Sex-Specific Expression of Gastrin-Releasing Peptide Receptor: Relationship to Smoking History and Risk of Lung Cancer

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Background: Activation of gastrin-releasing peptide receptor (GRPR) in human airways has been associated with a proliferative response of bronchial cells to gastrin-releasing peptide and with long-term tobacco use. The GRPR gene is located on the X chromosome and escapes X-chromosome inactivation, which occurs in females. Increasing evidence demonstrates that women are more susceptible than men to tobacco carcinogenesis. We hypothesized that the susceptibility of women to the effects of tobacco may be associated with airway expression of GRPR. Methods: We analyzed GRPR messenger RNA (mRNA) expression in lung tissues and cultured airway cells from 78 individuals (40 males and 38 females) and in lung fibroblasts exposed to nicotine in vitro. Nicotinic acetylcholine receptors in airway cells were assayed by use of radioactively labeled nicotine and nicotine antagonists. A polymorphism in exon 2 of the GRPR gene was used to detect allele-specific GRPR mRNA expression in some individuals. Statistical tests were two-sided. Results: GRPR mRNA expression was detected in airway cells and tissues of more female than male nonsmokers (55% versus 0%) and short-term smokers (1–25 pack-years [pack-years = number of packs of cigarettes smoked per day multiplied by the number of years of smoking]) (75% versus 20%) (P = .018 for nonsmoking and short-term smoking females versus nonsmoking and short-term smoking males). Female smokers exhibited expression of GRPR mRNA at a lower mean pack-year exposure than male smokers (37.4 pack-years versus 56.3 pack-years; P = .037). Lung fibroblasts and bronchial epithelial cells exhibited high-affinity, saturable nicotinic acetylcholine-binding sites. Expression of GRPR mRNA in lung fibroblasts was elevated following exposure to nicotine. Conclusions: Our results suggest that the GRPR gene is expressed more frequently in women than in men in the absence of smoking and that expression of this gene is activated earlier in women in response to tobacco exposure. The presence of two expressed copies of the GRPR gene in females may be a factor in the increased susceptibility of women to tobacco-induced lung cancer. [J Natl Cancer Inst 2000;92:24–33]

Lung cancer is the leading cause of death from cancer for men and women in the United States and accounts for approximately 14% of new cancer cases (1). Although lung tumors are classified into many different histopathologic subtypes, the predominant risk factor for all tumor types is cigarette smoking (2). A number of studies [reviewed in (3)] have shown that gastrin-releasing peptide (GRP), a member of the bombesin-like peptide family, plays an important role in neoplasia by stimulating cell proliferation. In normal tissues, bombesin-like peptides stimulate growth of bronchial epithelial cells and endometrial stromal cells and have been implicated as important regulators of human lung development, including promotion of cell proliferation and epithelial differentiation in fetal lung development (4–6). The effects of GRP are mediated through the family of G-protein-coupled bombesin receptors, which includes the gastrin-releasing peptide receptor (GRPR), the neuromedin B receptor, and the bombesin receptor subtype 3 (BRS-3) and subtype 4 (BB4, which has been identified in amphibians only).

Growth stimulation by bombesin-like peptides has been shown to play an important role in human carcinogenesis. GRPRs and/or bombesin-dependent growth stimulation have been observed in human prostate, breast, and gastric carcinomas (7–13). In the case of lung cancer, GRP has been well documented as an autocrine growth factor for both small-cell lung cancer and non-small-cell lung cancer (NSCLC), and bombesin-like peptides can promote proliferation of lung tumor cell lines (3,14). Expression of messenger RNA (mRNA) for the three bombesin-like peptide receptors has been observed in NSCLC, and it has been shown that transfer of the GRPR gene to immortalized bronchial epithelial cells confers an increased growth response to GRP (15). Elevated levels of GRP have been observed in the urine of asymptomatic cigarette smokers compared with normal nonsmokers [P = .007; (16)]. Expression of mRNA for GRPR is also associated with long-term smoking (3). These studies suggest that bombesin-like peptides and their receptors play a role in the promotion of lung carcinogenesis.

Important differences in susceptibility to lung cancer exist between men and women (17–20). The risk for all major lung cancer types is consistently higher in women than in men at every level of exposure to cigarette smoke; odds ratios for an association of lung cancer with smoking are 1.2-fold to 1.7-fold higher for women than for men, depending on the histologic type of lung cancer (17). Factors such as differences in baseline exposure, smoking history, or body size do not account for the increased risk, which is likely due to a higher susceptibility to the effects of tobacco carcinogens in women (17–20). The airways of females also exhibit a higher degree of bronchial responsiveness to cigarette smoke compared with those of males of all age groups, and airways of females appear more susceptible to adverse effects of cigarette smoke than those of males (21). The gene encoding the GRPR, which mediates the prolif-
erative effects of bombesin-like peptides in the lung (3), is located on the X chromosome and has been shown to escape X inactivation in somatic cell hybrids produced from the fibroblasts and lymphoblasts of normal women (22). The GRPR gene is located on the distal end of the p arm of the X chromosome, adjacent to the pseudautosomal region, which is known to contain clusters of genes that escape X inactivation. Thus, women may have two actively transcribed alleles of the GRPR gene, compared with one in men.

