

T_H2-like Chemokine Patterns Correlate with Disease Severity in Patients with Recurrent Respiratory Papillomatosis

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Recurrent respiratory papillomatosis (RRP), characterized by the recurrent growth of benign tumors of the respiratory tract, is caused by infection with human papillomavirus (HPV), predominantly types 6 and 11. Surgical removal of these lesions can be required as frequently as every 3 to 4 wks to maintain a patent airway. There is no approved medical treatment for this disease. In this study, we have characterized the T_H2-like chemokine profile (CCL17, CCL18, CCL20, CCL22) in patients with RRP and asked whether it was modulated in patients who had achieved significant clinical improvement. CCL17, CCL18 and CCL22 messenger RNAs (mRNAs) were increased in papillomas compared with clinically normal laryngeal epithelium of the RRP patients. Overall, CCL20 mRNA expression was not increased, but there was intense, selective CCL20 protein expression in the basal layer of the papillomas. Patients with RRP expressed more CCL17 ($p = 0.003$), CCL18 ($p = 0.0003$), and CCL22 ($p = 0.007$) in their plasma than controls. Plasma CCL18 decreased over time in three patients enrolled in a pilot clinical trial of celecoxib, and the decrease occurred in conjunction with clinical improvement. There was a significant correlation between sustained clinical remission in additional patients with RRP and reduced levels of CCL17 ($p = 0.01$), CCL22 ($p = 0.002$) and CCL18 ($p = 0.05$). Thus, the change in expression of these three plasma T_H2-like chemokines may, with future studies, prove to serve as a useful biomarker for predicting disease prognosis.

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INTRODUCTION

Recurrent respiratory papillomatosis (RRP), characterized by the recurrent growth of premalignant tumors of the upper respiratory tract, is caused by infection with human papillomavirus (HPV), predominantly types 6 and 11. Extension into the lower airway occurs in approximately 17% of patients (1,2). Malignant conversion occurs in approximately 3% of patients with RRP (3,4), and is much more likely in those with pulmonary involvement. Recurrence frequency is variable between patients, but relatively constant within most patients (5). In severe disease, surgical removal of

the lesions can be required as frequently as every 3 to 4 wks, leading to a lifetime requirement of greater than 150 surgical procedures to maintain a patent airway. There is no approved medical treatment for this disease.

We reported previously that RRP is characterized by a biased adaptive immune response, with a T_H2-like predominance (6–8). Both IL-10 and IL-4 are upregulated in papillomas and in peripheral blood mononuclear cells exposed to HPV-11 E6 protein, and the tissues and cells express a concomitant decrease in IFN- γ , IL-12 and IL-18 (7). However, the immunologic mechanism(s) that governs

the variation in disease severity and the T_H2-like bias to HPVs remains unresolved. Of note, 6.9% of men and women aged 14 to 69 have oral or airway HPV infection (9) yet the vast majority of these individuals never develop RRP. The incidence in the United States among children (under age 14) is estimated to be 4.3/100,000 (10) and among adults, 1.8/100,000 (11). This suggests that the HPV-specific, T_H2-like bias may be unique to these patients.

Toward understanding the immune mechanism(s) that prevents patients with RRP from clearing or controlling their HPV infection, we previously performed a paired messenger RNA (mRNA) microarray study that characterized the repertoires of genes expressed in papillomas versus those expressed by autologous clinically normal laryngeal tissues (6). Among the results was evidence that there was differential chemokine mRNA expression by the papilloma tissues, which suggested that the virus might be able to polarize the patients' innate immune responses. Chemokines elicit and

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guide leukocyte movement, support angiogenesis (12) and participate in the balance of T_H1 -like versus T_H2 -like responses maintained by macrophages (13,14). We had also previously identified a robust expression of cyclooxygenase-2 (COX2) and its downstream product prostaglandin E_2 (PGE₂) throughout the airway tissues of patients with RRP compared with controls, mediated by constitutive activation of the EGFR/Rac1 pathway (15). PGE₂ can bias the adaptive immune response away from an effective T_H1 -like pattern (16), and can enhance expression of T_H2 -like chemokines by innate immunocytes (16,17). Therefore, both viral and host factors could modulate the innate response in these patients.

