ORIGINAL RESEARCH

Potential Involvement of the Epidermal Growth Factor Receptor Ligand Epiregulin and Matrix Metalloproteinase-1 in Pathogenesis of Chronic Rhinosinusitis

Tetsuya Homma[1,2], Atsushi Kato[1], Masafumi Sakashita[1,3], Tetsuji Takabayashi[1,3], James E. Norton[1], Lydia A. Suh[1], Roderick G. Carter[1], Kathleen E. Harris[1], Anju T. Peters[1], Leslie C. Grammer[1], Jin-Young Min[4], Stephanie Shintani-Smith[4], Bruce K. Tan[4], Kevin Welch[4], David B. Conley[4], Robert C. Kern[4], and Robert P. Schleimer[1]

[1]Division of Allergy and Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois; [2]Division of Allergology and Respiratory Medicine, Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan; [3]Division of Otorhinolaryngology Head and Neck Surgery, Department of Sensory and Locomotor Medicine, University of Fukui, Fukui, Japan; and [4]Department of Otolaryngology, Northwestern University Feinberg School of Medicine, Chicago, Illinois

Abstract

Chronic rhinosinusitis (CRS) is a heterogeneous chronic inflammatory disease of the nose and paranasal sinuses that presents without or with nasal polyps (CRSwNP). Notable features of CRSwNP are the frequent presence of type 2 allergic inflammation and high prevalence of *Staphylococcus aureus* (SA) colonization. As inflammation persists, sinus tissue undergoes epithelial damage and repair along with polyp growth, despite active medical management. Because one feature of damaged tissue is enhancement of growth factor signaling, we evaluated the presence of epidermal growth factor receptor (EGFR) ligands and matrix metalloproteinases (MMPs) in CRS. The objectives of this study were to analyze the expression of EGFR ligands and MMPs in patients with CRS and to investigate the possible role of SA on epithelial activation. Sinonasal tissues were collected during surgery from control subjects and patients with CRS. Tissues were processed as described previously for analysis of mRNA (RT-PCR) and proteins (ELISA) for the majority of EGFR ligands within the tissue extracts. CRS tissue was used for evaluation of the distribution of epiregulin (EREG), an EGFR ligand, and MMP-1 by immunohistochemistry. In parallel studies, expression of these genes and proteins was analyzed in cultured primary airway epithelial cells. Elevated expression of EREG and MMP-1 mRNA and protein was observed in uncinate and polyp tissue from patients with CRSwNP. Immunohistochemistry study of clinical samples revealed that airway epithelial cells expressed both of these proteins. Cultured primary human airway epithelial cells expressed MMP-1, and MMP-1 was further induced by stimulation with EREG or heat-killed SA (HKSA). The induction of MMP-1 by HKSA was blocked by an antibody against EREG, suggesting that endogenous EREG induces MMP-1 after stimulation with HKSA. EREG and MMP-1 were found to be elevated in nasal polyp and uncinate tissues in patients with CRSwNP. Elevated expression of EREG and MMP-1 may be related to polyp formation in CRS, and colonization of SA might further enhance this process.

Keywords: chronic rhinosinusitis; epidermal growth factor receptor; epiregulin; matrix metalloproteinase-1; *Staphylococcus aureus*

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Correspondence and requests for reprints should be addressed to Robert P. Schleimer, Ph.D., Division of Allergy and Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, 240 East Huron, Room M-318, McGaw Pavilion, Chicago, IL 60611. E-mail: rpschleimer@northwestern.edu

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Clinical Relevance

We demonstrate elevated expression of the tissue repair and remodeling factors, epiregulin (EREG) and matrix metalloproteinase (MMP)-1, in sinonasal tissues from subjects with chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP). *In vitro* studies showed the induction of MMP-1 by heat-killed *Staphylococcus aureus* (SA) in epithelial cells, and blocking antibody studies implicated EREG in MMP-1 induction. SA colonization is prominent in CRS, and SA thus might amplify the expression of EREG and MMP-1 from the epithelial cells in patients, promoting remodeling of sinonasal tissues. Targeting EREG and/or epidermal growth factor receptor ligand–induced MMPs may have some utility in preventing polyp formation in patients with CRSwNP.

Chronic rhinosinusitis (CRS) is characterized by persistent symptomatic inflammation of the nasal and sinus mucosa, and is one of the most common chronic diseases in adults in the United States (1–3). Although the etiology and pathogenesis of CRS remain controversial, both bacterial and fungal infections have been theorized to play a role, in association with defective innate immune responses of the epithelial barrier (2, 4). CRS is typically classified into CRS with nasal polyps (NPs; CRSwNP) and CRS without NPs (CRSsNP) (3). Sinonasal tissue from most patients with CRSwNP displays a type 2 cytokine profile with pronounced infiltration of eosinophils (5–7). Disease management of patients with CRSwNP is often unsatisfactory, and symptoms can persist despite medical treatment and surgical intervention (1).

The epidermal growth factor (EGF) ligand family consists of several ligands, including EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor (TGF)-α, amphiregulin (AREG), epiregulin (EREG), and neuregulin. EGF ligands and their receptor, the EGF receptor (EGFR), regulate cellular proliferation, differentiation, and migration to coordinate repair of damaged epithelial cells (8). Elevated levels of EGFR ligands have been shown in a variety of airway disorders, such as bronchial asthma and chronic obstructive pulmonary disease (9–11). EGFR itself has been reported to be up-regulated in airway epithelial cells in asthma, chronic obstructive pulmonary disease, and CRS (12, 13). Upon EGFR activation, airway epithelial cells produce a variety of cytokines, chemokines, and tissue-repairing/-remodeling–related genes, such as MUC5AC and matrix metalloproteinases (MMPs) (14, 15).

Increased expression of MMPs has been reported to be closely related to the remodeling and polyp-forming processes that occur in allergic airway tissue. Previous studies showed elevation of MMP-1, -2, -7, -8, and -9 in sinonasal tissues from patients with CRS (16, 17). MMPs are induced *in vitro* in airway epithelial cells by a wide variety of stimuli, including HB-EGF, TGF-β, IL-17A, leukotriene D4, and respiratory syncytial virus (18–20). Among EGFR ligands, TGF-α and EGF were reported to be elevated in subjects with CRS when compared with control subjects (12, 21), but most of the ligands in the family have not been evaluated in CRS.

