Nonsteroidal Anti-inflammatory Drugs Modulate Gene Expression of Inflammatory Mediators in Oral Squamous Cell Carcinoma

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Abstract. *Background:* Most patients with head and neck cancer receive nonsteroidal anti-inflammatory drugs concomitant with oncogenic treatment in order to control cardiovascular diseases and chronic inflammatory processes. Inflammation is closely related to neoplastic development and the release of inflammatory cytokines and chemokines represents a crucial event in this relationship. The aim of the present study was to evaluate the effect of acetylsalicylic acid (ASA) and celecoxib treatment in the gene expression pattern of cytokines and chemokines in squamous cell carcinoma (OSCC) cell lines. *Materials and Methods:* Cells were treated with plasmatic concentrations of ASA and celecoxib and were submitted to cell viability assay and immunoenzymatic assay to investigate interleukin 6 (IL6) production. Treated cells were collected and a gene expression array was performed using the reverse transcriptase-quantitative polymerase chain reaction. *Results:* Both treatments provoked a discrete inhibitory effect on cell viability and modulated IL6 production. The mRNA expression of several cytokines, chemokines, chemokine receptors, and other chemotaxis-related genes were modulated after treatment with ASA and celecoxib. *Conclusion:* Plasmatic doses of ASA and celecoxib altered the expression of IL6 and the gene expression of chemokines (ligands and receptors) and cytokines in a dose- and time-dependent manner.

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Cancer-related inflammation can be derived from extrinsic and intrinsic factors that determine the development and evolution of the tumor. On one hand, some etiological factors of cancer, such as viruses, bacteria and auto-immune diseases, can promote a chronic inflammatory response that induces malignant neoplastic transformation. On the other hand, intrinsic factors inherent to neoplastic cells, such as activated oncogenes and tumor-suppressor pathways, cause an inflammatory response, in which neoplastic cells release cytokines and growth factors. Independent of the origin of the inflammation, inflammatory status improves neoplastic cell surveillance by induction of proliferation and resistance (1).

Due to the marked role of inflammation in tumor aggressiveness, some anti-inflammatory drugs have been tested as targeted antineoplastic therapies. In head and neck cancer (HNC), cyclo-oxygenase (COX) inhibition by some nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, has been associated with a lower risk for cancer development and progression (2). Experimental studies have demonstrated that exposure to NSAIDs confers a protective effect against HNC (3) and inhibits the metastatic potential of head and neck squamous cell carcinoma (HNSCC) cells (4). However, there is no consensus as to whether NSAIDs, mainly acetylsalicylic acid (ASA) and celecoxib (CLX), reduce the risk of HNC (5-7).

Paying particular attention to oral squamous cell carcinoma (OSCC), some aspects of the role of inflammation on tumor progression have, indeed, been unraveled. It is known, for example, that the inflammatory cytokines interleukin 1 beta (IL1 β), IL6, tumor necrosis factor (TNF α) and the chemokines C-C motif chemokine ligand 5 (CCL5), C-X-C motif chemokine ligand 8 (CXCL8), CXCL10 play an important role in OSCC development and progression, as well as on radioresistance (8-10). However, the effect of NSAIDs on OSCC-related cytokines has been poorly addressed.

Experimental studies with OSCC cell lines treated with ASA and CLX have focused on the effect of these drugs on adhesion molecules, cell migration and invasion (11), as well as on the inhibition of cell growth (12, 13). To date, as far as we are aware only one study has evaluated the effects of these NSAIDs on the modulation of inflammatory cytokines and growth factors in OSCC (8). The authors observed that after treatment with ASA (2 mM), celecoxib (25 μ M) as well as other nuclear factor kappa B (NF- κ B) inhibitors, a considerable decrease in IL6 and IL8 was observed, although no modulation of other cytokines was found.

In the present study, we aimed to evaluate the effect of ASA and CLX on the gene expression of cytokines and chemokines in OSCC cell lines.

Materials and Methods

Cell lines. OSCC cell lines (SCC4 and SCC9; American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% L-glutamine and 1% antifungal and antibiotic solution (Sigma-Aldrich St. Louis, MO, USA). The cells were maintained at 37°C in an atmosphere containing 5% CO₂ and 95% humidity until they reached 70-80% confluence.

