantly higher PGE$_2$ concentration than CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells or CD44$^{\text{low}}$ cells (Figure 2A). In addition, COX2 mRNA expression was significantly elevated in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells, compared with CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells or CD44$^{\text{low}}$ cells (Figure 2B).

Endogenous PGE$_2$ inhibits 5-FU-induced apoptosis of CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells

To investigate the effect of CD44$^{\text{high}}$/ESA$^{\text{low}}$ cell-derived PGE$_2$ on CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells, direct co-culture assays were performed by culturing the two cellular populations together (Figure 3A). GFP-tagged CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells and CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells were co-cultured in the presence of 5-FU, followed by determination of the percentage of apoptotic cells within the GFP-negative CD44$^{\text{high}}$/ESA$^{\text{low}}$ population by FACS. The percentage of apoptotic cells within a culture of CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells significantly decreased in the presence of CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells, relative to CD44$^{\text{high}}$/ESA$^{\text{high}}$ cultures grown in the absence of CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells (Figure 3A). Next, indirect co-culture of CD44$^{\text{high}}$/ESA$^{\text{low}}$ and CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells was performed using Transwell cell culture inserts, in the presence of 5-FU (Figure 3B). The percentage of apoptotic cells within a culture of CD44$^{\text{high}}$/ESA$^{\text{low}}$/PGE$_2$ cells was significantly reduced when CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells were indirectly co-cultured with CD44$^{\text{low}}$/ESA$^{\text{low}}$ cells (Figure 3B). Furthermore, the percentage of apoptotic cells within a culture of CD44$^{\text{high}}$/ESA$^{\text{high}}$/PGE$_2$ cells was significantly increased by the addition of
Anti-apoptotic effect of PGE$_2$ on CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells

![Diagram](Image)

**Figure 6.** CD44$^{\text{high}}$/ESA$^{\text{low}}$ cell-derived prostaglandin E$_2$ (PGE$_2$) inhibits 5-fluorouracil (FU)-induced apoptosis in CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells. PGE$_2$ release from CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells may contribute to enhanced resistance to 5-FU-induced apoptosis in CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells. (Statistical significance levels of P < 0.05, P < 0.01, and P < 0.001 are indicated by *, ** and *** respectively).

SC-51089 and AH-6809 in the presence of 5-FU, when CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells were indirectly co-cultured with CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells (Figure 3C). These results suggest that CD44$^{\text{high}}$/ESA$^{\text{low}}$ cell-derived PGE$_2$ may contribute to the inhibition of 5-FU-induced apoptosis in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells.

**PGE$_2$ induces NR4A2 expression via PKA dependent manner in CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells**

We previously revealed that exogenous PGE$_2$ induces NR4A2 expression in a PKA-dependent manner in oral SCC cells [7]. Therefore, we aimed to examine the effect of PGE$_2$ on NR4A2 expression in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells. CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells exhibited a slight increase in NR4A2 mRNA expression in the presence of PGE$_2$ (Figure 4A). However, NR4A2 mRNA expression was significantly upregulated in the presence of PGE$_2$ in CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells. Moreover, PGE$_2$-induced NR4A2 expression was inhibited by the addition of PKA inhibitor in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells (Figure 4B).

**NR4A2-knockdown enhances 5-FU-induced apoptosis in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells**

Finally, we examined the effect of NR4A2 siRNA knockdown on 5-FU-induced apoptosis in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells. Following siRNA knockdown of NR4A2, the percentage of apoptotic cells was significantly increased (Figure 5A). In addition, we examined expression of apoptosis-related genes, such as anti-apoptotic Bcl2 and pro-apoptotic Bax, during NR4A2 siRNA knockdown in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells. Bcl2 mRNA expression was significantly downregulated after siRNA knockdown of NR4A2 (Figure 5B). In contrast, Bax expression was significantly upregulated after siRNA knockdown of NR4A2 (Figure 5C).

