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Original article

Epidermal growth factor receptor – but not histamine receptor – is upregulated in seasonal allergic rhinitis

Background: We were interested in exploring the molecular mechanisms underlying the observed difference in histamine (H) responsiveness between seasonal allergic rhinitic (SAR) and nonrhinitic (NR) subjects. We hypothesized that SAR subjects express higher nasal mucosal histamine receptor 1 (H1R) and 2 (H2R) levels than do NR subjects. In addition, we examined expression of genes involved in regulating the glandular response, including epidermal growth factor (EGF), EGF receptor (EGFR), and mucins (Muc5Ac and Muc5B).
Methods: Fourteen subjects, seven SAR and seven NR, were provoked during pollen season with doubling doses of H (0.125–8.0 mg/ml). Nasal airway resistance (NAR) was measured by active posterior rhinomanometry. Provocation was halted when NAR exceeded 150% of baseline. Prior to provocation, nasal scrapings were obtained and mRNA quantified using two-step real-time PCR.
Results: The mean PD_{50} (concentration of H producing a 50% increase in NAR) was significantly lower in the SAR than NR group (0.36 *vs* 1.32 mg/ml; $P < 0.05$). The ratio of relative gene copy numbers between the SAR and NR groups were as follows: H1R, 0.85 ($P = 0.52$); H2R, 0.67 ($P = 0.35$); EGF, 1.02 ($P = 0.93$), and EGFR, 103.5 ($P < 0.05$).
Conclusions: There were no significant differences in H1R or H2R mRNA levels between SAR and NR subjects in-season, despite observed differences in H reactivity. SAR subjects, however, did show a significant elevation in EGFR expression, consistent with the observation of mucus hypersecretion in allergic rhinitis.

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Key words: allergic rhinitis; epidermal growth factor; epidermal growth factor receptor; histamine; histamine receptor; messenger RNA; mucin; nasal provocation.

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Nasal congestion (decreased nasal airway caliber) results from vasodilation, plasma leakage, and mucus secretion in the nasal mucosa. These events are triggered by a variety of endogenous pharmacologic agents including histamine, bradykinin, prostaglandins, leukotrienes, and neuropeptides (1). Provocation studies indicate that allergic rhinitic subjects exhibit greater histamine-induced congestion than do normal control subjects. These physiological changes have been demonstrated using acoustic rhinometry, rhinostereometry, and rhinomanometry as objective endpoints (2–4). Among various possibilities, the mechanism of action resulting in this hyperresponsiveness to histamine could involve an upregulation of histamine receptor levels. In addition, heightened neurogenic reflexes and/or differential expression of vasoactive and/or mucus secreting factors may reflect or be involved in mediating an enhanced downstream response to histamine among allergic rhinitics.

Pharmacologic blockade of both histamine receptors 1 (H1R) and 2 (H2R) have been shown to inhibit vascular-mediated increases in nasal airway resistance (NAR) and peripheral venodilation after histamine challenge (5, 6). Along with exhibiting higher baseline NAR measurements, perennial allergic rhinitics demonstrate higher H1R and H2R mRNA transcript levels in nasal scrapings than do normal controls (7, 8). HR expression has not, however, been examined in seasonal allergic rhinitis (SAR) patients.

In addition to vascular reactivity, allergic rhinitis is characterized by mucus hypersecretion. Expression of various genes, including epidermal growth factor (EGF), EGF receptor (EGFR), and mucins (e.g. Muc5Ac), has been studied in allergic airways when compared to controls, and evidence exists for a role of these genes in allergic mucus hypersecretion (9–14). EGF (and EGFR) have been shown to regulate mucin production in asthmatics and may play a similar role in rhinitic subjects (9). Takeyama et al. (10) observed both goblet cell metaplasia and EGFR positive staining in the tracheobroncial tree of rats after ovalubumin sensitization, and selective EGFR inhibitors prevented this process. Lee et al. (11) demonstrated a role for EGFR in the regranulation of nasal goblet cells of rats. Both EGFR and Muc5Ac mRNA were increased in airway epithelial cells

exposed to cigarette smoke in another study by Takeyama et al. (12). Although in an animal study Muc5/5Ac mRNA was increased in the lower airways of mice sensitized to olvalbumin (a presumed downstream effect to EGFR), in a clinical study, investigators did not observe differences in MuC5/5Ac gene expression in nasal turbinate scrapings of allergic rhinitics *vs* controls (13, 14).