Drug treatment. The cells were incubated for 1, 6 and 12 h under concentrations of 1, 3 and 6 mM for ASA (Aspirin; Sigma-Aldrich) and 1, 3 and 6 μ M for CLX (Pfizer, New York, NY USA). These concentrations were determined based on plasma concentrations in humans in which these drugs exert an anti-inflammatory effect (14).

ASA was prepared in ethanol and CLX in dimethyl sulfoxide (DMSO). The following experimental groups were set up: a) Control: cells incubated with normal medium; b) ethanol group: cells incubated with ethanol at the same concentrations used in the ASA group (0.1, 0.3 and 0.6%); c) ASA group: cells treated with ASA at concentrations of 1, 3 and 6 mM for 1, 6 and 12 h; d) DMSO group: cells incubated with DMSO at the same volume used in the CLX group (0.1, 0.3 and 0.6%); e) CLX group: cells treated with CLX at 1, 3 and 6 μ M for 1, 6 and 12 h.

Immunoenzymatic assay (Luminex[®] xMAP[™] system). Bio-Plex ProTM Human Cytokine 17-plex Assay (Bio-Rad GmbH, Munich, Germany) was used for the analysis of the inflammatory cytokines produced by the SCC4 and SCC9 cell lines in the Control group. Supernatant was obtained from the cell cultures and measured according to the manufacturer's instructions using a Bio-Plex Pro II wash station and Bio-Plex Array Reader (Bio-Rad). Data was calculated by the Bio-Plex Manager software.

Cell viability assay. Cell viability was performed in quadruplicate using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) for SCC4 and SCC9 cell lines in all groups. In a 96-well plate, 5 $\times$ 10³ cells were serum-free cultivated for 24 h. After this period, RPMI+10%FBS medium was added and cells were cultivated for an additional 12 h. After treatment with ASA and CLX, the MTT (5 μ g/ml) was added and after 2h of incubation, the absorbance at 490 nm was determined at

1, 6, 12 and 24 hours using a spectrophotometer (ELX 800; Bio-Tek Instruments Inc, Winooski, VT, USA). Three independent experiments were performed for both cell lines.

Immunoenzymatic assay for IL6. In the analysis of the cytokine profile for both OSCC cell lines, the highest values were found for IL6 and IL8 (see Results). It was previously reported that CLX has a modulatory effect only on IL6, not altering the IL8 concentration in inflammatory diseases (15, 16). Therefore, the effect of ASA and CLX on IL6 released by SCC4 and SCC9 cells was verified. To do this, we used a human IL6 ELISA kit (Novex[™]; Invitrogen, Carlsbad, CA, USA). Three independent experiments using the treatments described above were performed in quadruplicate according to manufacturer's recommendations.

RNA extraction, cDNA synthesis and polymerase chain reaction (PCR) array and RT-qPCR. RNA was extracted using (Thermo Fisher, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of the RNA were determined in a spectrophotometer (NANODROP 2000; Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 1 μ g of total RNA was reverse transcribed using RT2 First Strand Kit (Qiagen, Hilden, Germany). For gene expression analysis, the RT2 Profiler PCR Array system PAHS-022ZA (Qiagen) was used with 96-well plates containing primers for 84 genes (39 chemokine ligands, 36 chemokine receptors, 13 cytokines involved in chemotaxis, five cytokine receptors involved in chemotaxis and four other genes involved in chemotaxis), as well as five different endogenous control genes, a control for genomic DNA contamination, control of reverse transcriptase and positive control of the PCR reaction, according to the manufacturer's instructions. Reactions were performed using the 7,500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and the cycling conditions were: 10 min at 95°C, (95°C for 15 s, 60°C for 60 s) (40 cycles), followed by dissociation curve analysis.

The analysis of the differential expression genes was performed using RT2 Profiler PCR Array Data Analysis version 3.5, provided by the manufacturer through the website www.SABiosciences.com/pcrarraydataanalysis.php. Normalization was achieved using the best endogenous gene selected by the software. This software classifies the average threshold cycle of the gene as follows: A: relatively high (>30) in either the control or the test sample; B: relatively high (>30), meaning that its relative expression level is low in both the control and test samples, and the p-value is either unavailable or relatively high (>0.05); C: either not determined or greater than the defined cut-off value (default 35); and 'OKAY' (significant difference in gene expression between the samples). Only genes classified as 'A' or 'OKAY' by the software and with a fold change of ≥ 2 or ≤ -2 were considered as being differentially expressed.

