Glycine-Extended Gastrin Promotes the Growth of Lung Cancer

Theodore J. Koh, John K. Field, Andrea Varro, Triantafillos Liloglou, Pat Fielding, Guanglin Cui, JeanMarie Houghton, Graham J. Dockray, and Timothy C. Wang

1Gastroenterology Division and Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts; 2Molecular Oncology, Roy Castle International Centre for Lung Cancer Research, The University of Liverpool, Liverpool, United Kingdom; 3Molecular Genetics and Oncology Group, Department of Clinical Dental Sciences, The University of Liverpool, Liverpool, United Kingdom; and 4The Physiological Laboratory, University of Liverpool, Liverpool, United Kingdom

ABSTRACT
The less processed forms of gastrin have recently been shown to act as trophic factors for both normal and malignant colonic cells. Although incompletely processed forms of gastrin such as glycine-extended gastrin and progastrin are also expressed in human lung cancers, the clinical significance of this expression has not been addressed. Consequently, we investigated the effects of overexpression of glycine-extended gastrin in a mouse strain that is prone to developing lung cancer and also examined the expression of incompletely processed gastrins in primary human lung cancers. We found that transgenic overexpression of glycine-extended gastrin in FVB/N mice resulted in a significant increase in the prevalence and growth of bronchoalveolar carcinoma. In addition, a substantial subset of human lung cancers was found to express progastrin and/or glycine-extended gastrin. Overexpression of glycine-extended gastrin by human lung cancers was associated with a significantly decreased survival. Taken together, these results suggest that glycine-extended gastrin may play a role in the growth and progression of some human lung cancers.

INTRODUCTION
Worldwide, lung cancer is the leading cause of cancer-related mortality. In the United States, lung cancer represents the leading cause of death from cancer with an estimated 171,500 new cases in 1998, resulting in 165,500 deaths (1). Over 40,000 new cases are diagnosed each year in the United Kingdom (2). Unfortunately, by the time lung cancer is detected, it is often difficult to treat, resulting in poor (~14% in the United States and ~5% in the United Kingdom) 5-year survival rates (2, 3). A better understanding of the genes involved in the pathogenesis of lung cancer would theoretically lead to novel therapeutic targets in the treatment of lung cancer. The development of lung cancer, as with many other cancers, is a multi-stage process involving alterations in multiple oncogenes (e.g., K-ras), tumor suppressor genes (e.g., p53), and DNA mismatch repair genes (e.g., MSH1, hMLH1, and MSH2; Refs. 4–7). However, although several of these early genetic changes have been well studied, many of the downstream targets of these signaling pathways have been less thoroughly examined. Some of these downstream targets are growth factors (such as epidermal growth factor, transforming growth factor α, and platelet-derived growth factor) secreted by lung epithelial cells, as well as a variety of neuroptides such as bombesin/gastrin-releasing peptide (particularly in small cell lung cancer), vasopressin, bradykinin, neurotensin, and gastrin (8).

It has long been recognized that gastrin in its amidated form is an important regulator not only of acid secretion but also mucosal growth, primarily for the fundic mucosa of the stomach (9, 10). Gastrin has been shown to function as a growth factor for the gastrointestinal mucosa in vivo and for gastric cancer cell lines in vitro. However, it is likely that gastrin has important growth factor functions outside of the gastrointestinal tract. For example, it has previously been shown that gastrin is also expressed in the majority of human lung cancers (11). Although the biological significance of gastrin expression in lung cancer has not yet been fully elucidated, early studies have been intriguing. Zhou et al. (12) reported that patients with small cell carcinoma, adenocarcinoma, and squamous cell carcinoma of the lung had elevated levels of gastrin in both their serum as well as in their bronchoalveolar lavage fluid compared with normal controls. In addition, they found that elevated serum gastrin levels correlated with worse prognosis; in addition, gastrin levels fell after curative resection and became elevated again in the setting of new metastases (13). In contrast, other studies have shown no prognostic value for gastrin measurements in the serum or bronchoalveolar lavage fluid in lung cancer patients (14).

Lung cancers, as with most malignancies, lack the neuroendocrine-processing enzymes to make the fully processed amidated form of gastrin. Thus, the vast majority of gastrin synthesized by lung cancers is in the form of the less-processed gastrin intermediates, progastrin, and glycine-extended gastrin (11). Interestingly, it has also been demonstrated that normal lung tissue expresses very low levels of these gastrin-processing intermediates (11). Although in the past it was assumed that incompletely processed gastrins possessed no biological function, more recent studies have shown that these less processed forms of gastrin can act as growth factors for the normal colon (15–17), as well as gastrointestinal cell lines (18, 19). Although additional work is required to characterize relevant receptors, it is clear that progastrin and glycine-extended gastrins have low affinity for the gastrin-CKK-B (CKK-2) receptor.

