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UP-REGULATION OF IL-6, IL-8 AND CCL2 GENE EXPRESSION AFTER ACUTE INFLAMMATION: CORRELATION TO CLINICAL PAIN

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Abstract

Tissue injury initiates a cascade of inflammatory mediators and hyperalgesic substances including prostaglandins, cytokines and chemokines. Using microarray and qRT-PCR gene expression analyses, the present study evaluated changes in gene expression of a cascade of cytokines following acute inflammation and the correlation between the changes in the gene expression level and pain intensity in the oral surgery clinical model of acute inflammation. Tissue injury resulted in a significant up-regulation in the gene expression of Interleukin-6 (IL-6; 63.3-fold), IL-8 (8.1-fold), chemokine (C-C motif) ligand 2 (CCL2; 8.9-fold), chemokine (C-X-C motif) ligand 1 (CXCL1; 30.5-fold), chemokine (C-X-C motif) ligand 2 (CXCL2; 26-fold) and annexin A1 (ANXA1; 12-fold). The up-regulation of IL-6 gene expression was significantly correlated to the up-regulation on the gene expression of IL-8, CCL2, CXCL1 and CXCL2. Interestingly, the tissue injury induced up-regulation of IL-6 gene expression, IL-8 and CCL2 were positively correlated to pain intensity at 3 hours post-surgery, the onset of acute inflammatory pain. However, ketorolac treatment did not have a significant effect on the gene expression of IL-6, IL-8, CCL2, CXCL2 and ANXA1 at the same time point of acute inflammation. These results demonstrate that up-regulation of IL-6, IL-8 and CCL2 gene expression contributes to the development of acute inflammation and inflammatory pain. The lack of effect for ketorolac on the expression of these gene products may be related to the ceiling analgesic effects of non-steroidal anti-inflammatory drugs.

Keywords

Gene expression; acute inflammatory pain; cytokines; chemokines; NSAIDs

Introduction

Tissue injury initiates the liberation of various inflammatory mediators and hyperalgesic substances including prostaglandins (PGs), cytokines and chemokines [7, 9], which integrate the inflammatory response. Leukocyte migration to the injured area, a characteristic of the inflammatory response, is associated with pain and tenderness, and is involved in wound healing [19, 64, 75]. Chemokines (chemotactic cytokines) are small, secreted proteins that organize trafficking of leukocytes under normal conditions and in response to tissue damage

[76]. In general, they are not stored within cells but are synthesized in response to a variety of agents, including proinflammatory cytokines [18]. IL-6 plays a role in controlling leukocyte recruitment pattern during acute inflammation [24]. In-vitro studies showed that IL-6 secretion is induced by many other inflammatory mediators including IL-1 β , tumor necrosis factor- α (TNF- α) and PGE² [66]. In turn, IL-6 induces the chemokines CCL2 and IL-8 [55]. Inflammatory cytokines, including IL-6, are also involved in the modulation of pain [13]. In inflammatory pain models, IL-6 induces a short-lasting prostaglandin (PG) dependent hyperalgesia [9] and local analgesia via opioid secretion, probably from inflammatory cells [11, 51, 65]. Most, if not all, nucleated cells are capable of synthesizing IL-6 at variable rates [13]. Chemokines, particularly IL-8, CCL2 and CXCL1 also participate in inflammatory hyper-nociception in experimental animals. However, the mechanisms underlying this hyper-nociceptive effects remain unclear, for review see [70].