The prevalence of *Staphylococcus aureus* (SA) colonization was found to be elevated in upper airways of patients with CRSwNP when compared with normal subjects, and is often cited as evidence of a link between bacterial colonization and CRS pathogenesis (22). SA enterotoxins can activate polyclonal T cell responses, and structural constituents of SA activate Toll-like receptor 2 expressed on airway epithelial cells, and can induce chemokines and cytokines from airway epithelial cells (23, 24). Recently, we have shown that heat-killed SA (HKSA) induced both EGFR ligands and MMPs from airway epithelial cells (25). These findings suggest that SA may promote repair responses, and perhaps polyp formation, in CRS by inducing EGFR ligands and MMPs, from airway epithelial cells.

Because expression of EGFR ligands in CRS tissue has not been extensively studied, the aim of this study was to assess the expression of EGFR ligands in CRS and explore the possible mechanisms that lead to appearance of potential polyp-promoting factors, such as MMP-1, from airway epithelial cells. Furthermore, we investigated the possible role of SA, a well recognized CRS-related pathogen, using *in vitro* studies with airway epithelial cells to evaluate the hypothesis that SA might induce factors involved in epithelial repair and, possibly, polyp growth.

Materials and Methods

Patient Recruitment and Clinical Sample Collection

Patients with CRS were recruited from the Allergy–Immunology and Otolaryngology Clinics of the Northwestern Medical Group (Chicago, IL) and the Northwestern Sinus Center at Northwestern Medical Group. All subjects gave informed consent, and the study protocol was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine. All subjects met the criteria for CRS as defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force (3, 26). Sinonasal and NP tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS and control patients undergoing skull-base surgery. Details of the subjects' characteristics are included in Table 1. Further details are provided in the online supplement.

Cell Culture and Treatments

Primary normal human bronchial epithelial (NHBE) cells, from at least three different donors, were purchased from Lonza (Walkersville, MD). Human primary nasal epithelial cells (NECs) were collected from uncinate tissue (UT) by curettage with a Rhinoprobe (Arlington Scientific, Springville, UT) under a Northwestern University Feinberg School of Medicine Institution Review Board–approved human subject research protocol. NHBE cells and NECs were seeded in collagen-coated 12-well plates, and were maintained in serum-free bronchial epithelial cell growth medium (Lonza). Before stimulation, NHBE cells and NECs were cultured in BEGM without hydrocortisone for at least 24 hours. Submerged NHBE cells were stimulated with 100 ng/ml of EREG (R&D Systems, Minneapolis, MN) or 5×10^8 particles/ml of HKSA (Invitrogen, Carlsbad, CA) for 24 hours and then the total RNA was isolated. Because live SA did not yield consistent results when stimulating epithelial cells, we employed the more defined stimulus, HKSA. NECs were also stimulated with HKSA for 24 hours and then the total RNA was isolated. For inhibition studies, NHBE cells were treated

Table 1. Subject Characteristics

Characteristics	Control (*n* = 43)	CRSsNP (*n* = 44)	CRSwNP (*n* = 65)	Polyp from Subject with CRSwNP Polyp
Male/female	23/20	21/23	44/21	
Age, median (range), yr	45.5 (16–69)	35.5 (18–67)	44.5 (19–75)	
Atopy (yes/no/unknown)	3/38/2	14/21/9	32/22/11	
Asthma (yes/no/unknown)	1/41/1	5/39/0	29/36/0	
Methodology used				
Tissue RNA, *n* (M/F)	18 (9/9)	18 (6/12)	28 (20/8)	28 (18/10)
Age, median (range), yr	48 (16–62)	35.5 (20–67)	43 (27–57)	43.5 (23–72)
Tissue protein extract, *n* (M/F)	9 (8/1)	10 (5/5)	20 (12/8)	20 (8/12)
Age, median (range), yr	39 (22–61)	37.5 (22–66)	46.5 (20–71)	46 (20–71)
Immunohistochemistry, *n* (M/F)	13 (5/8)	7 (3/4)	10 (6/4)	19 (10/9)
Age, median (range), yr	52 (16–67)	36 (21–53)	51 (32–75)	45 (32–75)
Nasal epithelial cell RNA, *n* (M/F)	15 (7/8)	21 (13/8)	21 (13/8)	18 (12/6)
Age, median (range), yr	45 (23–69)	33 (18–57)	51 (19–71)	48.5 (22–70)

Definition of abbreviations: CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps.

with 100 nM of the specific EGFR inhibitor, AG1478 (Sigma-Aldrich, St. Louis, MO), or DMSO control added 1 hour before the stimulation. Anti-EREG or isotype control antibody (5 μg/ml) was applied 1 hour before the stimulation (25).

Real-Time RT-PCR

Total RNA was isolated from epithelial cells using NucleoSpin RNA II Isolation Kit (Macherey-Nagel, Bethlehem, PA) and cDNA was synthesized from RNA using SuperScriptII reverse transcriptase (Invitrogen). Real-time RT-PCR (RT-PCR) was performed with the TaqMan method using an Applied Biosystems 7,500 sequence detection system (Life Technologies, Grand Island, NY), as described previously (27, 28). The mRNA expression levels were normalized to the housekeeping gene, β-glucuronidase, or β-actin. Further details are provided in the online supplement.

ELISA

Concentrations of EREG (Cloud-Clone Corp., Houston, TX) and MMP-1 (R&D Systems) in tissue homogenates and in supernatants of cultured cells were determined with a commercially ELISA kit, as detailed in the online supplement. The concentrations of EREG and MMP-1 in the tissue homogenates were normalized to the concentration of total protein.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as described previously (29). Briefly, blocked sections were incubated with either polyclonal anti-human EREG antibody (R&D Systems) or polyclonal anti-human MMP-1 antibody (Abcam, Cambridge, MA) at 4°C overnight. After washing, sections were incubated with ABC reagent (avidin–biotin–horseradish peroxidase complex; Vector Laboratories, Burlingame, CA) followed by diaminobenzidine reagent (Invitrogen). Sections were observed after they were counterstained with hematoxylin. Details of the methods for IHC and semiquantitative analysis of EREG are provided in the online supplement.

Statistical Analysis

All data are presented as the mean (±SEM), unless otherwise specified. Differences between the groups were analyzed with the Kruskal-Wallis ANOVA with Dunnett's *post hoc* testing and Mann-Whitney *U* test. Correlations were assessed using the Spearman rank correlation. The data from culture experiments were analyzed using both parametric Student's *t* test and nonparametric Kruskal-Wallis ANOVA with Dunnett's *post hoc* test statistical analyses, with similar results; data from nonparametric testing are shown. In all cases, *P* less than 0.05 was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) software.

Results

Patient Characteristics and Expression of EGFR Ligands in Patients with CRS

Sinonasal and polyp tissues were collected from subjects with CRSsNP ($n = 44$) or CRSwNP ($n = 65$) and control subjects ($n = 43$) to determine the expression of EGFR ligands and MMP-1 (Table 1).