In this study, we have characterized the T_H2 -like chemokine profile in patients with RRP, asked whether the profile correlated with disease severity, and asked whether that profile changed when severity changed. We found an elevated T_H2/T_H1 -like chemokine balance in patients with RRP that correlated with disease severity. The inducible T_H2 -like chemokine CCL20 was expressed selectively in the basal keratinocyte layer of papillomas, where infiltrating immunocytes would first gain access to HPV antigen-expressing cells. We also found that plasma levels of the T_H2 -like chemokines CCL17, CCL18 and CCL22 were reduced in concert with sustained clinical remission.

MATERIALS AND METHODS

Patients

Studies were approved by the North Shore-LIJ Health System Institutional Review Board. Biopsies were collected of papilloma and autologous clinically normal airway epithelium (adjacent tissue) from patients with RRP and from control airway tissues from patients without RRP undergoing surgery at Long Island Jewish Medical Center. Blood was drawn prior to induction of anesthesia. Disease severity scores were calculated as described previously (5,18) and classified as either mild/moderate (score <0.06), or severe (score ≥0.06 or tracheal involve-

ment). Severity has been associated previously with altered immunologic responses in RRP, while age of disease onset, gender, or infection with HPV6 versus HPV11 has not correlated (7,8).

Celecoxib Studies

Design of the double-blinded placebo-controlled celecoxib studies for treatment of RRP has been described previously (15). Briefly, patients are randomized to either drug or placebo for 1 year and then switched to the other drug for a second year. The pilot study has been completed, and the blind broken. The Phase IIb trial (ClinicalTrials.gov identifier NCT00571701) (19) is ongoing and the blind has not been broken. At the time of this study, 38 patients were enrolled, 23 patients had sufficient clinical data to assess changes in disease status, and seven were free of disease for at least two 3-month intervals. Multiple plasma samples, at irregular intervals, were obtained from the three patients enrolled in the pilot study. Plasma samples were obtained at regular 3-month intervals in the Phase IIb study. All samples were stored at -80°C. T_H2 -like chemokine levels in plasma samples were measured as described below.

Quantitative PCR

Expression of the T_H2 -like chemokines *CCL17 (TARC)* (20), *CCL18 (DC-CK-1, PARC, AMAC-1, MIP-4)* (21,22), *CCL20 (LARC, MIP-3a)* (23–27), and *CCL22 (MDC, STCPI, ABCD-1)* (17,28–32) and the T_H1 -like chemokines *CCL19 (MIP-3b, exodus-3)* and *CCL21 (6Ckine)* (33–36) were measured by quantitative reverse transcriptase PCR (qPCR) with gene specific primers and an 8-mer oligonucleotide probe from the Universal Probe Library Set (Roche, Mannheim, Germany) (37) as shown in Table 1. Samples were amplified according to the manufacturer's directions with the BioRad for Probes PCR kit (Bio-Rad Laboratories, Hercules, CA, USA), using *ROX* as a normalization standard. Real-time PCR was performed using an Applied Biosystems 7900 HT thermocycler (Life Technologies, Carlsbad, CA, USA) with SDS 2.4 software,

and results were analyzed using the $\Delta\text{-}\Delta C_t$ method (38). Six to 19 samples of each type of tissue, derived from different patients, were analyzed for each chemokine.

Immunohistochemistry

Eight- μ m sections of paraffin blocks of papillomas and "adjacent" normal laryngeal epithelium were obtained from five patients with severe RRP, processed using standard methods and stained to identify the location of CCL18 and CCL20. Briefly, sections were incubated with either anti-CCL18 polyclonal goat-biotin or anti-CCL20 polyclonal goat-biotin antibodies (R&D Systems, Minneapolis, MN, USA; 1:50 dilution), and detected with Streptavidin-Cy3-conjugated secondary anti-biotin antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:200 dilution). Slides were mounted with UltraCruz Mounting medium (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), observed through a Zeiss Axiovert 200M-inverted microscope, images analyzed using the AxioVision V4.7.2 software (Carl Zeiss Microscopy LLC, Thornwood, NY, USA), and pseudocolor images produced with ImageJ 1.46o (NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>) and Photoshop 7.0 (Adobe, San Jose, CA, USA) software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Chemokines were measured in the plasma of RRP patients and controls by DuoSet (CCL18) or Quantikine (CCL17, CCL20, CCL21 or CCL22) ELISA (R&D Systems) according to the manufacturer's directions.