We hypothesized that the additional active copy of the GRPR gene in women may result in more frequent expression of GRPR mRNA in nonsmoking women and/or an increased frequency of activation of GRPR expression in female smokers. In this study, we have examined GRPR mRNA expression in cultured normal airway cells and tissues from male and female subjects with various smoking histories. As a control, we have also examined mRNA expression of the gene encoding the related receptor, neuromedin B receptor. The neuromedin B receptor was found previously not to be associated with smoking or proliferative response to bombesin-like peptides (3) and is located on an autosome [chromosome 6; (23)], in the same group of subjects.

SUBJECTS AND METHODS

Human Subjects

Experiments were conducted with the use of tissues and cultured cells from 78 individuals (Table 1). Tissues were collected under an approved institutional review board protocol. Biopsy specimens were obtained sequentially from patients who agreed to participate in the study and who signed an informed consent form. Included were 71 lung resection or bronchoscopy patients, five lung transplantation patients, and two normal lung donors. Of the 71 resection or bronchoscopy patients, 58 were diagnosed with lung carcinoma (including 26 with adenocarcinoma, 20 with squamous cell carcinoma, three with bronchoalveolar carcinoma, two with large-cell carcinoma, one with malignant carcinoid tumor, and six with undifferentiated or mixed cell carcinomas), five with a benign lung tumor, three with carcinoma from a distant organ that metastasized to the lung, and three with emphysema or chronic obstructive pulmonary disease. One resection patient was diagnosed with lymphoma, and another was diagnosed with a squamous cell carcinoma of the head and neck. Of the five lung transplant recipients, three had cystic fibrosis, one had pulmonary hypertension, and one had scleroderma. Medical histories were reviewed for information on diagnosis, age, sex, and smoking history. The exposure to smoking was estimated as none (for lifetime nonsmokers), short-term (1–25 pack-years [pack-years = number of packs of cigarettes smoked per day multiplied by the number of years of smoking]), or long-term (>25 pack-years).

Cells and Tissues

Biopsy specimens of the mainstem bronchus and peripheral lung were obtained at the time of resection, bronchoscopy, lung transplantation, or lung donation. Airway and peripheral lung biopsy specimens did not show pathologic changes. Primary cultures of human bronchial epithelial cells and lung fibroblasts were established from the biopsy tissues in media selective for each cell type as previously described (3). Cells were grown in short-term culture and assayed for GRPR expression at passage 1, within 6 weeks of tissue donation. In several cases, a portion of the peripheral lung or bronchus was snap-frozen in liquid nitrogen at the time of tissue procurement. For some patients, a blood specimen was also obtained, and the peripheral blood lymphocytes (PBLs) were isolated and viably frozen for future analysis.

Nucleic Acid Isolation and Analysis

RNA was isolated from snap-frozen lung tissue, cultured cells, and PBLs by the acid phenol method (24). DNA was isolated for polymerase chain reaction (PCR) from cells with the use of standard methods (25). PCR amplification was done in a reaction volume of 25 μL with the use of 50–200 ng of template, 10 pmol of each primer (one primer end-labeled with 32P-1.5 mg of each deoxyribonucleotide triphosphate, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 0.25 U Taq polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Amplification of the GRPR mRNA following oligo-dT-primed reverse transcription (RT) of total RNA was performed with primers GRPR-1 (5'-CTCCCGTGAAAGATGACCTGG) and GRPR-2 (5'-ATCTTCTCATTGGGATGAGGAG) for 35 cycles (of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds). These primers result in a product of 389 base pairs (bp) and are designed to span an intron–exon boundary so that trace amounts of contaminating genomic DNA cannot be amplified under the PCR conditions used.

Southern blots of the RT–polymerase chain reaction (RT–PCR) products were hybridized with a 32P-labeled internal probe (GRPR-D: 5'-CACCTCCA-GTCTCATTTGTC). Primers, PCR conditions, and probe for the amplification and detection of neuromedin B receptor mRNA were as previously described (3).

The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified as a positive control for RT–PCR with the use of primers GAP-1 (5'-GTCAACCGATTGGTGCTGTATT) and GAP-2 (AGTCTTCTGGGT-GCAATGAT) for 25 cycles (of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute). Primers are designed to span an intron–exon boundary so that trace amounts of contaminating genomic DNA cannot be amplified under the PCR conditions used. The GAPDH PCR product (520 bp) was detected by Southern blot hybridization with a gene-specific oligonucleotide probe (GAP-3: 5'-CGATGTGCTGGCGCTGAGTACAT).