We assessed the gene expression of TGF-α, AREG, EGF, HB-EGF, and EREG by RT-PCR in UT from subjects with CRSsNP or CRSwNP and control subjects, as well as in NP tissue from patients with CRSwNP (Figures 1A–1E). β-glucuronidase was used as a reference gene to normalize mRNA expression. Although there were trends for elevated TGF-α and AREG, levels for TGF-α, AREG, EGF, or HB-EGF were not found to be elevated (Figures 1A–1D). However, mRNA for EREG was found to be significantly elevated in UT from patients with CRSwNP and even more so in NP tissues (Figure 1E). To confirm this observation at the protein level, we generated detergent extracts from homogenates of UT and NP tissues and measured the concentration of EREG protein by ELISA. In agreement with the mRNA data, EREG protein was strongly elevated in UT and NP tissue from the patients with CRSwNP (Figure 1F). In addition, there were positive correlations between EREG and MMP-1 protein, as expected (data not shown).

The Expression of MMP-1 and EREG in Sinonasal Tissues

We next analyzed microarray data that were generated previously to compare MMPs (MMP-1, -2, -3, -7, -9, and -11) and EGF family gene expression (TGF-α, AREG,

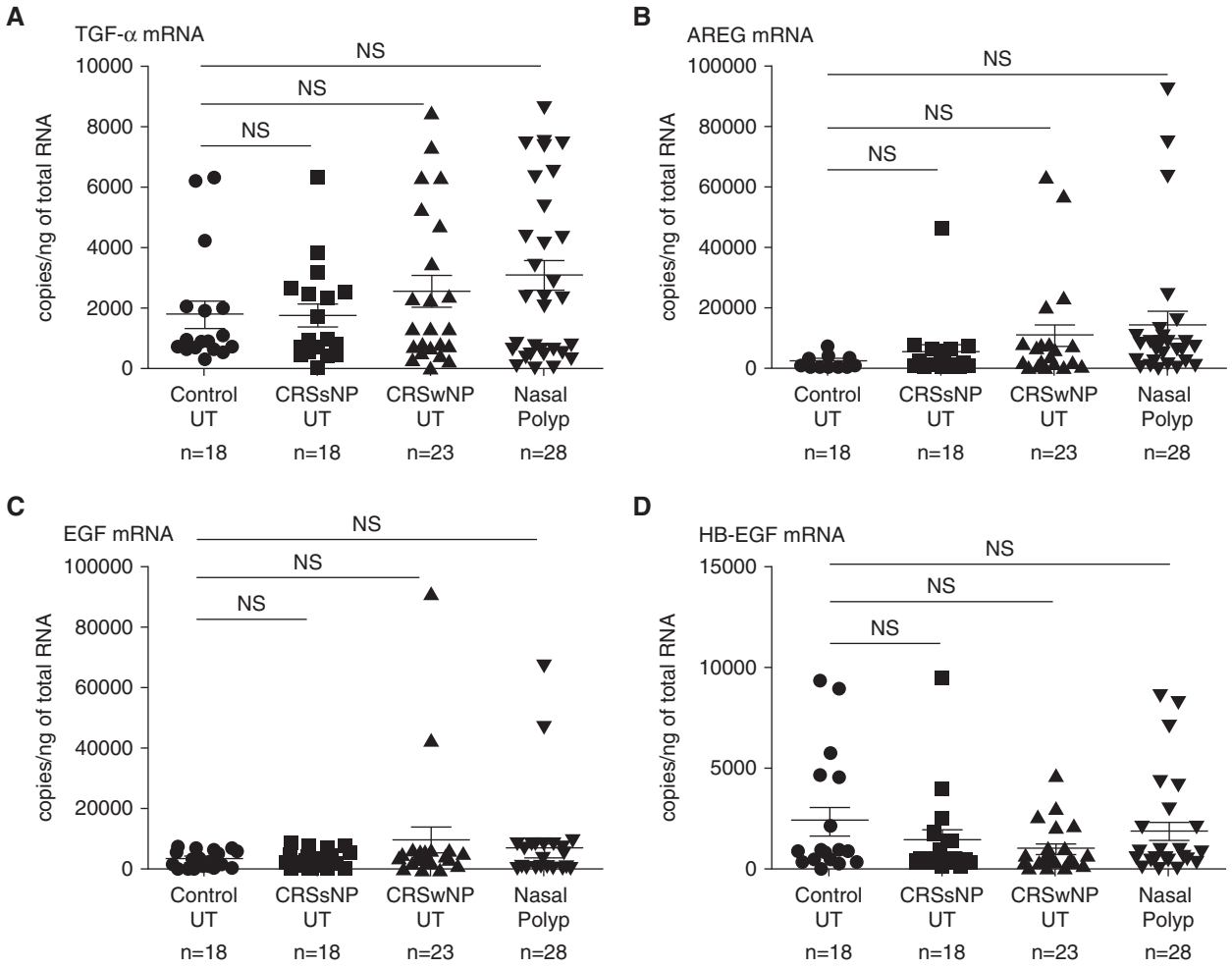

Figure 1. Total RNA was extracted from uncinate tissue (UT) and nasal polyp (NP) tissue. Expression of (*A*) transforming growth factor (TGF)-α, (*B*) amphiregulin (AREG), (*C*) epidermal growth factor (EGF), (*D*) heparin-binding EGF-like growth factor (HB-EGF), (*E*) epiregulin (EREG), and (*G*) matrix metalloproteinase (MMP)-1 mRNA were analyzed by RT-PCR. The expression of mRNA was normalized to the housekeeping gene β-glucuronidase. Expression of (*F*) EREG and (*H*) MMP-1 protein in tissue homogenates of UT and NP tissue was measured using ELISA. The concentrations of measured proteins were normalized to the concentration of total protein. Data represent mean (±SEM); $^*P < 0.05$. CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; NS, not significant.

EGF, HB-EGF, and EREG) in UT from patients with CRSsNP and control subjects, as well as in UT and NP tissues from patients with CRSwNP (30). Among the targeted genes, MMP-1 and EREG were found increased in NP tissues from patients with CRSwNP in comparison to levels seen in UT from control subjects, confirming the findings with RT-PCR. We consequently chose MMP-1 among the metalloproteases for our further studies. MMP-1 mRNA was found to be elevated in UT from patients with CRSwNP and in NP tissues (Figure 1G). This observed elevation was confirmed at the protein level by measuring MMP-1 protein in detergent extracts from homogenates of UT and NP tissues by ELISA (Figure 1H).