Multiplex ELISA

Chemokines were measured in plasma from the subset of patients enrolled in the Phase IIb celecoxib trial with a highly sensitive, customizable, multiplex, cytokine/chemokine array (Aushon Biosystems, Billings, MA, USA) (39). All assays were performed by the vendor in duplicate each time, on at least two different runs, to validate this assay.

Table 1. Real-time PCR primers and probes.

	Gene	Sense primer (5'→3')	Antisense primer (5'→3')	UPL probe
Cntl	<i>GAPDH</i>	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	60
T _H 2	<i>CCL17</i>	GGCTTCTCTGCAGCACATC	GGAATGGCTCCCTTGAAGTA	27
	<i>CCL18</i>	AGATTCCACAAAAGTTCATAG TTGAC	GATCTGCCGGCCTCTCTT	88
	<i>CCL20</i>	GTGGCTTTTCTGGAATGGAA	CAACCCCAGCAAGGTTCCT	117
	<i>CCL22</i>	CGTGGTGAAACACTTCTACTGG	CCTTATCCCTGAAGGTAGCAA	51
T _H 1	<i>CCL19</i>	AGTGGCACCAATGATGCTG	GTACCCAGGGATGGGTTTCT	74
	<i>CCL21</i>	AGAAAGGAAAGGGCTCCAAA	AGGCTTCAAGCGTTGGTG	01

UPL, Universal Probe Library.

Statistical Methods

Descriptive statistics and analysis of variance (ANOVA) were performed with InStat 3.01 (GraphPad Software, San Diego, CA, USA). Plasma chemokine levels were compared by a nonparametric ANOVA, Kruskal-Wallis test. Chemokine multiplex ELISA data from those patients in the Phase IIb clinical trial who achieved remission for at least two successive 3-month intervals (n = 7) were analyzed via mixed model repeated measures (MMRM) analyses, using SAS 9.1 (SAS Institute, Cary, NC, USA), where each of the chemokines (CCL17, CCL18 or CCL22) was modeled as a function of time, with the severity score group (mild/moderate versus severe) serving as a time-dependent covariate. Data was assumed to have compound symmetry covariance structure, and was validated with several other covariance structures. Model estimation was performed using restricted maximum likelihood (REML), and balanced design was determined using the Kenward-Roger method (40) to calculate fixed effects and degrees of freedom. The variance-covariance matrix was used to determine the correlation between the relative number of days since clinical remission, the disease score and a given chemokine. To correlate CCL18 plasma levels to sustainability of remission, a 2 × 2 contingency table was constructed containing: a) the number of patients from the two clinical trials that did/did not maintain a downward slope in CCL18 expression after achieving clinical remission, as defined above; and b) the number of

patients that did/did not maintain a sustained clinical remission. This contingency table was analyzed by Fisher exact test.

RESULTS

Chemokine Expression by Laryngeal Tissues from RRP Patients and Controls

To further study the possible bias in T_H2-like chemokine mRNA expression in papillomas suggested in our earlier mRNA expression array study (6) and ask if it represented an HPV-induced change or also was characteristic of the airway of patients with RRP, we quantitatively compared the T_H1/T_H2-like chemokine mRNA repertoires expressed by papillomas (papilloma) and clinically-normal laryngeal tissues from RRP patients (adjacent) to laryngeal tissues from controls without RRP (true normal) (Figure 1). Approximately 50% of individual biopsies of clinically normal airway of RRP patients contain latent HPV DNA (41). Therefore we cannot exclude the possibility that latent HPV infection could contribute to altered chemokine expression in adjacent tissue. However, latency is not expected to alter cellular functions since essentially no viral expression is observed (42).