We hypothesized that increased sensitivity to histamine in SAR is accompanied by an increase in mucosal histamine receptor density. Further, we hypothesized that genes involved in the secretory response to histamine were also differentially expressed in SAR and normals. To test these hypotheses, we obtained superficial nasal scrapings from SAR and control subjects and then examined mRNA levels of H1R and H2R, as well as EGF, EGFR, Muc5Ac, and Muc5B – genes involved in mucus production and secretion. In addition, we documented histamine PD_{50} (provocative dose to produce a 50% increase in NAR) in the same rhinitic and control subjects, and attempted to correlate the above mRNA levels with nasal responsiveness to histamine.

Materials and methods

Subjects were recruited through posters and newspaper ads. Inclusion criteria included ages 18–69 years and 'general good health'; exclusion criteria were: (i) asthma, (ii) active smoking (within 6 months), and (iii) pregnancy or lactation. All subjects read and signed an informed consent document approved by the Committee on Human Research of the University of California, San Francisco. Questionnaires were reviewed on each applicant, who were in turn provisionally classified as having SAR, perennial allergic rhinitis (PAR), no rhinitis (NR), or 'other'. Skin tests (to 13 common aeroallergens/mixes) were compared with questionnaire responses for consistency, and subjects were classified as SAR, PAR or NR when concordant information was present. Only SAR and NR subjects underwent further testing.

Histamine provocation testing was conducted in Northern California, with 'in-season' testing being conducted in April/May. Doubling doses of histamine phosphate, ranging from 0.125 to 2.0 mg/ml, were administered 0.2 ml/nostril at 10-min intervals by a pump-spray unit. NAR was determined by active posterior rhinomanometry 5 min after histamine instillation, utilizing a commercial instrument (Model NR6-2, GM Instruments, Kilwinnig, UK). NAR was taken as the average of three measurements at ± 75 Pa. The provocation sequence was terminated when NAR reached at least 150% of its baseline level. The PD_{50} was later estimated by geometric interpolation between the last two histamine doses administered. Pulmonary function (expiratory peak flow) was monitored after each provocation dose for safety purposes.

Within the week prior to histamine provocation testing, nasal scrapings were obtained using a commercial curette (RhinoProbe, Arlington Scientific, Park City, UT, USA). Three samples were taken from each inferior turbinate. Samples were immediately transferred into a proprietary buffer in RNAse-free tubes, snap frozen in ethanol-dry ice, and stored at –80°C before analysis. RNA was extracted using a commercial kit and accompanying protocol (RNeasy, QIAshredder; Qiagen, Inc., Valencia, CA, USA). In brief, samples collected were thawed, vortexed, pipetted into QIAshredder column, and spun at 10 600 g for 2 min. Eluted specimens were diluted 1 : 1 with 70% ethanol, and loaded onto RNeasy column and spun for 2 min at 10 000 rpm. Flow-through was discarded; column subsequently washed and RNA was then eluted with 45 μl of deionized water. Residual genomic DNA was removed by adding 5 ml of 10× DNAse 1 buffer (Ambion, DNA-free kit) and 1 ml RNAse-free DNAse 1. Specimens were mixed gently and incubated at 37°C for 20–30 min. After DNAse treatment RNA was cleaned up on a second RNeasy column. Eluted RNA was stored at –80°C.