Epithelial Cells Expressed both MMP-1 and EREG in Sinonasal Tissue

To further characterize the expression of EREG and MMP-1 in sinonasal tissues and in NP tissues, we performed IHC analysis of surgical specimens from UT of control subjects and subjects with CRS to determine the presence and distribution of both EREG and MMP-1 proteins (Figure 2). EREG staining was prominent in UT and NP tissues from subjects with CRSwNP, whereas light to no staining was observed in UT from patients with CRSsNP and control subjects (Figures 2A–2D). The expression was mainly in the epithelial layer rather than infiltrating inflammatory cells (Figures 2A–2D). Semiquantitative analysis of EREG staining was performed, and elevated staining was seen in both UT from subjects with CRSwNP and in NP tissue (Figure 2F). In contrast, MMP-1 protein was detected in UT from both control subjects and subjects with CRS, as well as in NP tissues, and an increase in CRS tissue was not readily discerned. MMP-1 staining was broadly observed in structural cells, such as epithelial and glandular cells, and in inflammatory cells (Figures 2G–2J).

EREG and MMP-1 Expression in NEC Scrapings

With the observation from the IHC study that both EREG and MMP-1 staining was strong in epithelial cells, we evaluated the

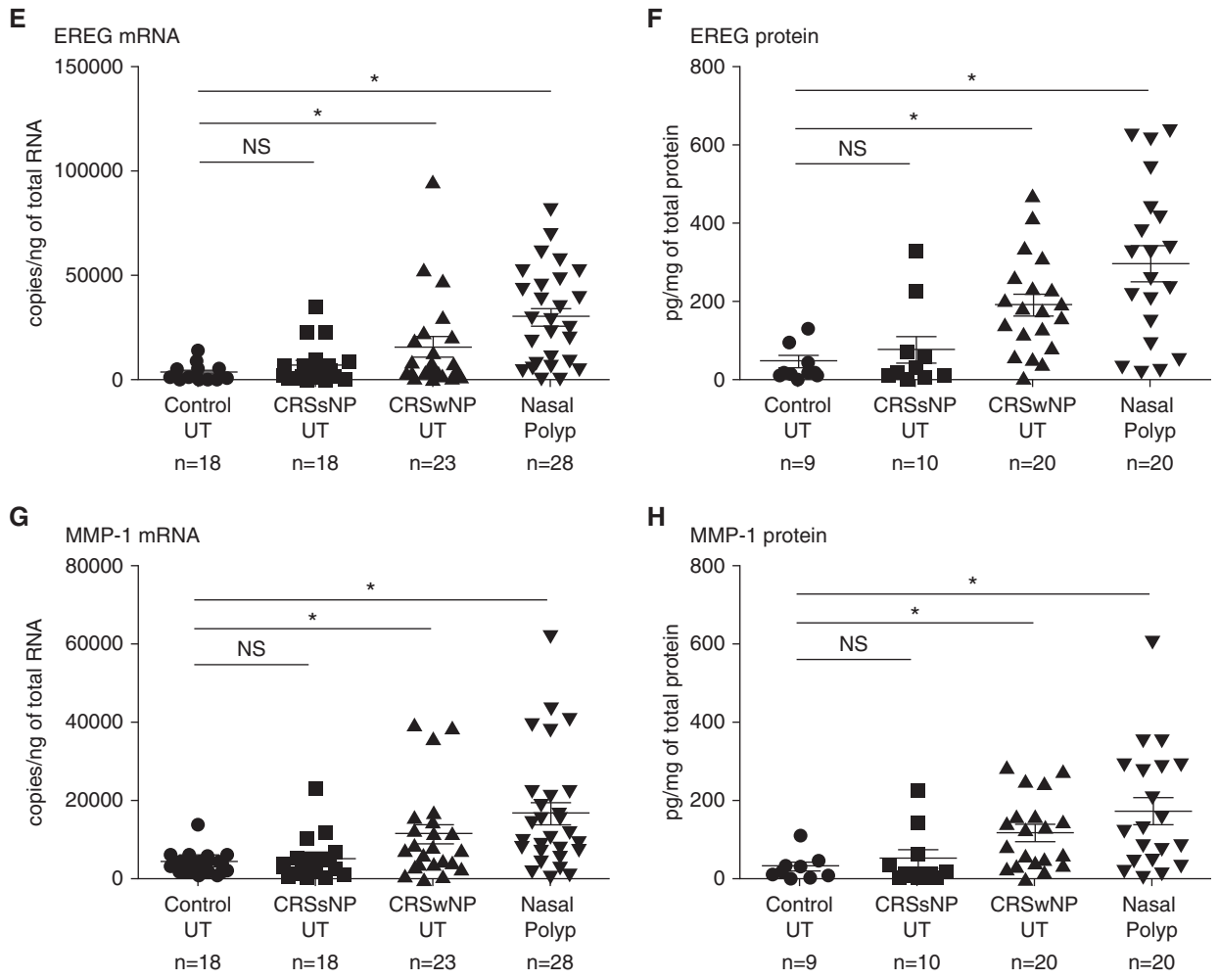

Figure 1. (Continued).

expression of EREG and MMP-1 mRNA in fresh NEC scrapings collected from patients with CRS and control subjects (Table 1). Consistent with the RNA and protein results in extracted tissue, EREG and MMP-1 mRNA were expressed at higher levels in epithelial scraping cells derived from CRSwNP, UT, and NP (Figure 3). In addition, the expression of EREG mRNA correlated significantly with the expression of MMP-1 mRNA in epithelial scraping cells ($r = 0.6643$, $P < 0.0001$; Figure 3D). In contrast, there were no changes in levels of mRNA for EGFR, an important EREG receptor, suggesting that NEC activation may have been due to elevated levels of ligand rather than as a result of overexpression of the EGFR (Figure 3C). These findings suggest the possibility that EREG and MMP-1 are elevated in CRS epithelium, and may play a prominent role in epithelial cell activation.

HKSA- and EREG-Induced MMP-1 from NHBE Cells

The preceding results suggest that epithelium is an important source of EREG and MMP-1 in nasal tissues, so we focused *in vitro* studies on activation of epithelial expression of these factors. We have previously reported that TGF-α, an EGFR ligand, induces expression of MMP-1 mRNA and protein from NHBE cells (25). We attempted to extend this previous observation by stimulating epithelial cells with EREG to test the hypothesis that EREG will also induce expression of MMP-1. EREG significantly induced both MMP-1 mRNA and protein from airway epithelial cells (5.5-fold, $n = 5$, $P < 0.01$; Figures 4A and 4B). This response was blocked by both an anti-EREG antibody and AG1478, a specific EGFR inhibitor. This finding suggests a hypothesis that the increased expression of MMP-1 *in vivo* in airway epithelial cells may, in part, be induced by EREG.