RT–PCR assays for GAPDH, GRPR, and neuromedin B receptor were performed in parallel with the use of the same reagents and RT reactions, and only RNA samples for which a GAPDH product was obtained were included in the data analysis. Use of GAPDH as a positive control for RT–PCR ensured that mRNA was of sufficient quality (size and purity) to be reverse transcribed and amplified by PCR. Representative results for GAPDH and GRPR are shown in Fig. 1. RNA samples for which no GAPDH product was visible in autoradiographs following Southern blotting were considered negative for GRPR mRNA expression. To verify that no GRPR band was present in negative samples, we carried out densitometry. For all RNA samples for which no band was visible (e.g., RNA samples from subjects 839 B, 845 B, 855 B, 860 B, and 881 B in Fig. 1), no absorbance peak was detected by densitometry, and the absorbance value found by densitometry was less than or equal to background (an empty lane on the autoradiograph film). We also verified that no mRNA for GRPR was present in negative samples by carrying out a second round of PCR or by using more starting RNA to show that further cycles of amplification or more template RNA would not produce a product. Because the detection method used relies on both amplification of complementary DNA (cDNA) reverse transcribed from RNA and Southern blotting, it is difficult to compare results from different blots run at separate times with the use of different probe preparations for detection. We, therefore, did not attempt to compare quantitatively the intensity of signals from positive reactions, but instead we classified subjects as either negative (nonexpressors) or positive (expressors), as in a previous publication (3). We did, however, notice that a subset of individuals displayed weak signals for GRPR, but not for GAPDH, compared with the majority of positive subjects. This subset (about 15% of the subjects) was made up of seven males and five females, the majority of whom had lower smoking exposures than the mean (see “Discussion” section).

Polymorphism Analysis

A polymorphism involving two nucleotides in exon 2 of the GRPR gene was detected in DNA isolated from fresh lung tissue and from cultured cells with the use of allele-specific oligonucleotide hybridization as described (26). The same method was used to detect the exon 2 polymorphism in GRPR mRNA, with the use of one of the primers GRPR-5 (5'-CATGGCATTGGGAGTATCGT) and GRPR-6 (5'-CCACGGGAGATGTGAAACTG) to amplify oligo-dT-primed total RNA, using an annealing temperature of 64°C. These primers result in a product of 428 bp and span exon 1 of the GRPR gene so that genomic DNA cannot be amplified. The RNA polymorphism was detected with the use of the hybridization conditions and probes described by Heidary et al. (26).

Nicotine-Binding Studies

Nicotine-binding experiments were performed as described (27) with the use of radiolabeled [N-methyl-3H]nicotine (specific activity, 81.5 Ci/mmol; New England Nuclear, Boston, MA). Briefly, lung fibroblasts or IB3-1 cells [immortalized human bronchial epithelial cells; (28)] were seeded into 24-well tissue
culture plates at a density of $1 \times 10^5$ cells per well. Normal, nonimmortalized human bronchial epithelial cells were plated at a density of $1 \times 10^6$ cells per 25-cm² tissue culture flask. Cells were exposed in quadruplicate at 4°C to increasing concentrations of $[N$-methyl-$^3$H]nicotine ($5$–$150$ nM) to determine total binding. For the assessment of nonspecific binding, 10 µM unlabelled nicotine was added. Specific binding of $[N$-methyl-$^3$H]nicotine was calculated by subtracting the mean nonspecific binding from the mean total binding (see Fig. 2). In competition experiments (see Fig. 3), binding of $[N$-methyl-$^3$H]nicotine was assessed in quadruplicate in the presence or absence of 0.1 nM or 1.0 nM unlabelled nicotine, tetraethylammonium chloride, (+)-tubocurarine, or hexamethonium. Nicotine was obtained from Sigma Chemical Co. (St. Louis, MO); other reagents were obtained from Research Biochemical International (Natick, MA).

Induction of GRPR Expression by Nicotine

Normal human lung fibroblasts were exposed to 0.1 µM or 1.0 µM nicotine in the culture medium for 1 day or 5 days. These concentrations reflect the tissue and serum levels of nicotine in the average smoker (29–31). Control cells received culture medium without nicotine. Cells were frozen in guanidinium buffer for RNA extraction at various time points after addition of nicotine. RT–PCR amplification and Southern blot hybridization using primers for GRPR mRNA and GAPDH mRNA were performed for each sample as described above. After blotting, hybridization, and exposure to X-ray film, the autoradiographs were scanned with the use of a densitometer, and the results are expressed as relative densitometric units (GRPR expression/GAPDH expression) for each sample (see Fig. 4).

Statistical Analysis

The following statistical tests were used where appropriate: Fisher’s exact test (comparing frequencies of GRPR expression in Table 2), two-tailed Student’s t test (comparing mean pack-years in Table 3), two-tailed Mann–Whitney test (comparing median pack-years in Table 3), and analysis of variance (comparing extent of binding of nicotine in Fig. 3).