We have recently shown in the oral surgery model, an increase in the protein and/or gene expression of several cytokines and chemokines, 48 hours after surgery [72, 73]. However, the pattern of cytokine and chemokine expression in response to inflammation and tissue injury is a dynamic process [24, 25, 39]. It is known that peripheral inflammation induces central sensitization, which is involved in the mechanism of inflammatory hyperalgesia. IL-6, but not TNF- α or IL-1 β , was recently reported as a messenger of inflammatory information from the periphery to the central nervous system in carrageenan model of inflammatory hyperalgesia [48]. Since TNF- α and IL-1 β are upregulated in the oral mucosal, 3 hours after surgery [35, 71]. It is therefore of interest to investigate the expression of IL-6 and other inflammatory cytokines and chemokines at this early time point following tissue injury.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications for pain [53, 60], with ketorolac effectively ameliorating postoperative pain after minor surgery [20, 35]. We have recently shown in the oral surgery model of acute inflammatory pain that COX inhibitors have effects beyond the inhibition of prostanoids synthesis that may contribute to their pharmacological actions [71-73]. Further, the cyclooxygenase-PG pathway mediates the inflammatory and hyperalgesic effects of some pro-inflammatory cytokines. It is of interest, therefore, to investigate the effect of NSAIDs on the expression of these cytokines.

The present study investigated the expression of selected cytokines and chemokines at an early time point following tissue injury and their relation to clinical pain, as well as the effect of COX-inhibition on the expression of these inflammatory mediators.

METHODS

Subjects, timeline of clinical procedures and biopsy collection

Subjects were 37 healthy volunteers (76% of Caucasian, 8% of African American, 16% of other race) aged between 16 to 29 years old, who required the surgical extraction of impacted third molars. The protocol was approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research, National Institutes of Health (NIH). Written informed consent was obtained from all participants before treatment. Pregnant or lactating females or patients with clinical signs of infection or inflammation at the extraction sites were not included in the study. Subjects randomly received either placebo or ketorolac (30 mg) intravenously 30 min before surgery. A 3 mm punch biopsy was taken from the oral mucosa overlying the impacted mandibular third molar prior to the first surgical incision and a second biopsy was taken from the opposite mandibular surgical site 3 hours post-surgery. Pain intensity reported from each patient was assessed using a 100 mm anchored visual analogue scale (VAS) with endpoints no pain and worst pain over the three hour post-

surgery observation at 20 min intervals. Subjects received intravenous midazolam (4.9 ± 0.4 mg) and 2% lidocaine (166.6 ± 18.2 mg) with epinephrine 1:100,000 prior to surgery. If subjects requested pain medication prior to the 3 h biopsy, they were given one dose of 100 mg tramadol. Biopsies were immediately frozen in liquid nitrogen and stored at -70°C until ready for RNA extraction.

Gene analysis using Affymetrix microarray

Twenty-four Affymetrix arrays (HG-U133 Plus 2.0, Affymetrix, $n = 6$ per treatment) were used in the study. As described previously [71-73], all microarrays were processed by one person in the same laboratory following standard operating protocols to minimize non-biological technical bias. Briefly, total RNA was extracted from each oral mucosal biopsy using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All extracted RNAs were purified using an RNeasy mini kit (Qiagen, Valencia, CA). The RNA quantity, purity and integrity were assessed by a NanoDrop spectrophotometer (ND-1000, Wilmington, DE) and Experion (BIO-RAD, Hercules, CA), respectively. A total of $5\text{ }\mu\text{g}$ of purified RNA was reverse-transcribed into cDNA using cDNA Synthesis kit (P/N 900431, Affymetrix, Santa Clara, CA) and the double-stranded cDNA was then purified using Sample Cleanup Module (P/N 900371, Affymetrix). Synthesized and purified cDNA was used as a template for synthesis of the biotin-labeled cRNA using GeneChip IVT Labeling Kit (P/N 900449, Affymetrix) and quantification of the labeled cRNA was calculated based on the adjusted cRNA formula. Subsequently, a total of $15\text{ }\mu\text{g}$ of fragmented cRNA from each biopsy was hybridized on each chip at 45°C for 16 hours in a GeneChip hybridization oven at 60 rpm. GeneChip operating software (GCOS 1.1, v1.2, Affymetrix) was used to scan the images and for data acquisition. To enable the comparison between arrays, a global scaling factor (target signal to 500) was used across all arrays to minimize the variables due to sample preparation, hybridization/staining or from different lots of arrays. Microarray quality control (QC) was evaluated for each Affymetrix array as described previously [72, 73].