Although the factors that may induce EREG expression in sinonasal tissues are not established, we have previously reported that airway epithelial cells produce MMP-1 and EGF upon stimulation with HKSA, and previous time course experiments showed that HKSA-induced MMP-1 expression was maximal at a 24-hour time point (25). Because the nose and sinuses of many patients with CRSwNP are colonized with SA (22), we tested whether HKSA can induce EREG in epithelial cells and whether the induction of MMP-1 might be secondary to EREG expression. HKSA stimulation induced both EREG mRNA (5.4-fold, $n = 5$, $P < 0.01$) and MMP-1 mRNA (22.9-fold, $n = 5$, $P < 0.01$) in NHBE cells (Figures 4C–4F). When the EGFR inhibitor, AG-1478, or anti-EREG neutralizing antibody were

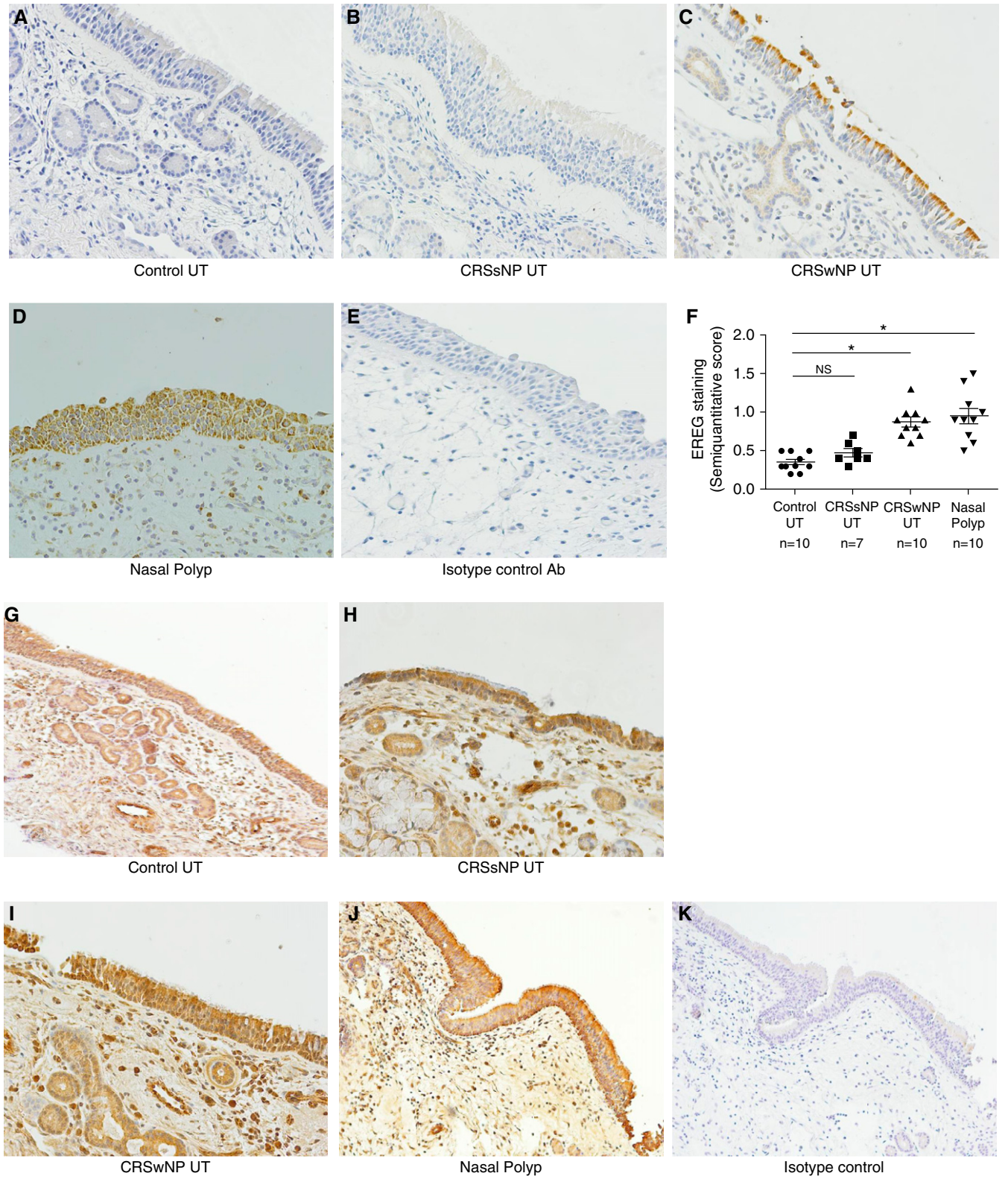

Figure 2. Immunohistochemical staining of (*A–E*) EREG and (*G–K*) MMP-1 was performed with respective anti–human cytokine antibodies. Representative immunostaining is shown for EREG in UT from (*A* and *G*) control subject, (*B* and *H*) patient with CRSsNP, (*C* and *I*) patient with CRSwNP, and (*D* and *J*) NP tissue. (*E* and *K*) Representative image of isotype control antibody staining in NP tissue is shown. Magnification, ×400. Semiquantitative analysis of EREG expression in UT from control subjects ($n = 10$), patients with CRSsNP ($n = 7$), and patients with CRSwNP ($n = 10$) and NPs ($n = 10$) was performed (*F*). $^*P < 0.05$. Ab, antibody.