Surprisingly, expression of the T_H2-like chemokines *CCL17* and *CCL18* was markedly reduced in “adjacent” tissue of patients compared with control “true normal” tissues, while *CCL20* and *CCL22* levels were essentially comparable (Figure 1). This suggests that laryngeal tissues of RRP patients may be polarized

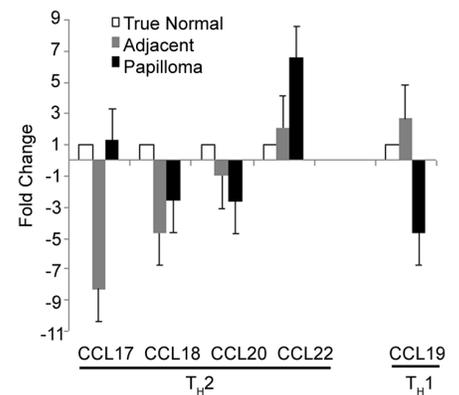


Figure 1. Chemokine levels are different in papilloma tissues, uninfected laryngeal tissues of RRP patients, and control laryngeal tissues. The T_H2-like chemokines *CCL17*, *CCL18*, *CCL20* and *CCL22*, and the T_H1-like chemokine *CCL19* were quantified by reverse transcription real-time PCR in papilloma tissues (black box; n = 9-16) and adjacent tissues from RRP patients (gray box; n = 12-19) and depicted as fold change compared with true normal laryngeal tissue from controls (white box; n = 6-7).

away from expressing at least some T_H2-like chemokines, and also that the constitutive expression of PGE₂ does not induce chemokine expression by laryngeal keratinocytes. In contrast, active HPV infection in the papillomas increased the levels of *CCL17* and *CCL22* markedly and possibly increased the level of *CCL18*, counteracting the anti-T_H2 bias. *CCL20* mRNA levels were not increased. The expression of the T_H1 chemokine *CCL19* was reduced markedly in papillomas compared with adjacent tissues from RRP patients and normal tissues from controls. Expression of *CCL21*, another T_H1-like chemokine, was not detectable in any of the tissues (data not shown). The net effect of these results suggests that active HPV infection shifts the local T_H1/T_H2-like chemokine balance toward a T_H2-like state in RRP patients.

Localization of T_H2-like Chemokine Expression in Papillomas

CCL18 and *CCL20* were the only T_H2-like chemokines we studied whose mRNAs were not expressed at markedly

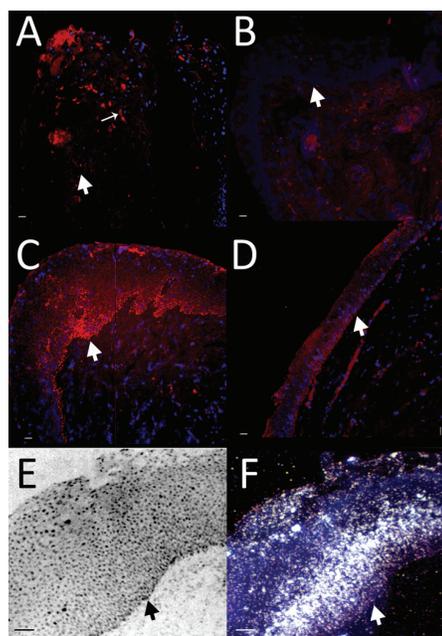


Figure 2. CCL18 localizes to scattered cells in the spinous layer of papilloma tissues and CCL20 localizes to the basal layer. CCL18 and CCL20 were detected by immunohistochemistry in papillomas and clinically normal adjacent epithelium of patients with severe RRP. (A) CCL18 stained scattered cells in the papillomas (thin arrow), but (B) showed only faint diffuse staining in normal adjacent tissues. (C) CCL20 was localized predominately to the basal layer of papillomas, adjacent to the basement membrane, while (D) normal adjacent tissue had uniform staining throughout the epithelium. (E,F) *In situ* hybridization, reprinted by permission from Steinberg, *et al.*, 1988 (1), shows HPV-6/11 viral expression in the suprabasal layer. A–D scale bar = 20 μ m; E and F scale bar = 100 μ m; large arrows point to the basement membrane.