RESULTS

To test the hypothesis that the additional active copy of the GRPR gene in women may result in an increased frequency of GRPR expression in women and/or an increased frequency of activation of GRPR expression in female smokers, we examined GRPR expression in normal airway cells and tissues from 78 men and women who underwent thoracic surgical procedures or bronchoscopies at the University of Pittsburgh Medical Center (Table 1). This included results from 21 subjects previously described (3). The specimens analyzed included bronchial epithelial cells and lung fibroblasts cultured from small airway biopsy specimens as well as fresh-frozen peripheral lung tissue. GAPDH expression was analyzed in parallel with GRPR expression; only RNA samples that were positive for GAPDH mRNA, indicating that the mRNA was of sufficient quality for RT–PCR, were included in this study.

A representative Southern blot showing subjects who were positive and negative for GRPR mRNA (389-bp product) is shown in Fig. 1, along with uniform positive results for GAPDH mRNA expression (520-bp product). In Fig. 1, both female subjects and one of six male subjects are positive for GRPR mRNA expression. The results of the analysis of all subjects from Table 1 are summarized in Table 2. By use of RT–PCR, GRPR mRNA expression was detected in 25 of 51 bronchial epithelial cell cultures, 16 of 20 fibroblast cultures, and eight of 14 specimens of peripheral lung (which included both epithelial and fibroblast cell types). These results are, to our knowledge, the first documentation of GRPR mRNA expression in human lung fibroblasts, although both fibroblasts and epithelial cells have been previously shown to respond to GRP (32). In seven patients, more than one specimen type was available for analysis, and GRPR mRNA expression was consistent in all instances for each subject between bronchial epithelial cells and lung fibroblasts or between lung fibroblasts and peripheral lung. In five patients, two different specimen types were positive; in two other patients, two different specimen types were negative. These data show that it is valid to combine results from different cell types to compare GRPR mRNA expression in a series of subjects.

Table 1. Subjects used for analysis of expression of gastrin-releasing peptide receptor (GRPR)*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patients</th>
<th>Mean age, y, ± standard deviation</th>
<th>Smoking history</th>
<th>Expression of GRPR messenger RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer (8 also had COPD)</td>
<td>29 females 29 males</td>
<td>66.4 ± 9.9 (range = 41–107 y)</td>
<td>7 nonsmokers 5 smokers &lt;25 PY 46 smokers &gt;25 PY</td>
<td>36 positive 22 negative</td>
</tr>
<tr>
<td>Cancer metastatic to the lung</td>
<td>3 females</td>
<td>67.7 ± 15.2 (range = 54–84 y)</td>
<td>1 nonsmoker 1 smoker &lt;25 PY 1 smoker &gt;25 PY</td>
<td>3 positive</td>
</tr>
<tr>
<td>Benign lung tumor</td>
<td>2 females 3 males</td>
<td>57.4 ± 8.3 (range = 48–66 y)</td>
<td>2 nonsmokers 2 smokers &lt;25 PY 1 smoker &gt;25 PY</td>
<td>2 positive 3 negative</td>
</tr>
<tr>
<td>Other cancer 1 lymphoma 1 SCCHN</td>
<td>2 males</td>
<td>69.5 (ages = 65 y, 74 y)</td>
<td>2 smokers &gt;25 PY</td>
<td>1 positive 1 negative</td>
</tr>
<tr>
<td>Emphysema/COPD (without cancer)</td>
<td>1 female 2 males</td>
<td>57.3 ± 14.0 (range = 46–73 y)</td>
<td>3 smokers &gt;25 PY</td>
<td>3 positive</td>
</tr>
<tr>
<td>Lung transplant recipients</td>
<td>3 females 2 males</td>
<td>33.8 ± 13.6 (range = 17–48 y)</td>
<td>5 nonsmokers</td>
<td>5 negative</td>
</tr>
<tr>
<td>Normal lung donors</td>
<td>2 males</td>
<td>17.5 (ages = 14 y, 21 y)</td>
<td>1 nonsmoker 1 smoker &lt;25 PY</td>
<td>2 negative</td>
</tr>
<tr>
<td>Summary</td>
<td>38 females 40 males</td>
<td>62.3 ± 15.0 (range = 14–107 y)</td>
<td>16 nonsmokers 9 smokers &lt;25 PY 53 smokers &gt;25 PY</td>
<td>45 positive 33 negative</td>
</tr>
</tbody>
</table>