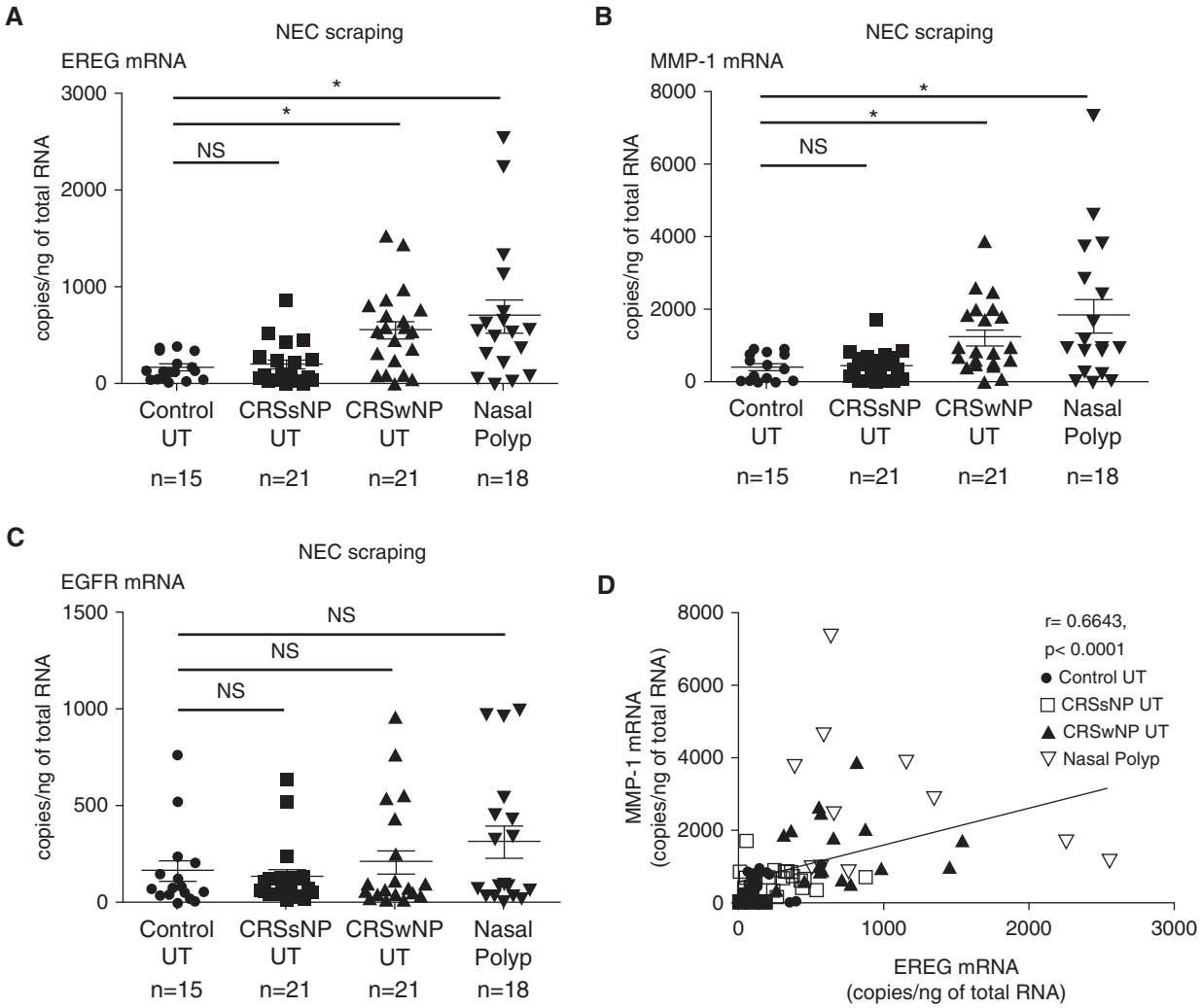

Figure 3. Total RNA was extracted from nasal epithelial cell scrapings from UT and NP tissues. Expression of (*A*) EREG, (*B*) MMP-1, and (*C*) EGF receptor (EGFR) mRNA was analyzed by RT-PCR. The expression of mRNA was normalized to the housekeeping gene β-actin. Data represent mean (±SEM); $^*P < 0.05$. The relationship of EREG and MMP-1 in nasal tissue was evaluated using RT-PCR. (*D*) The correlation shown was assessed using all values with the Spearman rank correlation test ($r = 0.6643$, $P < 0.0001$). *Solid circle*, control UT; *open square*, CRSsNP UT; *solid triangle*, CRSwNP UT; *open inverted triangle*, NP tissue. NEC, nasal epithelial cell.

added before HKSA stimulation, induction of MMP-1 mRNA and protein was inhibited, implying that HKSA induction of MMP-1 was dependent on the release of EREG and subsequent EGFR signaling (Figures 4C and 4D). Because the specific anti-EREG antibody inhibited the response nearly as well as the EGFR-inhibiting drug, we speculate that EREG is the primary member of the EGF ligand family responsible for the induction of MMP-1. Although NHBE cells and NECs are both airway epithelial cells, we confirmed in primary NECs the HKSA-induced expression of EREG mRNA (8.9-fold, $n = 8$, $P < 0.01$) and protein (10.0-fold, $n = 8$, $P < 0.01$) (Figures 5A and 5B).

Discussion

In the present study, we screened most of the family of EGFR ligands in tissue of patients with CRS and discovered elevated expression of EREG among these EGFR ligands. We also report that MMP-1 protein, an EGFR-induced gene, was elevated within CRSwNP UT and polyp tissue when compared with control UT. Although this is the first report of EREG in CRS, earlier studies have reported the presence and elevation of MMP-1 (31, 32). Furthermore, both EREG and MMP-1 mRNA were elevated in fresh NEC collected from patients with CRSwNP. Consistent with these findings, separate IHC studies revealed that both EREG and MMP-1 were mainly expressed in the epithelial cell layer, and a strong correlation was observed between the levels of expression of EREG and levels of MMP-1 *in vivo*. This correlation suggests that EREG may be responsible for the induction of MMP-1 *in vivo* in patients, a suggestion further supported by additional *in vitro* studies using an epithelial culture system in which we demonstrate that