RT-qPCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems) using SYBR[®]-Green mix (Applied Biosystems) and primers for the target genes C-X-C-Motif chemokine ligand 6 (CXCL6) (F: 5' agagctgcgttgcaactgtt 3'; R: 5' gcagtttaccatcggtttgggg 3'), CKLF-like MARVEL transmembrane domain containing 4 (CMTM4) (F: 5' ttcaatcgctactggctgctt 3'; R: 5' ccaggaatgtgttcactgcata 3'), C-C motif chemokine ligand 28 (CCL28) (F: 5' tgcacgga ggtttcacatcat 3', R: 5' ttggcagcttgcaacttcac 3') and platelet factor 4 variant 1 (PF4V1) (F: 5' agaaccacgcccgcacttccc; R: 5' aacgccaagaac agcatctc 3') and for the constitutive gene glyceraldehyde-3-phosphate

Figure 1. Concentration of cytokines and chemokines in SCC4 and SCC9 oral squamous cell carcinoma (OSCC) cell lines by the Bio-Plex Cytokine Assay. Both cell lines showed high levels of interleukin 6 (IL6) and IL8 protein production. G-CSF: Granulocyte colony-stimulating factor, GM-CSF: granulocyte-macrophage colony-stimulating factor, IFN γ : interferon gamma, IL17: interleukin 17, MCP1: monocyte chemoattractant protein-1, CCL4: C-C motif chemokine ligand 4, TNF α : tumor necrosis factor.

dehydrogenase (*GAPDH*) (F: 5' gcacctctgggctacactga 3'; R: 5'ccaccacccgtgtgctgta 3'), which was used for normalization of the target gene. The qPCR cycling conditions were as follows: 95°C for 10 min, (95°C for 15 s, 60°C for 60 s) (40 cycles) followed by dissociation curve analysis. Gene expression was calculated according to the Pfaffl mathematical model (17).

Results

OSCC cell lines express different cytokine profiles. This assay was performed to investigate the profile of cytokines, granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFN γ , IL1 β , IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12 (p70), IL13, IL17, and TNF α) and chemokines [monocyte chemoattractant protein-1 (MCP1) and C-C motif chemokine ligand 4 (CCL4)] produced by the SCC4 and SCC9 cell lines in the control group. Figure 1 shows the top 10 inflammatory mediators whose concentrations were higher than 10 pg/ μ l. SCC4 cells presented a higher production of G-CSF in relation to SCC9 cells. On the other hand, SCC9 presented high levels of GM-CSF, IFN γ , IL2, IL6, MCP1 and TNF α when compared to SCC4. IL6 was expressed at the highest levels, being produced mainly by SCC9 cells. IL8 was also expressed at high levels for both cell lines.

NSAIDs provoked a discrete inhibitory effect on cell viability. After 1, 6 and 12 h, no difference was observed between SCC4 and SCC9 cells treated with ethanol and with ASA at concentrations of 1, 3 and 6 mM (Figure 2). In addition, both cell lines treated with CLX at 1, 3 and 6 μ M showed no difference in cellular viability in relation to cells treated with DMSO after 1 and 6 h (Figure 3). However, after 12 h of treatment, cellular viability of the SCC9 cell line treated with CLX at 6 μ M significantly decreased when compared to those treated with DMSO at the same concentration

($p=0.005$) and when compared to SCC9 cells treated with CLX at 3 μ M ($p=0.02$) (Figure 3).

ASA and CLX at plasmatic concentrations modulate IL6 production. As shown in Figure 4A, there was no significant inhibitory effect on IL6 after 1 hour of exposure to ASA for any of the concentrations used. However, a significant increase in IL6 secretion was observed with ASA at 6 mM when compared do vehicle control ($p=0.0078$) after 6 h (Figure 4A). Twelve-hour treatment with ASA at 3 mM and 6 mM was associated with a significant decrease in IL6 when compared to their respective vehicle control ($p=0.0002$ and $p=0.03$, respectively) and with ASA at 1 mM ($p<0.001$) (Figure 4A). There were no significant differences between the effect of 3 mM and 6 mM for any of the exposure times.