*COPD = chronic obstructive pulmonary disease; PY = pack-years (number of packs of cigarettes smoked per day multiplied by the number of years of smoking); SCCHN = squamous cell carcinoma of the head and neck. Nonsmokers are individuals who never smoked.
As we reported previously (3), the overall frequency of GRPR gene expression in airway cells and tissues increased with higher exposure to tobacco smoke (Table 2). For all subjects combined, the prevalence of GRPR-positive subjects was 37.5\% (six of 16 subjects) in lifetime nonsmokers, 44.4\% (four of nine subjects) in short-term (1–25 pack-years) smokers, 72.2\% (13 of 18 subjects) in smokers in the exposure category of 26–49 pack-years, and 62.9\% (22 of 35 subjects) in smokers with 50 or more pack-years of exposure. A comparison of lifetime nonsmokers and short-term smokers with long-term smokers (>25 pack-years) revealed that there was a significant difference in frequency: 10 (40.0\%) of 25 positive compared with 35 (66.0\%) of 53 positive; \(P = .048\). Chi-squared analysis showed a trend in the data for increasing expression frequency as smoking exposure increased (\(P = .06\)). However, the impact of smoking is largely due to smoking effects in males compared with females, since females had a high frequency of expression in the absence of long-term smoking. Chi-squared test for trend as smoking increased showed statistical significance when males were analyzed separately (\(P = .012\)) but not when females were analyzed separately (\(P = .44\)).

We observed a large difference in the frequency of GRPR mRNA expression in the lung between females and males with limited or no smoking history. None of five male nonsmokers expressed GRPR mRNA, whereas six of 11 female nonsmokers were positive for expression (\(P = .09\), Fisher’s exact test; Table 2). A significant difference in frequency of mRNA expression was also observed between all females and males with a pack-year history of 25 years or fewer (including nonsmokers): One of 10 males expressed GRPR mRNA versus nine of 15 females (\(P = .018\), Fisher’s exact test). As pack-years of smoking increased, this difference between female and male smokers disappeared: Males and females with a smoking exposure greater than 25 pack-years showed no difference in the frequency of GRPR mRNA expression (19 of 30 males expressed the gene versus 16 of 23 females; \(P = .43\), Fisher’s exact test).

These results suggest that the GRPR gene is expressed more frequently in women in the absence of smoking and/or that expression is activated at an earlier time point in response to tobacco exposure in women than in men. Since data on environmental tobacco exposure were not available for these subjects, it is possible that passive smoke exposure affected the frequency of GRPR gene expression in nonsmokers and short-term smokers. Passive smoke exposure could influence the frequency of GRPR mRNA expression in females more than in males, since the possibility that nonsmoking females have a smoking spouse is higher than that for nonsmoking males. Effects on lung cancer risk of passive smoking from a spouse are relatively weak. Passive smoke from a spouse results in an increase in odds ratios for the risk of developing lung cancer to 1.0–1.3 compared with 1.0 for lifetime nonsmokers not exposed to passive smoke from a spouse (33–35), equivalent to fewer than 5 pack-years of active smoking. If GRPR-positive expression in the nonsmoking women in our study were due only to passive smoke, this would still place these nonsmoking subjects in the short-term smoking risk group and would not change the statistical analysis or the gender effect. However, it would mean that women, rather than showing increased frequency of GRPR gene expression in the absence of smoking, show heightened expression in response to tobacco smoke, compared with males.

A comparison of active smoking history with GRPR expression status in men and women shows that females, on average, express GRPR mRNA after significantly less exposure to tobacco smoke than males (mean, 56.3 pack-years for males versus 37.4 pack-years for females \(P = .037\), Student’s \(t\) test; median, 50 pack-years for males versus 42 pack-years for females \(P = .05\), Mann–Whitney nonparametric test; Table 3). No significant difference in pack-years of smoking was observed between males and females negative for GRPR mRNA expression (mean, 42.6 pack-years in males versus 28.5 pack-years in females \(P = .28\), Student’s \(t\) test; median, 35 pack-
years for males versus 30 pack-years for females \(P = .32\), Mann–Whitney nonparametric test).

Our data are also consistent with the increased susceptibility of females to lung cancer compared with males that has been reported in larger epidemiologic studies. An analysis of the pack-years of tobacco smoke exposure at the time of diagnosis in male and female lung cancer patients in our study demonstrated that the female subjects in our study developed lung cancer with significantly less tobacco exposure than did the males, as has been shown previously (17–20). The females with lung cancer among our subjects had a mean smoking history of 41.2 pack-years, compared with 59.9 pack-years for the males \(P = .032\), and the median exposure in pack-years was 50 for males and 42 for females \(P = .05\), whereas age at diagnosis showed no significant difference between males and females (Table 3). In addition, almost all of the nonsmokers in our study group who were diagnosed with lung cancer were female (six of 29 female lung cancer patients were nonsmokers, compared with one of 29 males; \(P = .05\), Fisher’s exact test). It has been previously observed that nonsmokers with lung cancer are two to three times more likely to be female than to be male (17). Of the 11 female nonsmoking subjects in our study, six expressed GRPR mRNA. Of these six nonsmoking females positive for GRPR expression, five were diagnosed with lung cancer. Only one of five nonsmoking females who did not express the GRPR gene in the airway was diagnosed with lung cancer. Among the 15 female nonsmokers and short-term smokers, there was a statistically significant association between expressing GRPR mRNA and being diagnosed with lung cancer (seven of nine females with lung cancer were GRPR mRNA positive in contrast to one of six females without lung cancer; \(P = .04\), Fisher’s exact test). This association was not observed in males, since male nonsmokers and short-term smokers did not express GRPR mRNA.