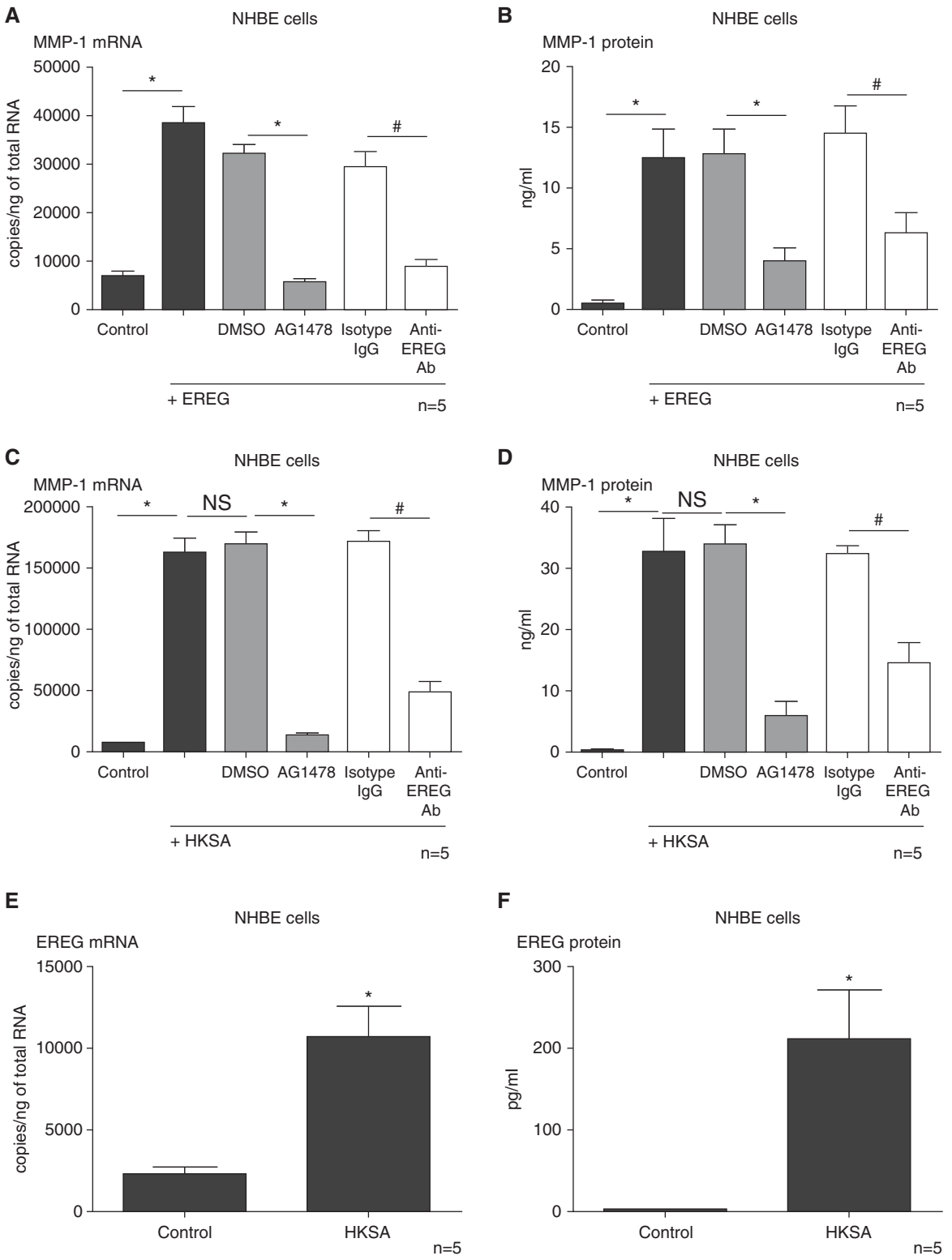

Figure 4. Submerged normal human bronchial epithelial (NHBE) cells were stimulated with either (*A* and *B*) EREG (100 ng/ml) or (*C–F*) heat-killed *Staphylococcus aureus* (HKSA; 5×10^8 particles/ml) for 24 hours. Cell lysates were harvested for RNA extraction to analyze (*A* and *C*) MMP-1 and (*E*) EREG mRNA expression by RT-PCR. Supernatants were collected for analysis of expression of (*B* and *D*) MMP-1 and (*F*) EREG protein by ELISA. Some submerged NHBE cells were pretreated with AG1478 (100 nM) or DMSO (0.01%), or anti-EREG antibody (5 μg/ml) or isotype IgG (5 μg/ml) for 1 hour before stimulation with (*A* and *B*) EREG or (*C* and *D*) HKSA for 24 hours. Data are shown as mean (±SEM) of three independent experiments. $^*P < 0.05$ when compared with media-only control; $^{\#}P < 0.05$ when compared with DMSO or isotype IgG treated cells.

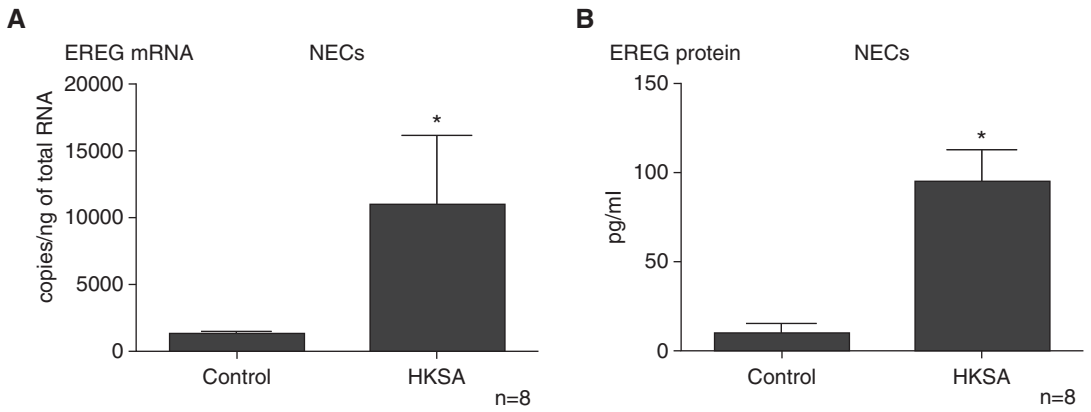

Figure 5. Submerged human primary NECs were stimulated with HKSA (5×10^8 particles/ml) for 24 hours. Cell lysates were harvested for RNA extraction to analyze (*A*) EREG mRNA expression by RT-PCR. Supernatants were collected to analyze expression of (*B*) EREG protein by ELISA. Data are shown as mean (±SEM) of eight independent experiments. $^*P < 0.05$ when compared with media-only control.

EREG stimulation led to significant induction of MMP-1 expression by airway epithelial cells.

Increased colonization by SA in the nasal cavity has been reported in subjects with CRSwNP, and a pathological role has been suggested for SA in CRS (22, 33–37). We therefore took a direct approach to identify the direct impact of SA on the epithelial cells with which they likely interact *in vivo* (25, 35). Indeed, HKSA induced both EREG and MMP-1 from NEC and NHBE cells, suggesting that SA may enhance the EGFR signaling indirectly by inducing EREG protein. To test whether the induction of MMP-1 by HKSA may be secondary to the induction of EREG, we used a specific inhibitor of EGFR signaling, AG1478, as well as a specific antibody against EREG. In both cases, induction of MMP-1 was inhibited, suggesting that HKSA induces MMP-1 indirectly via activation of the EGFR by EREG. Although a natural hypothesis from our findings would be that SA is responsible for induction of EREG and subsequent induction of MMP-1 and polyp formation, further studies will be required to definitively implicate SA in the induction of EREG and MMP-1 *in vivo*.