In relation to CLX, no significant differences were observed after 1 and 12 h with CLX at 1, 3 and 6 μ M (Figure 4B). After 6 h, treatment with CLX at 6 μ M was associated with a significant increase in IL6 ($p=0.008$) in relation to CLX at 3 μ M (Figure 4B). No modulation of IL6 was observed in SCC4 cell line treated with different concentrations of ASA or CLX after 1, 6 and 12 h ($p>0.05$) (data not shown).

Looking at the extent of inhibitory or stimulatory effects of the drugs, we noticed that the ASA at 3 mM for 12 h (IL6 mean of 267.68 ± 3.73 pg/ml) and CLX at 6 μ M for 6 h (IL6 mean of 365.19 ± 39.79 pg/ml) exerted the most marked inhibitory and stimulatory effects on IL6, respectively. Therefore, these concentrations and time incubations were chosen for evaluating gene expression in the subsequent assay.

SCC4 and SCC9 cells showed different chemokine and cytokine gene modulations after treatment with NSAIDs. The up-regulated and down-regulated genes observed in SCC4 and SCC9 cell lines after treatment with ASA and CLX, as

Figure 2. Cellular viability of SCC4 and SCC9 oral squamous cell carcinoma cell lines (OSCC) treated with ethanol (vehicle control) and acetylsalicylic acid (ASA) at 1, 3 and 6 mM for 1, 6 and 12 h. Data are the mean ($\pm$ standard deviation).

well as the fold-change values are described in Tables I and II. In SCC4 cells, ASA treatment reduced the expression of atypical chemokine receptor 4 (*ACKR4*), *CXCL10*, *CXCL11*, *CXCL3*, *CXCL6*, *CXCL8* and *PF4VI* genes, and increased the expression of G protein-coupled receptor 17 (*GPR17*). In SCC9 cells, ASA increased the expression of *CCL17*, *CCL20*, *CCL28*, *CCL3*, *CCL4*, *CCR10*, *CXCR2*, *CMTM4*, *IL1B* and *IL16*. In SCC4 cells, CLX reduced the expression of *ACKR3*, *CXCL6* and *PF4VI*. In SCC9 cells, there was an increase in the expression of *CCL28*, *CMTM1*, *CMTM3*, *CMTM4*, *CXCR6*, slit guidance ligand 2 (*SLIT2*), toll-like receptor 2 (*TLR2*), *TLR4*, *TNF α* and thymidine phosphorylase (*TYMP*). Globally, ASA and CLX tended to up-regulate the chemokine and cytokine genes in SCC9 cells and to down-regulate genes in SCC4 cells.

To confirm the results obtained by PCR array, we selected *CMTM4* and *CCL28* genes overexpressed in SCC9 cells and *CXCL6* and *PF4VI* genes down-regulated in SCC4 cells for validation by RT-qPCR of the differential gene expression observed after treatment with ASA and CLX by the PCR array. As demonstrated in Figure 5, *CMTM4* was up-regulated in SCC9 treated with CLX (fold-change 4.45, $p=0.0052$) and *CCL28* gene was overexpressed after treatment with ASA

(fold-change 7.67, $p=0.03$) and CLX (fold-change 3.23, $p=0.07$) when compared to control (Figure 5A and B). SCC4 cell line showed lower expression level of *CXCL6* after treatment with both ASA (fold-change: -10.25 , $p=0.002$) and CLX (fold-change: -4.59 , $p=0.05$) (Figure 5C). In addition, a significant decrease in the mRNA expression level of *PF4VI* was also observed after ASA (fold-change: -7.77 , $p=0.01$) and CLX (fold-change: -4.59 , $p=0.03$) treatments in relation to the control (Figure 5D).