Microarray data extraction and statistical analysis

The HG-U133 Plus 2.0 chip is comprised of 47,000 transcripts, including 38,500 well-characterized human genes and the expressed sequence tags (ESTs). As described previously [72, 73], Affymetrix raw data were acquired using GCOS Software to yield CEL files according to the user's instruction. The detection p-value (Wilcoxon's Signed Rank test) for each probe set determines the detection call that indicates if the transcript was present ($p < 0.05$) or absent. For the purposes of this study, we defined a probe set as present only if it was identified as present in at least three of the six samples. The candidate genes of interest were selected based on both statistical significance and fold changes using ArrayAssist software (v5.1.0, Stratagene Corp., La Jolla, CA) and SAS software (version 9.1, SAS Institute, Cary, NC). A paired t-test was used to compare the relative changes in gene expression following acute inflammation for each treatment group. Gene expression in pre-surgery normal tissue was used as baseline. The placebo group therefore represents the effects of acute inflammation compared to the interaction of drug treatment on acute inflammation. The changes in gene expression induced by acute inflammation from the placebo group were used to compare to that in the drug treatment groups. Only the transcripts showing significant differences ($p < 0.05$) following the drug treatments were considered for further analysis. The identity of each differentially expressed gene was functionally classified using the batch query in the NetAffx from <http://www.affymetrix.com/index.affy>.

Gene analysis using SuperArray and statistical analysis

A pathway-focused microarray was also used in this study to examine the genes associated with inflammatory cascade during acute inflammation (human-oligo Microarray, OHS-001, SuperArray Bioscience Corporation, Frederick, MD). A total of 2 µg of purified RNA obtained from each biopsy was used to synthesize cDNA and cRNA using the true-labeling-AMPTM RNA amplification kit (GA-010). After purification with a cleanup-kit (GA-012), a total of 10 µg of biotin-16-UTP labeled cRNA was hybridized overnight at 60°C, and the hybridized membrane was subjected to quantification of the conjugation-signals with streptavidin-linked alkaline phosphatase and CDP star (Chemiluminescent detection kit D-01). The array image was captured by Alpha-Innotech image station and image data were extracted by the GEArray Expression Analysis Suite (GEASuite).

Following background correction using the minimum average intensity over all the spots, raw data were normalized using the mean of intensity values of all the genes in interquartile range (25%-75%) on the array. The fold change for each gene between post-surgery tissue versus pre-surgery tissue was analyzed using paired t-test, and the differences among treatments were examined by exact test, where an exact *p*-value was estimated by Monte Carlo.

Verification by quantitative real-time PCR

The changes in gene expression selected from microarray analysis were validated using the same RNA samples used in the microarray analysis and additional RNA samples obtained from different subjects (*n* = 13-18 for each treatment group). As described previously [71-73], all reagents used in qRT-PCR were purchased from Applied Biosystems (Foster City, CA), and 2 µg of DNase-treated RNA (Qiagen, Valencia, CA) was used to synthesize cDNA using random primers from the High-Capacity cDNA Archive Kit (Catalog no. 4322171) according to the manufacturer's instruction. PCR was performed with cDNA template using the PCR Master Mix with AmpErase UNG (Catalog no. 4304437). Sequence-specific primers and TaqMan MGB probes were purchased from Assays-on-Demand Gene expression product. Quantification of gene expression was performed in a 20 µl reaction (384-well plate) on ABI Prism 7900 HT Sequence Detection System. Each sample was run in triplicate and 18S rRNA was used as endogenous control in all RT-PCR experiments. Negative controls were processed under the same conditions without a cDNA template. Data acquisition was conducted based on User Bulletin #2 software (v1.6, Applied Biosystems). The threshold cycle (Ct) of 18 rRNA was used to normalize target gene expression (Δ Ct) to correct for experimental variations. The relative change in gene expression (Δ Ct) was used for comparison of the gene expression in post-surgery tissue versus that in pre-surgery tissue using paired t-test. Linear regression analysis was used to examine the association between the fold changes in gene expression and the sum of patient reported pain intensity scores over the first 3 hours post surgery as measured by VAS, which was adjusted for age, sex and race. The association among these gene expressed was examined by Pearson correlation coefficients. The fold changes in gene expression with over 3 SD were truncated at 200 for the three samples (2 from IL-6 and 1 from CXCL1) in correlation analyses. All statistical analyses were conducted using SAS software (version 9.1, SAS Institute, Cary, NC).