higher levels in the papillomas than in the adjacent normal tissues from patients with RRP. We therefore asked whether active virus infection altered CCL18 and CCL20 distribution within the tissues. The CCL18 staining pattern in papillomas showed scattered positive cells in the upper spinous layer (Figure 2A; thin arrow). The frequency of these cells varied within different areas of a given papilloma and between patients. CCL18 expression in the

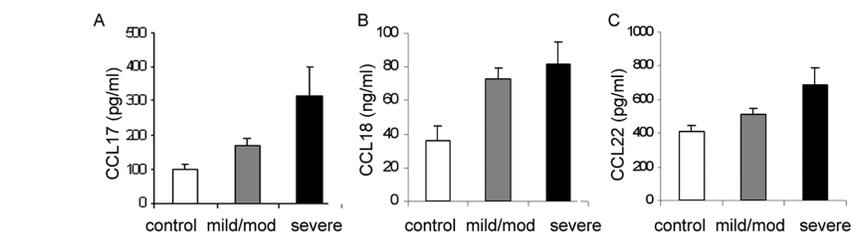


Figure 3. T_H2 -like chemokines are overexpressed in the plasma of patients with RRP and correlate with disease severity. (A) CCL17, (B) CCL18 and (C) CCL22 were measured in plasma of patients with RRP and controls. The concentration (mean \pm SEM) of each chemokine is shown. Concentrations of all three chemokines are higher in patients with RRP: CCL17 pg/mL (severe 317.9 ± 84.0 , mild/mod 169.7 ± 22.2 , control 98.0 ± 14.3 , $p = 0.003$); CCL18 ng/mL (severe 81.9 ± 12.8 , mild/mod 72.7 ± 6.7 , control 36.2 ± 8.8 , $p = 0.0003$); and CCL22 pg/mL (severe 686.5 ± 105.1 , mild/mod 512.8 ± 31.7 , control 411.4 ± 34.0 , $p = 0.007$). Levels are significantly different among patients with severe RRP ($n = 10$), patients with mild/moderate RRP ($n = 16$) and controls ($n = 14$), as determined by ANOVA, Kruskal-Wallis test.

adjacent tissues was barely detectable, but was diffuse throughout the epithelium (Figure 2B). By contrast, there was intense and selective CCL20 expression in the basal layer of the papillomas that extended more weakly into the lower spinous layers (Figure 2C). This pattern of staining was consistent in papillomas from multiple patients with severe RRP. CCL20 in adjacent tissues was diffusely uniform throughout the epithelial layers (Figure 2D). The high spinous cell/basal cell ratio in papillomas compared with normal laryngeal epithelium would explain the reduction in overall level of CCL20 mRNA in the papilloma tissue. Interestingly, we have shown previously that HPV 6/11 viral RNA is expressed strongly in the suprabasal layers (1) (Figures 2E,F), the converse to the CCL20 expression pattern. We were unable to detect the other T_H2 -like chemokines by immunohistochemistry, which could reflect their immediate release from the cells.

T_H2 -like Chemokines Are Enriched in the Plasma of RRP Patients, and Levels Correlate with Disease Severity

We then asked whether the T_H2 -like chemokine expression bias present locally in airway tissues of RRP patients was also seen systemically. We measured chemokines in the plasma of 16 patients with mild/moderate disease and 10 pa-

tients with severe disease compared with 10 controls without the disease. Patients with RRP expressed more CCL17 ($p = 0.003$), CCL18 ($p = 0.0003$) and CCL22 ($p = 0.007$) than patients without RRP (Figure 3). Neither CCL20, which is not constitutively expressed, nor the T_H1 -like chemokine CCL21, was expressed differentially in plasma of RRP patients and controls (data not shown). The assays were repeated in five subjects (three RRP patients with stable ongoing disease and two controls) over six time points spread out over 2 years, with less than 4% variability in the results for each individual when repeated measures were obtained over time. This supports our findings that inpatient immunologic parameters do not vary significantly over time independently of disease status (8), but we have seen a change in immune parameter when a therapeutic intervention changed the course of disease (43).