Total cellular RNA was checked for quality and quantified using a model 2100 Bioanalyzer (Agilent Technologies, Inc., Wilmington, DE, USA). mRNA was then quantified using two-step real-time PCR approach as described by Dolganov et al. (15). RT and TaqMan primers and probes for histamine receptors 1 and 2, EGF, EGFR, MUC5Ac, and MUC5b were designed using PrimerExpress software (ABI, Foster City, CA, USA); sequence information is available at http://asthmagenomics.ucsf.edu. Gene expression data were normalized to GAPDH (15).

PD_{50}s and relative gene copy numbers were, if necessary, log-transformed to satisfy normality and then analyzed by ANOVA using JMP (SAS Institute, Cary, NC, USA) on a Macintosh G4 computer (Apple Computers, Cupertino, CA, USA). Two-tailed analyses were employed with statistical significance defined as $P < 0.05$. The hypotheses tested were: (i) SAR subjects have a lower mean PD_{50} than NR subjects; (ii) SAR subjects express more mRNA for H1R, H2R, EGF, EGFR, Muc5Ac, and Muc5B than do NR subjects; and (iii) individual mRNA expression for the above genes will inversely correlate with individual PD_{50} (i.e. higher histamine reactivity/lower PD_{50} correlates with increased transcript activity).

Results

The allergic rhinitic and control groups ($n = 7$ each) had similar demographic characteristics. The actual ages of subjects recruited were 20–55 years (mean 32.6) in the rhinitic group and 20–54 years (mean 36.1) among controls. Both demographics and skin test reactivity patterns of cases and controls are summarized in Table 1. All cases showed significant skin test reactivity to a regional grass pollen mix, with the total number of positive skin tests ranging from 1 to 7 (mean, 3.3). Skin test reactivity did not reach significant levels in any of the controls, all of which were asymptomatic.

The histamine provocation procedure was tolerated well; no significant changes in pulmonary peak flow were documented (data not shown). The mean ($\pm$SEM) histamine PD_{50} was significantly lower in SAR compared to control subjects (0.36 ± 0.18 and $1.32 + 0.50$ mg/ml, respectively; $P < 0.05$). However, H1R and H2R mRNA transcript levels were not significantly different between the two groups (Table 2), and for individual subjects, H1R and H2R expression was not correlated with log PD_{50} ($P = 0.96$ and 0.59, respectively). Of the genes involved in mucus production and secretion, neither EGF nor mucin (Muc5Ac and Muc5B) mRNA differed between subgroups. Expression of mRNA for EGFR, on the other hand, was significantly greater in rhinitics than in controls (relative gene copy numbers: $5\,435\,000 \pm 2\,439\,000$ and $52\,500 \pm 32\,500$, respectively; $P < 0.05$). Individual

Table 1. Skin test reactivity of subjects

Subject	Rhinitis	Gender	Age	Saline	Histamine	Der F	Der P	Early trees	Feathers	Late trees	Grass mix	Weed mix	Mold mix	Dog	Cock-roach	Bermuda grass	Alternaria	Cladosporium	Cat	Rat	Mouse
1	Yes	Female	25	0	2	0	0	2	0	2	2	1	0	0	0	0	1	0	0	0	0
2	Yes	Female	28	0	1	±	1	1	0	0	2	±	1	0	1	1	0	0	1	0	0
3	Yes	Male	39	0	2	2	2	0	0	0	2	0	0	0	0	0	0	0	2	0	0
4	Yes	Male	21	0	2	0	0	0	0	0	2	±	0	0	0	0	1	1	0	0	0
5	Yes	Male	40	0	1	0	0	2	0	2	2	0	0	0	0	0	0	0	0	±	0
6	Yes	Male	20	0	1	0	0	0	0	0	1	0	0	0	0	2	0	0	1	0	0
7	Yes	Male	55	0	2	0	0	1	0	1	2	0	0	0	0	2	0	0	0	0	0
8	No	Female	28	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	±	±
9	No	Female	20	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	No	Female	38	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	No	Male	38	0	1	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	No	Male	54	0	2	0	0	0	0	0	0	0	0	0	±	0	0	0	0	0	0
13	No	Male	22	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
14	No	Male	53	0	1	0	0	0	0	0	0	0	0	0	0	0	0	±	0	0	0

Shaded cells = 'significant' skin test reactivity = diameter of wheal ≥ histamine control.
Numeric scoring: '1+' = <2 mm wheal; '2+' = 2–4 mm wheal.