Discussion

To our knowledge, this is the first study to perform a screening of chemokine ligands, chemokine receptors, cytokines and cytokine receptors involved in OSCC, after treatment with ASA and CLX. The present work showed that ASA and CLX, used in concentrations near plasmatic levels in humans, were able to modulate the mRNA expression of several inflammatory proteins in two OSCC cell lines. We found important differences between the effects of ASA compared to CLX on inflammatory protein expression, as well as between SCC4 and SCC9 cells, as we discuss below.

Figure 3. Cellular viability of SCC4 and SCC9 oral squamous cell carcinoma (OSCC) cell lines treated with dimethyl sulfoxide (vehicle control) and celecoxib (CLX) at 1, 3 and 6 μ M for 1, 6 and 12 h. Data are the mean ($\pm$ standard deviation). *Significantly different from dimethyl sulfoxide (DMSO) at 0.6% ($p=0.005$) and CLX at 3 μ M ($p=0.02$), Tukey's test.

Firstly, we analyzed the cytokine panel released by OSCC cell lines without any treatment. For example, we found that IL6 was produced at high levels by SCC9 cells, but at moderate levels by SCC4 cells. In OSCC, the high expression of IL6 is related to worse prognosis (18, 19), and increased invasion, metastasis, and chemoresistance (20, 21). The two cell lines used in the present study were chosen for their ability to simulate the various OSCC phenotypes; which probably influenced the results (22).

Although the two cell lines expressed the cytokines differently, there were no important differences in relation to cell viability after NSAID treatments. In general, the drugs did not drastically inhibit cell growth of either cell line, even after 12-h exposure. Another study also demonstrated that ASA did not inhibit OSCC cell growth at concentrations up to 30 mM for 6 days of exposure (13), differing from another investigation in which ASA reduced OSCC cell growth at 2 mM after 72 h (8). CLX showed a higher potency for cell growth inhibition compared to other NSAIDs, with concentrations ranging from 10 to 50 μ M (13), and an exposure time of 72 h (8, 23). We probably did not observe any notable cell growth

inhibition due to the use of low drug concentrations and reduced exposure times.

The potential of cytokine inhibition by COX inhibitors was evident mainly after 12 h of drug exposure. We detected that the inhibitory effect of ASA on IL6 increased in accordance with the exposure time and concentration, but this tendency was not observed for CLX. On the contrary, some CLX concentrations improved IL6 production, mainly after 1 and 6 h. This fact was not reported in the study performed by Meyer *et al.* (8), but the concentrations and exposure times were higher than those used in the present study. Regarding the opposite effect of CLX at concentrations of 3 and 6 μ M on IL6 production, we believe that it may be related to the loss of the anti-inflammatory effect of the drug when used at higher doses, as reported by Niederberger *et al.* (24). Interestingly, in the study by Zhao *et al.*, in patients with osteoarthritis who were treated with several doses of CLX, a dose of 100 mg led to better control of inflammatory symptoms than did a dose of 200 or 500 mg (25).

Although the CLX administration period is every 12 h (25), no significant effect on IL6 cytokine production after 12 h of treatment was observed in this study. Gallelli *et al.* reported a drastic reduction of IL6 in synovial fluid in patients who

Figure 4. Interleukin 6 (IL6) concentration released by SCC9 oral squamous cell carcinoma (OSCC) cells after treatment with acetylsalicylic acid (ASA) (A) and celecoxib (CLX) (B) after 1, 6 and 12 h. Data are the mean $\pm$ standard-deviation. Significantly different from #corresponding ethanol control ($p=0.0078$, 0.0002 and $p=0.03$; *ASA at 1 mM ($p<0.001$) and **CLX at $3\text{ }\mu\text{M}$ ($p=0.008$), Tukey's test.

received CLX every 12 h (16). Intriguingly, in a clinical study conducted in 2008, the use of oral CLX every 12 h for 12 weeks reduced the severity of epithelial dysplasia in patients with oral premalignant lesions (26). Recent clinical studies used CLX every 12 h in metronomic chemotherapy regimens and achieved satisfactory results in increasing tumor progression-free survival in patients with HNC (27).