Our data also show that all female lung cancer patients who express GRPR mRNA had a significantly lower mean and median pack-year tobacco exposure at diagnosis than men with lung cancer (both those who express and those who do not express GRPR; Table 3). Again, these results suggest that expression of GRPR mRNA in women is associated with their increased risk of lung cancer (mean, 39.4 pack-years for women versus 62.7 pack-years for men; \(P = .026\) [GRPR positive and GRPR negative]; Table 3). GRPR-positive women with lung cancer had a median of 44 pack-years of exposure compared with a median of 52 pack-years in GRPR-positive men with lung cancer (\(P = .03\)). Among all lung cancer patients, the group with the lowest mean tobacco exposure was women who expressed GRPR mRNA in their lung (39.4 pack-years; Table 3). In view of the large range of tobacco exposure in the 29 female lung cancer patients in this study (0–110 pack-years), we did not observe that the lower mean smoking history of GRPR-expressing women with lung cancer compared with GRPR-nonexpressing women with lung cancer was statistically significant \(P = .677\).

As a control to determine if sex-specific expression of GRPR was related to its location on the X chromosome, we also examined expression of the gene encoding the neuromedin B receptor, another G-protein-linked receptor in the family of bombesin-like peptide receptors, which is located on an autosomal chromosome (chromosome 6). We were able to perform this analysis on reverse-transcribed reaction products stored from 37 male subjects and 28 female subjects used in the GRPR analysis (83.3% of all subjects). The stored RT reaction products were analyzed for neuromedin B receptor mRNA in parallel with a new analy-
Nicotine, an important component of tobacco smoke, has been shown to directly induce expression of the genes encoding proenkephalin, diazepam binding inhibitor, and neutrophil elastase in cells that express nicotinic acetylcholine receptors, the sites through which nicotine exerts its biologic effects (29–31). It has also been shown that exposure to nicotine results in increased expression of a reporter gene under the control of the GRP gene promoter in U937 cells (36). It has recently been reported that human bronchial epithelial cells express nicotinic acetylcholine receptors (37). Because of the association that we observed between smoking history and GRPR mRNA expression, we hypothesized that nicotine might induce GRPR expression in the human airway and that human lung fibroblasts, like human bronchial epithelial cells, might express nicotinic acetylcholine receptors.

We examined binding of tritiated nicotine to human lung fibroblasts from one donor, human bronchial epithelial cells cultured from six donors, and the immortalized human bronchial epithelial cell line IB3-1. Consistent with a previous report (37), we found saturable binding sites for nicotine in all bronchial epithelial cell cultures (data not shown). In bronchial epithelial cell cultures, the number of specific binding sites per cell ranged from $5.4 \times 10^4$ to $15.1 \times 10^4$. Saturable, specific binding for nicotine was also found in cultured lung fibroblasts (Fig. 2). The $K_d$ (i.e., dissociation constant) for binding was approximately $30 \text{nM}$. The number of saturable binding sites was estimated at $1.3 \times 10^4$ per cell in lung fibroblasts. We further determined the specificity of these nicotinic-binding sites by examining competition for binding with nicotinic antagonists (Fig. 3). At either $0.1 \text{nM}$ or $1.0 \text{nM}$, tetraethylammonium chloride, (+)-tubocurarine, and hexamethonium all displaced $[\text{N-methyl-}^3\text{H}]\text{nico}
tine to a statistically significant greater extent than unlabeled nicotine ($P = .03$ for tetraethylammonium chloride, $P = .001$ for (+)-tubocurarine, and $P = .01$ for hexamethonium—two-tailed Student’s $t$ test), as would be expected for authentic nicotinic acetylcholine receptors. These results suggest that human lung fibroblasts, as well as human bronchial epithelial cells, express nicotinic acetylcholine receptors and that, through these receptors, nicotine contained in cigarette smoke can have direct biologic actions in the airway. A recent report (38) has noted that nicotine stimulates branching morphogenesis in embryonic mouse lung cultures. Since GRP is known to be crucial to branching morphogenesis in the developing lung, it is possible that the effects of nicotine are mediated through induction of GRP and/or its receptor (39).

To determine if nicotine can alter expression of GRPR mRNA in airway cells, we examined the effect of nicotine exposure on GRPR expression in cultured lung fibroblasts. Exposure...
sure of cultured human lung fibroblasts to nicotine at levels found in the bloodstream of smokers (0.1 μM or 1.0 μM) resulted in an approximately 12-fold increase in GRPR mRNA expression compared with that in control subjects [29–31]; Fig. 4]. These results suggest that nicotine in cigarette smoke may be an important modulator of GRPR gene expression in the human airway.