Furthermore, recent studies have suggested that the less processed forms of gastrin play a role in the pathogenesis of colorectal cancer. Transgenic mice that overexpress progastrin are more prone to colon carcinomas induced by azoxymethane than wild-type controls (20, 21). Mice that have elevated serum levels of glycine-extended gastrin through either infusion or insertion of a transgene are more prone to intestinal polyposis in the APC min mouse model (22, 23), whereas gastrin deficiency generated by either homologous recombination or through an immunogen that raises a gastrin-immunoneutralizing antibody results in a decreased number of polyops in the APC min mouse model (22, 23). Finally, it has been shown that glycine-extended gastrin can promote the invasiveness of human colon cancer cells (24).

Interestingly, we found that transgenic mice overexpressing glycine-extended gastrin (MT1/G-Gly) also spontaneously develop occasional bronchoalveolar carcinomas at 1 year of age (25). These tumors occurred in a genetic background (FVB) that has been reported to show spontaneous development of lung cancer (26). To explore fully the role of gastrin in the pathogenesis of lung cancer, we analyzed further the MT1/G-Gly mice in terms of tumor prevalence, tumor burden, and proliferation rates compared with wild-type FVB...
mice. In addition, we correlated these findings with expression patterns in human lung cancer specimens.

MATERIALS AND METHODS

Generation of MT1/G-Gly Mice. A mouse metallothionein promoter-human glycine-extended gastrin cDNA transgene was used to generate transgenic mice that overexpress glycine-extended gastrin in the FVB background as described previously (25). This transgene resulted in elevated levels of glycine-extended gastrin at the RNA level in all tissues examined (colon, kidney, liver, lung, pancreas, and stomach) and of peptide in the serum, with the predominant circulating form being G34-Gly. One line (7483) was primarily used for this study, although lung cancers were noted in all four MT/G-Gly-transgenic lines that were generated (25).

Animals were housed in microisolator, solid-bottomed polycarbonate cages and fed a commercially prepared pelleted diet and given water ad libitum. The mice were all maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility under barrier conditions as virus antibody free mice for the duration of the experiment. The protocol was approved by the animal care committee (Institutional Animal Care and Use Committee) of the University of Massachusetts Medical School.

Analysis of Murine Lung Tumors. Nineteen MT1/G-Gly mice and an equal number of strain-matched (FVB) control mice were sacrificed at 18 months. Twelve MT1-G-Gly mice and 16 FVB mice were sacrificed at 12 months. The lung tissue was then either fixed in 4% paraformaldehyde overnight for eventual embedding with paraffin or in OCT compound and frozen on dry ice. Blocks were sectioned at 5 μm for histological and immunohistochemical analysis and thoroughly examined for lung lesions. A minimum of five sections were examined for each lung. Routine H&E staining was performed for all sacrificed mice, and the number of tumors/lung was quantified according to size and histological phenotype. Ductular hyperplasia/dysplasia is defined as a hyperproliferative state of the bronchial epithelium with or without the presence of dysplastic cells (as evidenced by cytological atypia, loss of nuclear polarity, increased nuclear to cytoplasmic ratio, increased basophilic staining of the nucleus, and increased number of mitoses) with preservation of the underlying bronchial architecture. Adenomas were defined as a circumscribed lesion maintaining glandular architecture lined by dysplastic epithelium that distorts the underlying bronchial architecture. Adenocarcinomas were defined as macroscopically apparent lesions (>5 mm) with invasion of neoplastic cells into surrounding structures, including blood and/or lymphatic vessels.

Patients. Tumor samples were obtained from consenting patients undergoing surgical resection for lung cancer at the Cardiothoracic Centre Liverpool NHS Trust. A diagnosis of squamous carcinoma of the lung was made in 109 patients (mean age, 65.5 years; range, 42.7–87.5 years) and adenocarcinoma of the lung in 143 (mean age, 67.1 years; range, 48.8–82.6 years). This study was undertaken with ethical approval. The specimens formed part of an archival collection.

Immunohistochemistry. For murine tissues, proliferating cell nuclear antigen staining was performed from paraffin-embedded sections from all slides where tumors were found, sectioned at 5 μm, and then deparaffinized in xylene through alcohol. The slides were then placed in 2 m HCl for 1 h at room temperature. The slides were washed in PBS, incubated with horse serum for 30 min, washed again in PBS, and then underwent incubation with a mouse proliferating cell nuclear antigen antibody (working dilution 1:100; DAKOPATTS, Copenhagen, Denmark) and then stained using the Animal Research Kit (DAKOPATTS). The proliferating cell nuclear antigen labeling index was determined as the number of immunopositive cells multiplied by 100 and divided by the total number of cells/high-powered field in either tumors or surrounding normal tissue.