RESULTS

Gene expression profile and regulatory networks at the onset of clinical pain following tissue injury

Using the pathway-focused microarray, we found that 25 genes associated with the inflammatory cascade were up-regulated following tissue injury, including cytokines,

chemokines and their receptors (Fig. 1). Of these, IL-6 (4.4-fold, $p=0.04$), CCL2 (3.7-fold, $p=0.02$) and CXCL2 (3.3-fold, $p=0.01$) were significantly up-regulated after tissue injury ($n = 9$ per group of pre- and post-surgery). Gene expression of CXCL3 (3.4-fold), IL8 (2.12-fold), TNF (1.9-fold), TNFRSF1B (2.0-fold, tumor necrosis factor receptor superfamily, member 1B), and C3 (1.7-fold, complement component 3) were also increased following tissue injury but did not reach statistical significance due to the large range of inter-individual variations.

HG U133 Plus 2.0 arrays were then used to further study gene expression beyond the inflammatory cascade response to tissue injury as well as the effect of ketorolac, a widely used NSAID, on these gene expression responses. Consistent with the data from the pathway focused microarray, tissue injury induced a significant up-regulation on the gene expression of IL-6 (3.3 fold, $p=0.01$), IL-8 (4.2 fold, $p=0.04$), CCL2 (4.4 fold, $p=0.01$), CXCL2 (7.1 fold, $p=0.002$) 3 hrs following tissue injury (Table 1). Furthermore, the gene expression of CXCL1 (2.6 fold, $p=0.03$), ANXA1 (22-fold, $p=0.001$) and CYR61 (2.0-fold, $p=0.04$, encoding cysteine-rich, angiogenic inducer 61) were also increased at 3 hrs post-surgery. Ketorolac treatment showed modulatory effects on the tissue injury induced up-regulation of the gene expression of CXCL1, CXCL2, IL-8 and CYR61, but not on the gene expression of IL-6, CCL2, or ANXA1 (Table 1).

Verification of changes in gene expression by quantitative RT-PCR

The changes in expression of the genes selected from microarray were further verified using qRT-PCR. The tissue injury-induced up-regulation on the gene expression of IL-6 (63.3-fold, $p<0.0001$), IL-8 (8.1-fold, $p<0.001$), CCL2 (8.9-fold, $p<0.0001$), CXCL1 (30.5-fold, $p<0.001$), CXCL2 (26-fold, $p<0.0001$) and ANXA1 (12-fold, $p<0.0001$) was consistent with the microarray data in the direction of change. Consistent with the microarray results, ketorolac treatment did not change the gene expression of IL-6, CCL2 and ANXA1 following acute inflammation as compared to that in the placebo-treated group (Figure 2). The changes in gene expression of IL-8, CXCL1 and CXCL2 after treatment of ketorolac were consistent with the results from microarray in the direction of change but did not reach statistical significance.

CYR61 as well was up-regulated by tissue injury (3.1-fold, $p<0.0005$), with no significant effect by ketorolac treatment (data not shown). On the other hand, the gene expression of peroxisome proliferator activated receptor alpha (PPARA) was not significantly affected by tissue injury or ketorolac treatment (data not shown).

Correlation between pro-inflammatory cytokine gene expression and clinical pain intensity

To examine whether the up-regulation in the gene expression of inflammatory cytokines is associated with the patient-reported pain intensity, the association between the gene expression and pain on the VAS over the first 3 hours post-surgery was examined using Pearson correlation. Interestingly, the tissue injury induced up-regulation on the gene expression of IL6 ($r=0.68$, $p=0.005$), IL8 ($r=0.52$, $p=0.049$) and CCL2 ($r=0.64$, $p=0.014$) were positively correlated to the pain intensity reported from patients in the placebo group (Figure 3). This correlation was not observed in the ketorolac treatment group, as expected, since ketorolac significantly lowered the pain scores reported by these patients.