We also performed a gene expression screening of inflammatory cytokines and their receptors, after treatment of cells with ASA and CLX. In general, the SCC9 cell line exhibited up-regulation of genes and SCC4 cells showed down-regulation. We noted that chemokines, mainly CCs (CCL3, -4, -17, -20, and -28) and CXCs (CXCL3, -6, -8, -10, -11, and -16), were particularly modulated by NSAIDs, mainly

Table I. *Genes differentially expressed in SCC4 and SCC9 cell lines treated with acetylsalicylic acid (ASA) relative to the control group.*

Cell line	Gene	Description	Fold-change
SCC4	<i>GPR17</i>	G Protein-coupled receptor 17	8.16
	<i>PF4V1</i>	Platelet factor 4 variant 1	-3.53
	<i>CXCL10</i>	C-X-C motif chemokine ligand 10	-2.46
	<i>ACKR4</i>	Atypical chemokine receptor 4	-2.41
	<i>CXCL8</i>	C-X-C motif chemokine ligand 8	-2.41
	<i>CXCL3</i>	C-X-C motif chemokine ligand 3	-2.22
	<i>CXCL11</i>	C-X-C motif chemokine ligand 11	-2.13
	<i>CXCL6</i>	C-X-C motif chemokine ligand 6	-2.08
	<i>IL1B</i>	Interleukin 1 beta	13.30
	<i>CXCR2</i>	C-X-C motif chemokine receptor 2	7.07
SCC9	<i>CCL4</i>	C-C motif chemokine ligand 4	5.93
	<i>CCL20</i>	C-C motif chemokine ligand 20	5.01
	<i>CCL3</i>	C-C motif chemokine ligand 3	4.61
	<i>CCL28</i>	C-C motif chemokine ligand 28	4.13
	<i>CCL17</i>	C-C motif chemokine ligand 17	3.82
	<i>CCR10</i>	C-C motif chemokine receptor 10	3.75
	<i>CMTM4</i>	CKLF-like MARVEL transmembrane domain-containing 4	3.23
	<i>IL16</i>	Interleukin 16	3.06

Table II. *Genes differentially expressed in SCC4 and SCC9 cell lines treated with Celecoxib (CLX) relative to the control group.*

Cell line	Gene	Description	Fold-change
SCC4	<i>PF4V1</i>	Platelet factor 4 variant 1	-3.40
	<i>ACKR3</i>	Atypical chemokine receptor 3	-2.15
	<i>CXCL6</i>	C-X-C motif chemokine ligand 6	-2.03
SCC9	<i>CMTM3</i>	CKLF like MARVEL transmembrane domain containing 3	38.04
	<i>TYMP</i>	Thymidine phosphorylase	25.25
	<i>CXCR6</i>	C-X-C motif chemokine receptor 6	8.77
	<i>TNFA</i>	Tumor necrosis factor	8.01
	<i>TLR4</i>	Toll-like receptor 4	6.31
	<i>CMTM1</i>	CKLF like MARVEL transmembrane domain containing 1	5.78
	<i>CMTM4</i>	CKLF like MARVEL transmembrane domain containing 4	4.18
	<i>CCL28</i>	C-C motif chemokine ligand 28	3.92
	<i>SLIT2</i>	Slit guidance ligand 2	3.82
	<i>TLR2</i>	Toll-like receptor 4	3.37

by ASA. *In vitro* and *in vivo* studies have found an association between CCL2, -3, -7 and -20 expression with OSCC invasion, migration and lymph node metastasis (28-31). Of the CXC family, the members most studied in OSCC are CXCL8 and CXCL12, whose expression has been related to increased cell proliferation, angiogenesis, invasion, migration and metastasis (9, 32, 33). Interestingly, in response to ASA treatment, the

SCC4 cell line showed a decreased expression of the CXCL chemokines (CXCL3, -6, -8, -10, and -11) and the SCC9 cell line showed increased expression in the CC family (CCL3, -4, -17, -20, and -28). Chemokine receptor genes were also modulated in the SCC9 line by the treatments performed in the present study. Treatment with ASA increased the expression of CXCR2 and CCR10 and with CLX the expression of CXCR6 was increased. The high affinity binding of CXCR1 and CXCR2 to chemokine CXCL8 in OSCC cell lines showed an important effect on increased proliferation, migration, invasion and production of metalloproteinases (34). Regular use of ASA reduced CCL15 and increased CCL13 and -17 in older patients with cancer (35), but the relationship of this modulation with tumor prognosis has not yet been established.