Change in Disease Severity Modulates Systemic T_H2 -like Chemokine Expression

We used plasma samples from the three patients who had been enrolled in a pilot clinical trial of celecoxib as a novel medical treatment for RRP (15) to ask if treatment with the selective COX-2 inhibitor celecoxib would modulate CCL18 levels and whether changes would correlate with

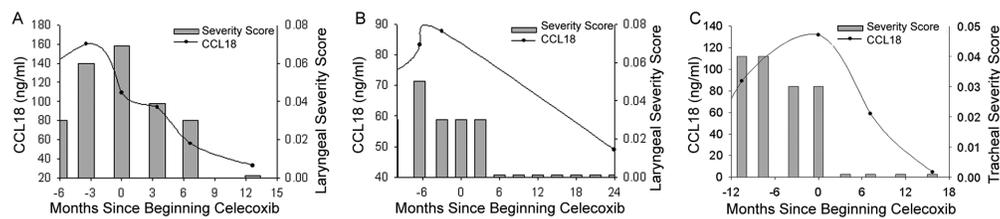


Figure 4. Plasma CCL18 levels decrease in concert with improved clinical status in patients treated with celecoxib, a COX-2 inhibitor. Three patients (A,B,C) in the pilot clinical trial had CCL18 levels (solid line) measured by a single-plex ELISA in plasma samples collected at varying times during the study. Clinical improvement, denoted by reduction in severity score (gray bars), correlated with decreasing CCL18 concentrations.

clinical improvement. A marked decrease in plasma CCL18 was noted in all three patients (Figure 4). The decrease occurred in conjunction with clinical improvement, but patients B and C were both disease free well before the CCL18 levels reached their lowest levels, suggesting that the chemokine reduction reflected, but did not drive, clinical response.

To further investigate the correlation between change in disease severity and change in chemokine levels, we analyzed the plasma from the seven patients enrolled our current Phase IIb celecoxib trial who were in complete remission (absence of disease) for at least two consecutive 3-month time intervals. Since the ongoing clinical trial remains blinded, we could not correlate chemokine levels with inception of therapy or even know if the patients were receiving celecoxib at the time their disease changed. However, we did know that their disease status had changed. We measured levels of three T_H2-like chemokines, CCL18 and also CCL17 and CCL22, at multiple sequential time points surrounding the time at which remission occurred. We correlated the chemokine levels with the number of months since onset of remission, using a test of repeated measures with severity group at the time of each sample as a covariant. Data were depicted graphically and linear regression lines of best fit were determined for each patient (Figure 5). Reduction in CCL17 ($p = 0.01$) and CCL22 ($p = 0.002$) plasma levels strongly correlated in this model with months since clinical remission, while changes in CCL18 levels ($p = 0.8$) did not.

We noted that patients followed one of two patterns of chemokine change. In pattern I, patients had downward slopes for all three chemokines (patients #2, #3 and #5). In contrast, those with pattern II (patients #1, #4, #6 and #7) had no decline in CCL18 despite clinical improvement, coupled with a shallow downward or level slope for one or more of the other two chemokines. These patterns associated with sustainability of response (Table 2). All three patients with pattern I had sustained remission, while three of the four patients with pattern II had recurrence of disease by the last time point analyzed. The correlation between sustained clinical remission and CCL18 pattern was significant ($p = .05$). Thus, analyzing the change in expression of these three T_H2-like chemokines may be able to predict sustained improvement.

DISCUSSION

Chemokines have multiple functions. They attract leukocytes to sites of inflammation, regulate leukocyte homing, and have a role in angiogenesis and tumor growth (44). RRP is a virally induced disease characterized by growth of premalignant tumors, with an apparent failure of the host immune system to control the infection. We have found that the pattern of expression of T_H1-like and T_H2-like chemokines in patients with RRP paralleled the increased T_H2-like cytokine milieu that we have shown previously (7,8,45). We also found that changes in chemokine expression correlated with change in disease severity, consistent with the hypothesis that chemokines play

a role in the HPV-specific immune dysregulation of this disease.