Correlation among the changes in expression of genes related to inflammatory cytokines

To further examine whether these genes encoding inflammatory cytokines have a similar expression profile and whether they participate in a common or intersecting biological pathway in this acute inflammatory model, the Pearson correlation coefficients were calculated among these genes. Because there was no significant difference in gene

expression level between the treatments (placebo and ketorolac), the two treatment groups were pooled to calculate the correlation coefficient for each gene. As shown in Table II, the up-regulation of IL-6 gene expression was highly correlated to the up-regulation of the gene expression of IL-8 ($r=0.57$, $p=0.001$), CCL2 ($r=0.74$, $p<0.001$), CXCL1 ($r=0.48$, $p=0.014$) and CXCL2 ($r=0.63$, $p=0.001$). Moreover, the up-regulation of CCL2 gene expression was significantly correlated with that of CXCL1 ($r=0.52$, $p=0.006$) and CXCL2 ($r=0.68$, $p<0.0001$), with the up-regulation of CXCL1 expression being highly correlated with the up-regulation of CXCL2 ($r=0.79$, $p<0.0001$).

DISCUSSION

In the present study, an up-regulation of IL-6, IL-8, CCL2, CXCL1, CXCL2 and ANXA1 gene expression was demonstrated in the oral mucosal biopsies 3 hours after oral surgery at the onset of acute inflammatory pain. We have shown earlier an increase in TNF- α [71], IL-1 β and PGE² [35] in the same model at the same time point which, integrated with the present data, helps to create a picture of the inflammatory cascade associated with tissue injury in this clinically well-defined model (Figure 4). Furthermore, we show a correlation between pain intensity and IL-6, IL-8 and CCL2 gene expression as well as correlations between gene expression of different cytokines and chemokines.

Interaction of cytokines and chemokines after acute inflammation

Inflammation, a highly coordinated process, is characterized by leukocyte migration into injured tissues [26]. The transition of leukocytic infiltrate from neutrophils to monocytes is a critical step in the successful resolution of inflammation [41] that is regulated by IL-6 [24, 28] through orchestrating the chemokine-directed attraction and apoptotic clearance of leukocytes [38, 41, 55]. IL-8, CXCL1 and CXCL2 are the main chemotactic mediators involved in neutrophil recruitment [31]. CCL2, on the other hand, is a potent chemoattractant for monocytes [50]. IL-6 and IL-8 are considered early markers of the inflammatory cascade [44]. We show here, a correlation between the gene expression of IL-6 and other measured chemokines. The gene expression of IL-8, however, was not correlated to any of them, suggesting that IL-6 but not IL-8 induces the expression of CCL2, CXCL1 and CXCL2. It is known that various cell types respond to IL-6 [23, 66], which magnifies the importance of its role in inflammatory conditions. Our clinical data is in agreement with earlier *in vitro* and animal studies [24, 41, 55]. However, it does not agree with other studies, where IL-6/sIL-6R attenuated the release of IL-8 and CXCL1 by IL-1 β and TNF- α stimulated human peritoneal mesothelial cells [24, 40].

Chemokines are implicated in a variety of acute and chronic inflammatory conditions [16]. Their role in inflammation is finely regulated through the specificity of chemokines secreted and the expression of their receptors under different inflammatory conditions [8]. IL-8, also known as CXCL8, is one of the most thoroughly studied chemokines. It is detected in many inflammatory conditions including rheumatoid arthritis [61] and ulcerative colitis [37]. The induction of IL-8 by other cytokines, especially IL-1 β and TNF- α has been described in several cell types [2, 32, 68]. Further, synergistic interaction between IL-1 β and TNF- α in induction of IL-8 has been suggested [68, 69, 74]. We have shown earlier in the oral surgery model, upregulation of both TNF- α and IL-1 β [35, 71], which might play a role in the upregulation of IL-8 reported here.