Regarding other inflammatory cytokines, ASA and CLX were associated with an increase in *IL1B* and *TNFA* gene expression, respectively. Both cytokines are able to activate NF- κ B, which is related to many pro-tumoral processes (36, 37). In HNC, elevated plasma levels of TNF α have been related to reduced survival of patients (38).

CLX also improved the gene expression of *TLR2* and *TLR4*. Recently TLRs have been implicated in both antitumor and protumoral events of carcinogenesis (39). In OSSC, the expression of TLR2, -4 and -9 was found to be involved in the development and progression of this tumor type (40). The effect of CLX on TLRs is poorly understood in cancer, including OSCC. ASA and CLX reduced the expression of TLR3 in OSSC cells in another study (8).

The highest fold-change detected in the present study was for SCC9 cells treated with CLX, and involved the *CMTM* family (CKLF-like MARVEL transmembrane domain). CLX up-regulated the expression of *CMTM3* by 38-fold; in addition, *CMTM1* and -4 were also up-regulated by CLX and ASA, but at a lower intensity. There are few studies addressing the role of these proteins in cancer. In OSCC, Zhang *et al.* (41) associated the expression of *CMTM3* and *CMTM5* with OSSC prognosis.

Atypical chemokine receptors (ACRs), more specifically *ACKR3* and *ACKR4* genes, were down-regulated in the SCC4 cell line after CLX and ASA treatment, respectively. The most common ACKR4 ligands are CCL19, CCL21, CCL25 and CXCL13 (42, 43). Increased expression of this receptor has been implicated as a negative regulator of tumor growth and metastasis (44, 45). However, this gene has never been previously investigated in OSCC, and the effect of NSAIDs on these receptors is unknown.

Finally, we observed a decrease in *PF4V1* gene expression by SCC4 cells treated with both ASA and CLX. The chemokine PF4V1 plays an important homeostatic role under physiological and pathological conditions (46). In a recent study, Jian *et al.* found a positive correlation between the expression of *PF4V1* and the survival of patients with lung cancer (47). Although several studies

Figure 5. Evaluation of the overexpressed genes *CKLF-like MARVEL transmembrane domain-containing 4 (CMTM4)* (A) and *C-C motif chemokine ligand 28 (CCL28)* (B) in SCC9 and the down-regulated genes *C-X-C and platelet factor 4 variant 1 (CXCL6)* (C) and *platelet factor 4 variant 1 (PF4V1)* (D) in SCC4 by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) after treatment with acetylsalicylic acid (ASA) and celecoxib (CLX). Three independent experiments were performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). [#]Significantly different from the vehicle control at $p < 0.05$, unpaired t-test with Welch's correction.

have shown that *PF4V1* is able to modulate tumor development (47), its role in OSCC is poorly understood.

In conclusion, plasmatic doses of ASA and CLX altered the expression of IL6 and the gene expression of chemokines (ligands and receptors), cytokines, and other protein-related inflammation molecules in OSCC cell lines in a dose- and time-dependent manner, with different patterns related to the type of OSCC cell line and drug concentration/exposure time. Further investigation is necessary to elucidate the mechanisms of action of NSAIDs on these proteins, especially in the context of patients with HNC.

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Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

Authors' Contributions

Daniella Moraes Antunes: carried out the literature research, experimental studies, data acquisition and its analysis, article preparation, editing and its review. Maria Fernanda Setúbal Destro Rodrigues carried out experimental studies, data acquisition and its analysis, article preparation, editing and its review. Douglas Magno Guimarães carried out experimental studies and data acquisition. Carina Magalhães Esteves Duarte carried out experimental studies and data acquisition. Lucyene Miguita: carried out experimental studies, data acquisition and its analysis. Luciana Corrêa participated in data acquisition and its analysis and critically reviewed the article. Ana Paula Ligeiro de Oliveira participated in data acquisition and its analysis. Kristianne Porta Santos Fernandes critically reviewed the article. Fabio Daumas Nunes conceived the study, participated in its design and coordination, carried out experimental studies, data acquisition and statistical analysis, article preparation, editing and its review.

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