Comparison of papilloma tissues to autologous clinically normal airway tissues as well as to tissues from controls with no history of RRP enabled us to ask which chemokine differences were driven by the papillomavirus infection and which were a reflection of the patient's local or systemic immune status that might impact on susceptibility to HPV-induced disease. One caveat to this approach is the fact that latent HPV infection is widespread in the airway of patients with RRP, and we cannot exclude the possibility that the latent infection affects chemokine expression, even though viral expression is essentially undetectable in latency (42).

Keratinocytes are known to express many of the same cytokines and chemokines as immunocytes (46), although the regulation of expression has not been studied as extensively in keratinocytes. Expression of the T_H2-like chemokines CCL17 and CCL22 was increased significantly in papilloma tissues compared to autologous clinically normal tissues (Figure 1). The T_H2-like cytokines IL-4 and IL-13, which we reported previously are increased in papillomas (7,47), are potent stimulators of CCL22 expression by monocytes (48–50) while the T_H1-like cytokine IFN- γ (absent in papillomas) suppresses CCL22 expression by monocytes, macrophages and dendritic cells (DCs) (49). CCL17 is expressed by alternatively activated macrophages (AAM ϕ s) and DCs, and the same T_H2-like cytokines that induce

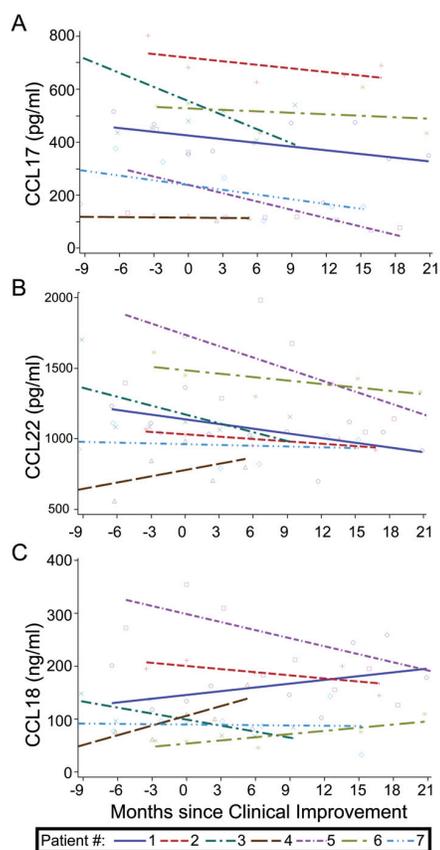


Figure 5. Plasma CCL17 and CCL22 levels decrease over time in patients who achieve remission of disease. Plasma samples collected every 3 months from seven patients (separate colors) enrolled in an ongoing celecoxib clinical trial who became disease-free for at least 6 months were analyzed for (A) CCL17, (B) CCL22, and (C) CCL18 concentrations via multiplex ELISA. Data is shown relative to time (months) after complete clinical remission. Linear regression was performed for each patient, and a test of repeated measures using severity group as a covariant was employed. CCL17 ($p = 0.01$) and CCL22 ($p = 0.002$) strongly correlated with the number of months since remission, CCL18 did not.

CCL22 expression promote generation of AAMφs (51). Others have suggested that elevated COX-2 expression leads to an overexpression of CCL20 through a PLCP1/PKCα/MEK1/2/ERK1/2-dependent pathway (52) and that alterations in the COX2/PGES/PGE₂ pathway affect T_H2-like chemokine ex-

Table 2. Relationship between chemokine pattern and sustained clinical response.

Chemokine pattern	Patient number ^a	Slope of CCL17 ^b	Slope of CCL22 ^b	Slope of CCL18 ^b	Sustained clinical response
I	Pt # 3	+++	+++	+++	Yes
	Pt # 5	+++	+++	+++	Yes
	Pt # 2	+	+	+	Yes
II	Pt # 6	+	+	-	Yes
	Pt # 1	+	+	-	No
	Pt # 7	+	+	+/-	No
	Pt # 4	+/-	-	-	No

^aPatient number refers to the patient identifier numbers in Figure 5.