We hypothesized that the second X-linked copy of the GRPR gene in women, if it is functional, might be a target for induction by nicotine or other transcriptional regulators. To test whether the increased frequency of GRPR mRNA expression in women is related to the gene’s location on the X chromosome and the fact that this locus escapes X-chromosome inactivation in women, we examined a polymorphism involving two nucleotides in exon 2 of the GRPR gene (26). We used allele-specific oligonucleotide hybridization to detect these polymorphisms in DNA and RNA isolated from cells cultured from normal lung. Our analysis of DNA from 26 females and two males confirms that males are hemizygous and that approximately 38% of women are heterozygous at position 450 (can be a C or T) and position 661 (also C or T), as previously described [26; data not shown]. We identified one female subject (sample 780) who is heterozygous at position 450 but homozygous at position 661; this recombinant genotype was also reported previously [Table 4; (26)]. For nine subjects (eight females and one male), both DNA and RNA were available for allele-specific oligonucleotide hybridization analysis (Table 4). For four heterozygous women, GRPR mRNA was detected from both alleles, indicat-

**Fig. 3.** Nicotinic antagonists compete effectively for binding of [N-methyl-3H]nicotine to immortalized human bronchial epithelial cells (IB3-1 cells). At either 0.1 nM or 1.0 nM, tetraethylammonium chloride (TEAC), (+)-tubocurarine, and hexamethonium (HEX) all displaced [N-methyl-3H]nicotine binding to a significantly greater extent than did equimolar nicotine. *P = .03, **P = .001, ***P = .01. d.p.m. = disintegrations per minute. Error bars represent 95% confidence intervals.

**Fig. 4.** Expression of gastrin-releasing peptide receptor (GRPR) messenger RNA (mRNA) in cells exposed to nicotine. A) Top row: Southern blot of GRPR reverse transcription–polymerase chain reaction products from untreated normal human lung fibroblasts and the same cells exposed to 0.1 μM or 1.0 μM nicotine for 1 day or for 5 days. Blots were hybridized with a 32P-labeled oligonucleotide probe for GRPR. Bottom row: Southern blot of amplification of mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the same reverse transcription reactions used for the GRPR polymerase chain reaction. Blots were hybridized with a 32P-labeled oligonucleotide probe for GAPDH. B) Densitometric analysis of the data shown in panel A. Results are expressed as relative densitometric units (GRPR expression/GAPDH expression) for each sample.
ing that, in these women, the GRPR gene copy on the inactive X chromosome might be expressed in the lung (Table 4).

As a first step in developing a clinically useful, minimally invasive assay that could be used for general screening of at-risk populations, we examined GRPR mRNA expression in PBLs. For four subjects, PBLs and lung cells or tissue were available; in each case, the expression pattern in PBLs was identical to that in the lung (three subjects expressed GRPR mRNA in the lung and PBLs; one subject was negative for GRPR mRNA expression in both tissues). This result indicates that the GRPR gene is expressed in lymphocytes and suggests that the expression pattern in PBLs, which are also exposed to nicotine from cigarette smoke, may reflect that of the lung. However, a large number of samples would be necessary to substantiate such an assumption with confidence.

**DISCUSSION**

We have presented evidence that a statistically significant higher proportion of female nonsmokers or light smokers (<25 pack-years) express GRPR mRNA in noncancerous airway cells and tissues than males with a similar smoking history. In a previous study of GRPR gene expression, more frequent expression of GRPR mRNA was observed in the normal respiratory epithelium of long-term smokers, regardless of whether the individuals were active smokers at the time of analysis, although this study involved only a few females (3). Such a relationship was not found for neuromedin B receptor (3). There was also a significant association between expression of mRNA for GRPR, but not for neuromedin B, and proliferative response to both GRP and neuromedin B (3). Human bronchial epithelial cell cultures from individuals with a smoking history of greater than 25 pack-years were more likely to respond to bombesin-like peptides in proliferation assays (3). The association between GRPR mRNA expression and smoking history suggests that components of tobacco smoke act directly or indirectly to affect GRPR gene expression. Our results suggest that nicotine may induce GRPR mRNA expression, consistent with our demonstration of high-affinity, saturable binding sites for nicotine on lung fibroblasts and bronchial epithelial cells. Women may be more sensitive to GRPR mRNA induction by nicotine, or there may be other unknown regulators of GRPR gene expression that can target the two gene copies in women.