Table 2. Ratios of gene expression in rhinitics *vs* controls

Hist1	Histamine receptor 1	0.85	NS
Hist2	Histamine receptor 2	0.67	NS
ADR1	Adenosine receptor 1	0.47	NS
ADR2	Adenosine receptor 2	1.23	NS
ADR3	Adenosine receptor 3	1.04	NS
EGF	Epidermal growth factor	1.02	NS
EGFR	Epidermal growth factor receptor	103.46	$P < 0.05$
Muc5Ac	Mucin 5Ac	1.36	NS
Muc5B	Mucin 5B	0.51	NS

relative gene copy numbers for EGFR *vs* individual log PD_{50} showed a nonsignificant inverse correlation ($P = 0.10$).

Discussion

We did not find that the lower mean PD_{50} among SAR is accompanied by increased histamine receptor mRNA levels in superficial nasal scrapings. Despite the small sample size, our negative findings are unlikely to be due solely to a lack of statistical power, as the central estimates for both H1R and H2R were in the opposite direction to that expected. These data are in contrast to two studies comparing PAR subjects with controls, which document higher H1R and H2R expression in superficial scrapings of the nasal mucosa of the former group (7, 8). These studies had only modestly larger sample sizes (10–11 subjects per group, compared to our seven), and utilized optical density of DNA staining on electrophoretic gels to quantify HR mRNA, rather than the more precise quantitative PCR technique utilized in our study. Based upon the effect size and variance published in those studies, we estimate that we had 70% power to detect a similar difference in our sample.

This apparent inconsistency could reflect differences in pathophysiology between SAR and PAR (16, 17). Alternatively, we may not have accessed the most relevant physiologic compartment, as we extracted HR mRNA from epithelial cells of the nasal lining, and histamine's vascular effects involve HR located in blood vessels. Of note, Okayama et al. (18) were unable to localize H1R in the epithelium or submucosal glands of the human nasal mucosa by autoradiographic binding, despite its localization in the submucosal vascular endothelium. Further, Ishibe et al. (19), examining biopsy specimens from both humans and guinea pigs, could not demonstrate differences in H1R density between allergic rhinitics and controls using the technique of radioligand binding, and concluded that H1R receptor density was not responsible for nasal hyperreactivity in rhinitis.

Full exploration of subgroup differences in nasal vascular reactivity to histamine would require examination of deeper (i.e. biopsy or excision) specimens, documentation of actual receptor protein (e.g. by *in situ* hybridization or autoradiography), as well as evaluation of 'downstream' and/or parallel mechanisms involved in the vascular response, including potential alterations in local nitric oxide production (20). Further, given histamine's ability to stimulate nociceptive nerves, vascular changes might occur as a result of neurally mediated reflexes, which can be modulated by factors unrelated to HR density (21). Studies of histamine responsiveness after pretreatment of the nasal mucosa with a local anesthetic or nitric oxide synthase blocker might shed light on these mechanisms.

In addition to reflecting vascular changes, nasal congestion results, to a lesser degree, from histamine-induced nasal secretions from serous and mucous glands. Of possible relevance in this regard, we documented significant elevations in EGFR (but not EGF, Muc5Ac, or Muc5B) mRNA in SAR compared to normal controls.

Increased expression of a gene involved in mucus secretion is consistent with observed upper airway hyperresponsiveness to histamine, although a more suitable physiologic correlation for such gene expression would have been volume (or mucin content) of secretions. Further, increased expression of EGFR may be related to a number of inflammatory parameters not documented in this study, so this finding must be interpreted with caution.

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