^b+++ , Steep downward slope; +, moderate downward slope; +/-, flat slope; -, inverse slope upward).

pression (53,54). However, PGE₂ does not appear to have the same effect on laryngeal keratinocytes, since concentrations of PGE₂ in papilloma and clinically normal airway tissues of RRP patients are quite comparable (data not shown).

CCL18, a highly abundant and constitutively expressed chemokine in normal plasma (22,55–58), is expressed by many innate immunocytes including DCs (13,59), AAMφs (13) and eosinophils (60). CCL18 is expressed prominently in asthma and other T_H2 disorders (54,60). Thus, it was surprising that we did not see a marked increase in CCL18 in the papilloma tissues. The vast majority of mRNA analyzed from the tissues is derived from the epithelial cells, not innate or adaptive immune system cells. Thus, even though we saw some cells in some papillomas that were strongly positive by immunohistochemistry (Figure 2A), the CCL18 mRNA would be diluted by the large amount of mRNA extracted from negative papilloma cells. It appears that the virus, the microenvironment in the tissues, or both acting together, stimulate papilloma cells to upregulate expression of CCL17 and CCL22, but not CCL18.

T_H2-like chemokines are important chemoattractants that guide T_H2-like cells and regulatory T cells (Tregs) to sites of inflammation (61) through their interaction with CCR4 (48,62). Increased expression of CCL17 and CCL22 could be the mechanism for our previous finding that

Tregs are enriched in papillomas (61). CCL22 is also a potent chemoattractant for DCs and natural killer (NK) cells (63–65), which are more abundant in papillomas (47,66). CCL20 mRNA levels did not appear to be upregulated in papillomas when analyzing total biopsy extracts, but the protein was clearly upregulated differentially in the basal layer of this stratified squamous epithelium (Figure 2C). CCL20 is an inducible chemokine that acts as a chemoattractant for NK cells (67) and immature DCs (24), and plays an important role in recruitment and activation of T_H2-like T cells. Basal cells are immediately adjacent to the basement membrane, thus CCL20 expression in these cells would form a strategic barrier to selectively admit T_H2-like, but not T_H1-like, T cells into the tissue. This would restrict access to the more suprabasal and spinous layer of keratinocytes where high-level HPV expression occurs in papillomas (Figures 2E,F) (1).

Plasma chemokine levels reflect a more systemic immune state of the RRP patients than analysis of laryngeal biopsy tissues. Plasma levels of CCL17 and CCL22 were clearly elevated in the RRP patients, in keeping with the papilloma tissue levels, but so was CCL18 (Figure 3). This disconnect suggests that the highly elevated plasma CCL18 levels in these patients are derived from another site, possibly lymphoid tissues. While the role of CCL18 has been de-

bated, strong evidence supports CCL18 as an antiinflammatory chemokine (22,54,60,68). Thus, we conclude that RRP is indeed a T_H2-like chemokine disease with a strong bias away from effective control of HPV infection, and that the bias correlates with disease severity.

The systemic T_H2-like chemokine bias in these patients is not “hard wired,” since plasma levels changed when disease severity improved.

CONCLUSION

On the basis of the first clinical study (15), we would conclude that celecoxib therapy induces both clinical response and reduction in CCL18 plasma levels and that CCL18 levels reflect clinical state rather than regulation by PGE₂ since initiation of treatment and clinical improvement clearly preceded the decline in chemokine expression to baseline for two of the three patients (Figure 4). Expansion of this research using seven patients from our current celecoxib study provided additional insights (Figure 5). There was a highly significant correlation between achievement of clinical remission and decline in plasma CCL17 and CCL22 levels, but not CCL18 levels. Rather, a sustained decline in CCL18 levels appeared to predict continued remission (Table 2). We postulate that immunologic events that contribute to clinical improvement involve modulation of the expression of CCL17 and CCL22, and that changes in CCL18 reflect the likelihood of sustaining the improvement. Monitoring plasma levels of the three T_H2-like cytokines (CCL17, CCL22 and CCL18) may, with further study, prove to be a useful tool for predicting disease prognosis in RRP.

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DISCLOSURE

BM Steinberg has a research grant of celecoxib and matching placebo from Pfizer for the clinical studies.

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