**Discussion**

Several types of cancer can produce pro-inflammatory cytokines such as PGE$_2$, thereby inducing localized infiltration of inflammatory cells [11-13]. PGE$_2$ is suspected to contribute to modulation of the cancer microenvironment (i.e., cell proliferation, anti-apoptosis, and angiogenesis, as well as migration and invasion of tumor cells) [14]. There is mounting evidence to support the oncogenic role of PGE$_2$ within the context of the COX2-PGE$_2$-EP1/2-dependent signalling pathway [15, 16]. Several studies have reported a significant relationship between COX2 expression and CSC properties [17-20]. For example, there is greater sphere-forming ability within breast cancer cells that upregulate expression of COX-2 and its receptor, EP4. Notably, sphere forming ability is suspected to be a significant feature of CSCs [18]. COX-2 stimulates self-renewal of cancer stem-like cells of glioma via PGE$_2$ [19]. In addition, CSC repopulation has been blocked by the inhibition of the COX2-PGE$_2$ signalling pathway in bladder cancers [20]. These results indicate that PGE$_2$ may play a significant role in the maintenance of CSCs in different types of cancers. In this study, PGE$_2$ was involved in the inhibition of 5-FU-induced apoptosis of CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells, indicating that PGE$_2$ may contribute to resistance to chemotherapeutics in HNSCC CSCs with epithelial character.

It remains unclear which type of cell (i.e., cancer cells, cancer-associated stromal cells, blood cells, or other normal cells) are the main source of PGE$_2$ in HNSCC. This study revealed that CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells exhibit a greater capacity to produce PGE$_2$, compared with other populations of cells. CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells with both CSC and mesenchymal properties may contribute to PGE$_2$ production in HNSCC. In addition, we found that 5-FU-induced apoptosis was significantly blocked in CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells when they were co-cultured with CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells. Thus, PGE$_2$ release from CD44$^{\text{high}}$/ESA$^{\text{low}}$ cells may confer anti-apoptotic capacity to CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells, likely via EP1/EP2 receptor activation. Previously, we found that...
CD44	extsuperscript{high}/ESA	extsuperscript{low} cells exhibit strong resistance to other chemotherapeutics (e.g., cisplatin and docetaxel) [9]. Therefore, CD44	extsuperscript{high}/ESA	extsuperscript{low} cells may be involved in the strong resistance to the conventional chemotherapy that is observed in HNSCC. CD44	extsuperscript{high} subpopulations with potent resistance to chemotherapeutics (e.g., CD44	extsuperscript{high}/ESA	extsuperscript{low} cells) may not only survive but also aid in the survival of other CD44	extsuperscript{high} subpopulations (e.g., CD44	extsuperscript{high}/ESA	extsuperscript{low} cells) in the presence of chemotherapeutics such as 5-FU.

NR4A2 is a member of the orphan nuclear hormone receptor subfamily and is implicated in a wide variety of biological processes [21, 22]. NR4As may be capable of inhibiting tumour suppressor signalling [23]. In this study, PGE	extsubscript{2}-induced NR4A2 expression was inhibited by a PKA inhibitor, suggesting that PGE	extsubscript{2} induces NR4A2 expression in a PKA dependent manner within CD44	extsuperscript{high}/ESA	extsuperscript{low} cells. The combination of Bcl2 upregulation and Bax downregulation, following NR4A2 siRNA knockdown, strongly supports the notion that NR4A2 contributes to anti-apoptosis signalling in CD44	extsuperscript{high}/ESA	extsuperscript{low} cells.

Collectively, our results indicate that CD44	extsuperscript{high}/ESA	extsuperscript{low} cells play a significant role in the induction of resistance to 5-FU within CD44	extsuperscript{high}/ESA	extsuperscript{low} cells, largely by providing PGE	extsubscript{2} (Figure 6). Our results suggest that cancer-associated PGE	extsubscript{2} may regulate chemotherapeutic resistance in HNSCC. Thus, CD44	extsuperscript{high}/ESA	extsuperscript{low} cell-targeted chemotherapies (or other potential therapies) may be effective in treatment of HNSCC.

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Disclosure of conflict of interest

None.

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Anti-apoptotic effect of PGE$_2$ on CD44$^{\text{high}}$/ESA$^{\text{high}}$ cells