Although previous reports by others have shown that the EGFR ligands, EGF and TGF-α, are elevated in CRS tissue (12, 21), our study is the first to screen the family of EGFR ligands. In our screen of EGFR ligands within tissue extracts from patients with CRS, we found that the most profoundly elevated family member in CRSwNP UT and NP tissues was EREG (Figure 1). Surprisingly, our results did not confirm previous reports of elevated expression of EGF or TGF-α in CRS tissue, although there was a trend toward higher levels of TGF-α (12, 21) (Figure 1). This discrepancy may come from the utilization of different control tissues. We have previously reported the importance of the location from which control tissue is derived (29, 30). In our current and previous studies, we chose UT as our control tissue, because it is both available and immediately adjacent to the site in ethmoid sinus from which typical polyps arise (38). It is possible that EGFR ligands may also be differentially expressed at various regions within the nasal cavity. Although we did not find elevated expression of EGFR ligands other than EREG in CRS tissue, we did detect basal expression of these ligands in the tissue. It is therefore possible that more than one family member contributes to the activation of EGFR *in vivo*, even though EREG is the only one the expression of which is highly increased in CRSwNP. Our studies with HKSA do suggest that EREG is the major driver of the response in the *in vitro* model. Regardless of the ligands driving the response, we speculate that EREG and other EGFR ligands promote a signaling process involved in both epithelial activation and repair in the context of CRSwNP.

Although growing evidence supports the importance of EGFR ligands in tissue repair and, potentially, in polyp formation, the specific functions of the individual EGFR ligands remain unclear (13). It has been shown that stimulation with HDM, *Aspergillus fumigatus*, SA, IL-17A, and even with mechanical compression can induce various EGFR ligands from cultured airway epithelial cells and also further induce expression of inflammatory chemokines in either an autocrine or paracrine manner (14, 25, 39, 40). These previous findings are complicated to interpret uniformly, but we are at least able to conclude, based upon our current findings, that EGFR ligand–dependent signaling is enhanced in CRS epithelial tissue.

At present, the mechanism by which NP tissue forms is not completely understood. We have recently reported that tissue plasminogen activator, which degrades fibrin mesh, is decreased in CRS, and factor XIII-A, a coagulation factor that cross-links and forms fibrin mesh, is elevated in polyp tissue (38, 41). Together, these changes are likely responsible for deposition of fibrin mesh in polyp tissue. The cleaved form of factor XIIIA is a transglutaminase, which can interact with αvβ3-integrin (CD51/CD61 complex), expressed by airway epithelial cells, and can mediate signaling (42). MMP-1 protein has been shown to be induced in dermal fibroblasts via αvβ3-integrin activation (43), suggesting that it may be worthwhile to evaluate whether factor XIIIA may induce MMP-1 protein from airway epithelial cells. Taking together our current data and that of previous reports, it is possible that coagulation factors, EGFR ligands, and MMPs are interacting factors involved in formation of the NPs. Further experiments are needed to address the nature of the interactions of these potential polyp-forming pathways. It may also be the case that EREG and MMP-1 are important

in the epithelial–mesenchymal transition (EMT) that has been demonstrated by many different groups in CRS, more so than the polyp formation itself (32, 44).

MMP-1, -2, -7, -8, and -9 have all been found by other investigators to be elevated in CRS tissue when compared with control subjects (16, 17). Among the several types of MMP, MMP-1 was the only one found to be elevated in NP, leading us to focus on MMP-1 in CRS tissues and airway epithelial cells. MMP-1 is a protease, and can degrade fibrillar collagen during the normal processes of wound healing, epithelial cell turnover, and EMT (45). In the airways, epithelial cells form the primary barrier against environmental stimuli, and epithelium in asthma and CRS is defective, with incomplete formation of tight junctions that normally prevent penetration by inhaled allergen or pathogens (46, 47). The overexpression of MMP-1 that we report here could be one of many factors responsible for the disruption of the epithelial barrier in CRS.

Our present findings, that EREG and MMP-1 are expressed in epithelium and elevated in affected tissue in patients with CRS, are consistent with a hypothesis that EREG may induce MMP-1 from epithelial cells in CRS, a hypothesis further supported by findings that EREG induced MMP-1 mRNA and protein *in vitro* in NHBE cells. We have previously reported EGFR-dependent induction of MMPs in epithelium through activation of the transcription factor, extracellular signal–regulated kinase (ERK) 1/2 (25). If this hypothesis is correct, one would expect to observe EGFR-related transcription factors to be activated in CRS tissue. Accordingly, Linke and colleagues (48) reported that the ERK1/2 pathway was activated in tissue from patients with CRSwNP. Based on the present findings, EREG is the most likely stimulus of this activation *in vivo*. EGFR activation can promote wound healing and tissue regeneration by induction of secondary EGFR ligands as well as proteolytic MMPs; however, EGFR activation can also induce EMT and decrease the integrity of the epithelial barrier, and thereby could be involved in CRSwNP pathogenesis in more than one way.

The primary goal of this study was to investigate possible mediators of epithelial activation and polyp formation that occur in CRS, with the hope of identifying pathways that are amenable to therapeutic intervention. Current medical therapy in CRS has been centered upon antiinflammatory steroids and antibiotics. The present study suggests that a possible therapeutic target worthy of consideration is mediated by the EGFR signaling pathway and further activating mitogen-activated protein kinases, such as ERK1/2 and c-jun N-terminal kinases. We have recently reported the up-regulation of oncostatin M within NP tissues, and point out that it is known that oncostatin M enhances the activation of signal transducer and activator of transcription 1, 3, and 5 and various mitogen-activated protein kinases, suggesting further enhancement of EGFR-activated kinases (49, 50).

Although we might expect that EGFR tyrosine kinase inhibitors or anti-EGFR antibodies would diminish epithelial activation in CRS, use of these compounds is limited due to toxicity (51, 52). Our data also suggest that a broadly active MMP inhibitor, such as marimastat, could be useful in CRS. This compound decreased airway hyperresponsiveness in subjects with asthma (53). Recently, van Zele and colleagues (31) showed that doxycycline, an antibiotic that is also a potent inhibitor of MMP expression, reduced polyp size in patients with CRSwNP.

In summary, we report that MMP-1 and EREG were elevated in affected tissues from patients with CRSwNP as well as in nasal epithelial cells collected from patients with disease. Companion *in vitro* studies showed that HKSA induced EREG, which in turn induced MMP-1 via EGFR signaling, providing a possible mechanism by which SA may enhance the EMT and perhaps polyp formation. The present findings suggest that local blockade of EGFR signaling at the mucosal surface, either by inhibiting EREG or EGFR, could diminish the induction of MMP-1 and other proteases that contribute to barrier dysfunction. In addition, recognition that SA may promote epithelial activation through this EREG/MMP-1–dependent process suggests that prevention of SA activation of epithelium may also be of benefit. ■

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