For human studies, samples obtained from paraffin-embedded tissue blocks were arrayed in a 20 × 20 grid (27, 28) and included positive controls of duodenum and stomach (Fig. 3, A–F). Sections were processed using antibodies reacting selectively with the main products of the gastrin gene. Antibody L382 was raised to a COOH-terminal sequence of human preprogastrin (93–101; i.e., GRRSAEDEN); it reacts with progastrin and its COOH-terminal peptides, G17, and COOH-terminal progastrin fragments indicating that this antibody reacts with the COOH-terminal Gly-extended gastrins but not progastrin or amidated gastrins. Antibody L425 is directed at the common COOH-terminal amidated sequence shared by the amidated gastrins and cholecystokinin, and absorption controls indicate that it does not react with

Fig. 1. Lung histopathology in MT1/G-Gly transgenic mice. MT1/G-Gly mice were followed for 12–18 months and then sacrificed, and their lungs were processed for histology. Lesions were scored according to standard pathological criteria (see “Materials and Methods”). This revealed the presence of areas of ductal hyperplasia/dysplasia (magnification, ×200; A), alveolar adenoma (magnification, ×200; B), and bronchoalveolar carcinoma (magnification, ×200; C).
RESULTS

MTI/G-Gly Mice Have Significantly Higher Prevalence of Lung Tumors. Transgenic mice overexpressing glycine-extended gastrin (MTI/G-Gly) in both serum and the lung in the FVB mouse strain have previously been described (25). These mice express gastrin at the mRNA level in all tissues examined and have elevated circulating levels of glycine-extended gastrin in the serum (85.0 ± 28.0 pg/ml) compared with FVB mice (<30 pg/ml; Ref. 25). The MTI/G-Gly mice developed ductular hyperplasia/dysplasia (Fig. 1A), alveolar adenomas (Fig. 1B), and bronchoalveolar carcinoma (Fig. 1C), suggesting a stepwise progression toward the development of bronchoalveolar carcinoma similar to that previously published in mice with somatic stepwise progression toward the development of bronchoalveolar carcinoma (29, 30).

At both 12 and 18 months, the MTI-Gly mice had a significantly higher prevalence of bronchoalveolar carcinoma than did the wild-type controls (16.7 versus 0%, P < 0.05 and 26.3 versus 10.5%, P < 0.05, respectively; Fig. 2A). Of the mice that developed bronchoalveolar carcinoma at 18 months, the MTI-Gly mice also had a higher tumor burden (number of cancers/mouse) when compared with wild-type mice (2.0 ± 0.63 versus 1.0 ± 0.0, P = 0.09). The tumors of the MTI/G-Gly mice also had a higher proliferation rate as measured by proliferating cell nuclear antigen staining when compared with the tumors found in the FVB mice (15.88 ± 2.37 versus 6.83 ± 0.45, P < 0.05; Fig. 2B).

Expression of Gly-gastrin and Prolagrin in Lung Cancer. Immunostaining was then performed on primary human lung cancers using antibodies against human progastrin, glycine-extended gastrin or amidated gastrin (Tables 1 and 2). Antibodies reacting with progastrin (L382) and Gly-gastrin (L373) revealed consistent staining of glycine-extended gastrins or progastrin. Endogenous peroxide was blocked using antibodies against human progastrin, glycine-extended gastrin (L382) and Gly-gastrin (L373) revealed consistent staining of glycine-extended gastrins or progastrin. Endogenous peroxide was blocked with 3% hydrogen peroxide and nonspecific protein blocked with 10% goat serum and 0.25% BSA in 0.05 M Tris-HCl. Primary antibodies were used for 1 h at dilutions of 1:1500 for L382 and 1:1000 for L373 and the Sako LSAB2 HRP labeling kit was used with diaminobenzidine substrate and hematoxylin staining. Sections were screened independently by three observers, including one highly experienced pathologist. The criteria for positive staining in the specimens was staining found in >10% of the tumor cells.

Data Analysis. For the murine studies, statistical analysis was performed using either the χ² test or a one-tailed Student’s t test. All results are expressed as mean ± SE. For the human studies, all analyses were performed using SPSS 10.0 for Windows. Associations were assessed using the χ² and Kaplan Meier for survival analysis. Survival analysis was calculated for the adenocarcinoma specimens on the 63 individuals for whom we had follow-up data.