The role of ANXA1 and CYR61 in the inflammatory cascade

The up-regulation of the anti-inflammatory mediator ANXA1 and the angiogenic factor CYR61 in the present study integrates with the inflammatory cascade findings from this and previous studies (Figure 4). ANXA1 with anti-inflammatory properties plays a role in the

resolution of acute inflammation by acceleration of PMN apoptosis [63]. The anti-inflammatory properties are modulated by different inflammatory mediators such as being up-regulated by IL-6 whereas down-regulated by TNF- α and IL-1 β [12]. It is therefore possible that ANXA1 mediates in part the IL-6 induced apoptotic clearance of leukocytes mentioned above. However, in the present study ANXA1 expression was not correlated to IL-6 expression. On the other hand, CYR61 is known to play a role in wound healing [33] and is expressed during cutaneous wound healing [58]. However, in contrast to our data, where CYR61 was up-regulated 3 hours after surgery, it was not detected in mice until the third day post-wounding [33]. Being expressed in response to tissue injury, CYR61 supports adhesion and stimulates chemotaxis [21]. Studies show that CYR61 can either induce or suppress apoptosis in a cell type-specific manner. Generally, CYR61 adhesion to endothelial cells promotes cell survival, whereas its adhesion to fibroblasts induces apoptosis [6]. It is also regulated by different inflammatory mediators, up-regulated by IL-1 β , IL-6 and TNF- α in human fetal osteoblast-like cells [59]. PGs as well regulate the expression of CYR61 [6, 36]. On the other hand, IL-1 β expression is up-regulated by CYR61 in fibroblasts [5]. Both ANXA1 and CYR61 expression have been shown to modulate local inflammation and matrix remodeling, modulation of ANXA1 and CYR61 therefore might have therapeutic potentials in the disease associated with inflammatory disorders.

Cytokine, chemokines and pain

The correlation between pain intensity and gene expression of IL-6, IL-8 and CCL2 at the site of inflammation, shown here in a well defined clinical model, confirms the role of these molecules in the pathophysiology of pain. Stimulation of chemokine receptors on nociceptors produces pain and suggests their involvement in inflammatory pain [47]. CCL2 has also been implicated in nociception as mice overexpressing CCL2 show enhanced nociceptive behavior to both thermal and chemical stimuli [42]. Earlier studies, however, show variable relationships between cytokines and clinical pain. Demonstration of correlations between CSF levels of IL-6 and postoperative pain [3], wound exudates levels of IL-6 and analgesic consumption after elective cesarean section [4], and IL-8 in seminal plasma and pain in chronic prostatitis/chronic pelvic pain syndrome [29] support a functional relationship. Conversely, IL-6 levels in seminal plasma varied inversely with pain severity in patients with chronic prostatitis/chronic pelvic pain syndrome [43]. IL-8 plasma levels did not correlate with joint pain in paclitaxel treated patients [52] and CSF concentrations of IL-8 did not correlate to the pain intensity in patients with lumbar disc herniation and sciatica [1] or patients undergoing total hip arthroplasty [3]. CCL2 also did not correlate with the pain subscore of the chronic prostatitis symptom index [14].

The chemotactic effect of chemokines may contribute to their involvement in pain since activated inflammatory cells are themselves a source of pro-inflammatory cytokines. Inhibition of neutrophil migration was shown to prevent hyper-nociception in rats [34]. Migrating neutrophils participate in mechanical hyper-nociception by releasing hyper-nociceptive mediators, such as PGE² [10]. However, local injection of CXCL1 and CXCL2/3 results in dose-dependent PMN recruitment but does not induce mechanical hyperalgesia in the absence of other inflammatory mediators [54], which agrees with the absence of significant correlation between CXCL1 or CXCL2 and pain in the present study. Taken together, this points to the importance of the integrated inflammatory cascade in the mediation of pain and hyperalgesia.