The risk for all major lung cancer types is consistently higher for women than for men at every level of exposure to cigarette smoke and appears to be caused by an innate increased sensitivity to the carcinogenic effects of tobacco smoke (17). The airways of females appear more susceptible to the adverse effects of cigarette smoke than those of males (17,21). A study of lung function in adolescent smokers (40) suggested that, although smoking slowed the growth of lung function in both girls and boys, the deficits are greater in girls. The sensitivity of female lungs to tobacco carcinogens may be responsible for the observation that smoking cessation or the amount of lifetime smoking exposure affects the distribution of specific histologic subtypes of lung cancer to a greater degree in women than in men (41). Women are also at increased risk for other smoking-related malignancies, including esophageal and gastric cardia adenocarcinomas and esophageal squamous cell carcinoma (42,43). The risk for oral cancer following 40 or more pack-years of smoking was increased twofold in men and fivefold in women compared with nonsmokers (44). In addition to an increased risk for women due to smoking-related factors, there is evidence for an inherited genetic predisposition (45). Wang et al. (46) observed that a family history of cancer significantly increases the risk of lung cancer in nonsmoking Chinese women. Familial clustering of lung cancer has been observed among females and among nonsmokers, suggesting the involvement of a genetic component (47–49). It is possible that GRPR expression is determined by both genetic and environmental factors that could be related to the elevated risk observed among women and in some families or populations.

A number of studies have identified gender differences at the cellular level. Higher levels of aromatic/hydrophobic DNA adducts were observed in female lung cancer patients compared with males, even though the level of exposure to tobacco carcinogens was lower among the females than among the males (50). A higher frequency of G-to-T transversion mutations in the p53 gene has been observed in females compared with males (50). Gender differences have also been identified in the expression of the CYP1A1 gene, with females exhibiting higher mRNA levels in normal lung tissue than males, although large individual differences in expression level were observed (51). Immunohistochemical staining of c-erbB-2 was identified more frequently in female lung cancer patients than in male lung cancer patients (52). While these observations suggest that there are important molecular differences between men and women that may be related to lung cancer risk, no mechanisms have yet been shown to have a direct role in the etiology of lung cancer.

Although much work has been done to characterize the role of the GRPR–ligand system in cell growth, important gaps remain in our understanding of this system. There is strong evidence to support the hypothesis that the GRPR–ligand system plays a role in lung carcinogenesis, yet no studies have directly addressed the mechanism by which this pathway is activated and remains active after tobacco exposure ceases (3). Our results suggest that the second expressed copy of the GRPR gene in females may be involved in the increased susceptibility of women to tobacco-induced lung cancer. The ability to express the GRPR gene in the adult airway, either de novo or as a result of tobacco exposure, may be an underlying predisposing factor for development of lung cancer. The lack of either a smoking or a gender effect in expression of the neuromedin B receptor gene suggests that the observed effects on GRPR are specific and related to the escape of that gene from X inactivation and its ability to be induced by nicotine.

### Table 4. Gastrin-releasing peptide receptor genotypes detected by allele-specific oligonucleotide hybridization

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*All subjects except the donor of sample 822 were female. Females with only one allele displayed may be homozygous or hemizygous.
The increased frequency of GRPR mRNA expression in non-cancerous airway cells and tissues from smokers with and without lung cancer suggests that GRPR expression may be an early event in the airway remodeling that takes place prior to development of lung cancer. Although it is still unclear whether GRPR expression alone is an important risk factor in healthy nonsmokers, GRPR mRNA expression may be a useful marker for increased lung cancer risk or prernoeplastic change (either separate from or a part of histologic change). We have shown that the GRPR gene is expressed in PBLs and that the expression pattern in PBLs, which are also exposed to nicotine from cigarette smoke, may reflect that of the lung. If blood can be demonstrated to be a good surrogate for airway cells, large-scale screening of GRPR expression in at-risk populations would be possible.

We did not attempt to quantify the extent of mRNA expression in the airway cells from subjects in this study. This is because of the inherent difficulties in quantitating reactions relying upon amplification of cDNA and detection by Southern blotting. However, we did observe a subset of 12 subjects who yielded weak GRPR RT–PCR products. The intensity of these weak GRPR products (determined by densitometry) was less than 10% of the intensity of GRPR products from the majority of positive subjects, whereas the intensity of GAPDH RT–PCR products was approximately equal. Among these 12 subjects, seven were male and five were female. The group included two female subjects who were nonsmokers and one male subject who was a short-term smoker, as well as six subjects with smoking histories of 50 pack-years or fewer. The mean smoking exposure for this group was 48 pack-years for males and 38 pack-years for females. This observation suggests that there may be differences in amount of GRPR mRNA produced that are related to tobacco exposure, which could be detected with the use of a quantitative PCR technique with an internal standard.

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Recent studies (53, 54) in which morphology-based methods were used to determine the frequency of low- and high-grade lesions in the lungs of male and female current and former smokers have been inconclusive. Possible problems in relying only on morphologic alterations to screen for prernoeplastic change include differences in pathologic interpretation and lack of objective criteria in interpreting fluorescence images. The inability to examine the peripheral lung, which is the usual site of adenocarcinoma development, may be critical in screening women for lung cancer, since lung cancer in women most often presents as adenocarcinoma (53). As a complement to morphologic criteria, molecular markers, such as GRPR expression, may prove to be useful in the detection of airway cells predisposed to lung cancer and may help to identify individuals at risk for developing lung cancer, especially women.

References