Discussion

In this study, we present evidence that glycine-extended gastrin can contribute to the growth and progression of lung cancer. Expression of glycine-extended gastrin was noted in a substantial subset (e.g.,
~18%) of human lung adenocarcinomas. Although overexpression of glycine-extended gastrin does not appear to change the differentiation status of the adenocarcinomas, overexpression of glycine-extended gastrin within the carcinoma does correlate with a significantly worse mean survival. Finally, overexpression of glycine-extended gastrin in a mouse strain that is prone to developing bronchoalveolar carcinoma resulted in an increased prevalence and number of bronchoalveolar carcinomas.

Tumors are known to secrete a variety of peptides and growth factors that stimulate in an autocrine fashion their own growth, with the secretion of gastrin-releasing peptide in small cell lung cancer perhaps being the best example. Recent studies have suggested that the less processed forms of gastrin, including glycine-extended gastrin, can act as trophic factors for normal tissue (10, 15, 16, 25), as well as cancer cell lines and tumors (18, 19). Overexpression of both progastrin and glycine-extended gastrin has been shown to induce colonic hyperplasia and accelerate the development of colon cancer (20–22). Although specific receptors have not yet been fully characterized for the incompletely processed gastrins, recent studies from a number of groups have shown that G-Gly can activate mitogen-activated protein kinase and other signaling pathways in gastrointestinal cells (31), whereas progastrin appears to activate pp60c-Src kinase (32). In addition, migratory effects of G-Gly have been reported on mouse gastric epithelial (IMGE-5) cells (31). However, although a
role for gastrin in gastrointestinal tumorigenesis appears to be well established, relatively less attention has been given to its role in other epithelial tumors.

The expression of gastrin by lung cancer was first recognized in 1989 by Rehfled et al. (11), but there has been little work with respect to the functional significance of this association. With respect to human lung cancer, there have been a number of microarray analyses that have been applied to tumor specimens with the goal of predicting survival or tumor behavior (33). To date, gastrin has not emerged as a major gene of interest from our expression profiling studies (34); however, it is conceivable that this association may have been missed because of posttranscriptional regulation or more likely to the need for subset analysis. In our studies, we were able to confirm the earlier reports that progastrin and, to a lesser extent, glycine-extended gastrin, are expressed in human lung cancers (11), and less than half of tumors were positive for either peptide. In this investigation, adenocarcinomas that expressed progastrin tended to have a more immature differentiation status, whereas only patients with G-Gly-expressing tumors had a change in their mean survival. It is worth pointing out that the poorly differentiated subgroup only constituted 10% of the total number of adenocarcinomas analyzed so that the correlation with differentiation should be treated with caution. In this type of clinical analysis, survival data has to be considered the most powerful clinical indicator, and we therefore place greater weight on the relationship between G-Gly-expressing tumors and clinical outcome.

Although a number of hypergastrinemic mice have previously been described, the MT1/G-Gly mice represent the first reported gastrin-dependent model of lung adenocarcinoma. This propensity for lung cancer development was confirmed in three independent MT1/G-Gly lines and is no doubt related in part to the background genetic strain (FVB/N) that is prone to developing bronchoalveolar carcinoma (26). However, mice in our laboratory that overexpress human progastrin or amidated gastrin (G17) in the FVB background do not appear to have an increased frequency of lung bronchoalveolar carcinomas (data not shown). Although these may suggest specificity of the lung cancer pathway for G-Gly as opposed to G-17 or progastrin, caution is needed in this interpretation because the MT1/G-Gly transgene not only results in increased plasma levels of G-Gly but also is unique in targeting gastrin expression specifically to the lung epithelium, raising the possible need for autocrine action of this growth factor.

The MT1/G-Gly mouse model is similar in some respects to the K-rasLA mouse, another mouse model of lung cancer that shows a more rapid progression to neoplasia (29). In fact, gastrin overexpression in lung adenocarcinoma may be a direct consequence of oncogenic ras mutations. Activating ras mutations have been reported to occur in up to 50% of human adenocarcinomas (35) and in the large majority of mouse lung tumors (36, 37). Furthermore, it has been shown in colon cancer cell lines that activated ras can increase gastrin expression (38), suggesting that the expression of gastrin seen in lung cancers may be secondary to activating ras mutations. Future studies may be needed to address the possible role of gastrin as a downstream mediator of the Ras signaling pathway in tumorigenesis.

In summary, we find that overexpression glycine-extended gastrin appears to increase the frequency of lung carcinomas in a transgenic mouse model. The tumors from glycine-extended gastrin overexpressing mice also have increased proliferation rates. Finally, a substantial minority of human adenocarcinomas also overexpress glycine-extended gastrin, and the overexpression of glycine-extended gastrin appears to be associated with a significantly decreased mean survival. Taken together, this suggests that overexpression of glycine-extended gastrin can play a physiological role in the pathogenesis of lung adenocarcinoma and adds further weight to the therapeutic goal of blocking glycine-extended gastrin in cancer patients.

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