Ketorolac and the cytokine/chemokine cascade

NSAIDs are the most frequently prescribed analgesics particularly following minor operative procedures or tissue injury [53, 60]. NSAIDs have been shown to influence several cytokines and chemokines besides their cyclooxygenase inhibitory effect [71-73], which

might interfere with their analgesic effect as well as with resolution of acute inflammation and wound healing. In the present study, ketorolac did not influence the gene expression of IL-6, IL-8, CCL2, CXCL1 and CXCL2 at an early time point after surgery, and in agreement with the 48 hours expression results [72], ketorolac did not have an effect on ANXA1 expression, either.

We recently showed that both ibuprofen and rofecoxib up-regulates IL-6 gene and protein expression 48 hours after surgery in the same clinical model [72]. This discrepancy might be due to ketorolac's effect on PDE4D enzyme. In LPS treated alveolar epithelial cells, inhibition of PDE4 enzyme by rolipram resulted in a potent inhibition of IL-6 production [22]. Thus, it is possible that ketorolac via down-regulating PDE4D enzyme [71], prevented the further over-expression of IL-6 seen at latter time points. Furthermore, the effect of different NSAIDs on IL-6 varies remarkably in different studies e.g. [17, 27, 46, 56, 67].

Likewise, IL-8 and CCL2 are also differentially regulated by NSAIDs. We have reported an increase in gene expression of IL-8 in the oral surgery model by rofecoxib [73]. NS-398 and nimesulide in high concentrations, on the other hand, inhibit IL-8 release in stimulated human neutrophils [30]. As for CCL2, indomethacin, meloxicam and SC-58125 enhance glomerular CCL2 mRNA levels in experimental glomerulonephritis in rat [57], while indomethacin, ibuprofen and NS-398 reduced TNF- α and IL-1 α induced CCL-2 expression in hepatic stellate cells [15].

In the present study, ketorolac treatment did not affect the gene expression of CXCL1 and CXCL2. Aspirin and celecoxib, but not SC-560 were shown to reduce CINC-1 (the rat counterpart of human CXCL1) production by hepatocytes [49]. In contrast, indomethacin and piroxicam did not affect CINC-1 expression in LPS-stimulated rat macrophages [62] or IL-1 β stimulated NRK-49F cells [45]. The failure of ketorolac to affect PPAR α gene expression here (data not shown), might play a role in its inactivity towards the expression of CXCL1 [49].

The present study describes the expression of several inflammatory mediators at a clinically relevant time point following tissue injury, the onset of acute pain. The failure of PGs inhibition by ketorolac to interfere with the up-regulation of any of these mediators, may explain, in part, the ceiling analgesic effect observed with the use of NSAIDs in painful inflammatory conditions. Inhibition of one or more of the cytokines and chemokines that are demonstrated by our findings to temporally correlate with pain onset may provide a novel strategy for analgesic pain therapy.

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Figure 1.

Gene expression profile of inflammatory cytokines and receptors after tissue injury in a clinical model of acute inflammatory pain. The data derived from analysis using GE SuperArray are expressed as median (n = 9). *, $p < 0.05$ for comparison of pre-surgery post-surgery tissue (paired t-test). Insert: Representative images from SuperArrays (OHS-110) showing the up-regulation of inflammatory cytokines and receptor after acute inflammation. #, expression of housekeeping genes.

Figure 2.

The changes in gene expression following acute inflammation and ketorolac treatment as assessed by qRT-PCR. The gene expression level is expressed as the average threshold cycle after normalization using 18 rRNA expression (Average Delt Ct). The open symbols represent the gene expression level from each subject in normal pre-surgery tissue and after tissue injury. The solid symbols represent mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ significant difference from the pre-surgery group (paired t-test).

Figure 3. Correlation between gene expression level (RQ) and pain intensity (VAS). The association between the gene expression and pain scale was examined using Pearson correlation at the time of onset of clinical pain and was adjusted for age, sex and race.

Figure 4.

Schematic diagram depicts the inflammatory cascade associated with tissue injury and acute inflammation and its relation to neutrophils and monocytes recruitment and to pain. The solid black lines indicate the data and information obtained from the current oral surgery model. The brown lines indicate the data and information obtained from previous studies; the solid lines indicate up-regulation and dashed lines indicate down-regulation. Tissue injury results in the up-regulation of prostaglandins, TNF- α , IL-1 β , IL-6, ANXA1 and CYR61. IL-6 in turn up-regulates the chemokines IL-8, CCL2, CXCL1 and CXCL2, which results in the accumulation of leukocytes. IL-6, IL-8 and CCL2 contribute to the pain process at this early time point. Refer to the text for further details.

Table 1

Participants demographic characteristics

Study Group	SuperArray	Affymetrix microarray		qRT-PCR	
	Placebo	Placebo	Ketorolac	Placebo	Ketorolac
N	9	6	6	18	17
Age	23.6 ± 6.4	20 ± 2.3	18 ± 3	19 ± 3.86	19 ± 2.45
Gender F/M	4/5	1/5	2/4	10/8	10/7
Extraction difficulty *	8 ± 0	7 ± 1.0	7 ± 0.98	7 ± 0.92	7 ± 0.95
Ethnicity					
Caucasian	1	6	6	14	12
African	3	--	--	1	2
others	5	--	--	3	3

*. Extraction difficulty is the sum calculated by assigning a score of (2) for soft tissue impactions, (3) for partial bony impactions and (4) for full bony impactions.

Table 2
Changes in gene expression following acute inflammation and ketorolac treatment

Gene Symbol	Gene Title	Placebo	Ketorolac
CCL2	chemokine (C-C motif) ligand 2	4.43 [*] up	3.27 [*] up
CXCL1	chemokine (C-X-C motif) ligand 1	2.63 [*] up	1.02 [#] up
CXCL2	Chemokine (C-X-C motif) ligand 2	7.12 [*] up	2.35 ^{##} up
IL1A	interleukin 1, alpha	2.17 [*] up	2.34 [*] up
IL1B	interleukin 1, beta	1.65 [*] up	1.05 [#] up
IL1RN	interleukin 1 receptor antagonist	4.13 [*] up	5.2 [*] up
IL6	interleukin 6 (interferon, beta 2)	3.3 [*] up	1.85 up
IL8	interleukin 8	4.2 [*] up	1.02 [#] down
IL7R	interleukin 7 receptor	4.6 [*] up	1.34 [#] up
PTGS1	prostaglandin-endoperoxide synthase (prostaglandin G/H synthase and cyclooxygenase) 1	1.83 [*] down	1.25 down
PTGS2	prostaglandin-endoperoxide synthase (prostaglandin G/H synthase and cyclooxygenase) 2	2.69 [*] up	1.67 up
ANXA1	Annexin A1	22 [*] up	11.25 [*] up
CYR61	cysteine-rich, angiogenic inducer, 61	2.20 [*] up	0.76 [#] down
ALOX12	arachidonate 12-lipoxygenase	8.87 [*] up	7.99 [*] up
ALOXE3	arachidonate lipoxygenase 3	2.08 [*] down	5.56 ^{##} down
CRISP3	cysteine-rich secretory protein 3	19.14 [*] up	9.04 [*] up
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	9.80 [*] up	5.65 [*] up
SERPINF1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	9.71 [*] up	9.55 [*] up
COL11A1	collagen, type XI, alpha 1	9.53 [*] down	5.99 [*] down
KRT2	keratin 2 (epidermal ichthyosis bullosa of Siemens)	2.16 [*] up	25 ^{##} down
KRT4	keratin 4	8.15 [*] up	6.27 [*] up
SOC3	suppressor of cytokine signaling 3	5.64 [*] up	3.36 [*] up
CD36	CD36 molecule (thrombospondin receptor)	5.5 [*] down	6.67 ^{##} down

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Gene Symble	Gene Title	Placebo	Keftorolac
LOR	Ioricrin	18.68* down	24.11*# down

* significant compared to pre-surgery level

significant compared to placebo group.