Hepatocyte Growth Factor Production by Neutrophils Infiltrating Bronchioloalveolar Subtype Pulmonary Adenocarcinoma: Role in Tumor Progression and Death

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ABSTRACT

Increased numbers of tumor-infiltrating neutrophils are linked to poorer outcome in patients with adenocarcinoma of the bronchioloalveolar carcinoma (BAC) subtype. Hepatocyte growth factor (HGF) is a pleiotropic cytokine operating through activation of the proto-oncogene c-met and is a factor of poor prognosis in various cancers. Reports that neutrophils produce HGF led us to investigate their participation in the aerogenous spread of tumor cells and the prognosis of BAC, through the effect of HGF on c-met-expressing tumor cells. Immunoactive HGF was detected in bronchoalveolar lavage fluid (BALF) supernatants from 34 of 36 patients, whereas it was undetectable in BALF from healthy controls. The HGF thus detected was locally produced, because HGF mRNA was expressed by the patients’ fresh alveolar cells, and HGF protein was detected in 24-h culture supernatants. In immunochemical studies of BALF cytospin preparations and tumor specimens from the patients, neutrophils were always HGF-positive, whereas alveolar macrophages and tumor cells gave inconsistent results. Alveolar neutrophil-derived HGFs induced significant, concentration-dependent migration of BAC-derived tumor cells in vitro, and this effect was inhibited by anti-HGF neutralizing antibodies. Granulocyte-macrophage colony-stimulating factor and tumor necrosis factor α (present in the lung tumor microenvironment) provoked HGF release from neutrophil intracellular stores, and the capacity of blood neutrophils from BAC patients to produce HGF was unaltered. Immunochemical studies of c-met expression in BALF cytospin preparations and tumor sections showed that most HGF receptor-bearing cells were tumor cells. High HGF levels in BALF supernatants were significantly associated with poorer outcome in patients with BAC and were an independent predictor of clinical outcome in multivariate analysis. Altogether, our results support the notion that BAC generates a local environment that attracts functionally normal neutrophils from peripheral blood and leads to neutrophil release of biologically active HGF on contact with HGF receptor-expressing tumor cells, thereby contributing to poorer patient outcome.

INTRODUCTION

Peripheral lung ADC3 is rapidly emerging as a leading type of pulmonary malignancy in the United States and Japan (1, 2). Lung ADC consists of a heterogeneous pattern of BAC and papillary, acinar, and solid ADCs rather than a pure histological subtype (3–5). Whatever the histological subtype, ADC is surrounded by small proliferative lesions, usually referred to as areas of atypical alveolar hyperplasia (6). This intrapulmonary spread, eventually leading to respiratory failure and death, is the hallmark of BAC-subtype ADC.

We have previously shown that tumor cells drive local neutrophil recruitment and persistence in the lung via cytokine release (7, 8) and that increased numbers of tumor-infiltrating neutrophils are linked to poorer outcome of patients with BAC-subtype ADC (8). HGF is a pleiotropic cytokine the biological effects of which are mediated by activation of the c-met proto-oncogene tyrosine kinase receptor (9). HGF is angiogenic (10) but also induces alveolar epithelial cell proliferation in vitro and promotes alveolar epithelial cell migration in vivo in various models of lung injury (11–13). HGF levels are also an independent prognostic factor in various epithelial cancers (14–17). The recent report that neutrophils produce mature (bioactive) HGFs (18) led us to investigate the possible role of neutrophils in the aerogenous spread of BAC tumor cells, and their possible prognostic influence, through the release of mature HGF and its effect on c-met-expressing tumor cells.

PATIENTS AND METHODS

Clinical Samples

Patients with BAC. Between January 1990 and January 2000, 52 patients with histologically proven primary lung ADC of the BAC subtype were treated and followed-up in our chest department. The diagnosis of BAC was based on previously published criteria (19). The degree of intra-alveolar proliferation, including papillary proliferation, and the presence of solid areas of moderately-to-poorly differentiated ADC did not exclude the tumor from the BAC category if areas of classic BAC were also present (19).

BAL was used as a diagnostic procedure in 41 cases. This study focused on the 36 cases in which stored BALF samples were available. Clinical and radiological findings at diagnosis are given in Table 1. The mean age of the patients was 65 ± 1 years (range, 42–87 years). The patients were classified according to the current International TNM Classification System for Lung Cancer (4) in which T4 designates nonassessable disease (e.g., pneumonia-like consolidations). Only one patient was classified M1 (extra-thoracic metastasis). Follow-up data were recorded until death. The cutoff date for the survival analysis was May 2001. The survival time was defined from diagnosis to death or to the cutoff date.

BAL. Briefly, 200 ml of sterile saline, in four 50-ml portions, were infused into the radiologically abnormal segment or lobe. Fluid was recovered by gentle suction and then was pooled and filtered through sterile gauze. Total and differential cell counts were performed on cytospin preparations stained with MGG. The presence of tumor cells was recorded. The remaining BALF was spun, and the supernatant was aseptically separated and stored at −80°C for cytokine measurements. The cell pellets were washed with PBS, centrifuged, and resuspended (see “Cell Culture” and “RNA isolation, reverse transcription, and PCR amplification”) for cell culture and RNA isolation. No samples were collected specifically for this study, and data were analyzed anonymously.

Control BALF supernatants were obtained from six subjects undergoing

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3 The abbreviations used are: ADC, adenocarcinoma; BAC, bronchioloalveolar carcinoma; BAL, bronchoalveolar lavage; BALF, BAL fluid; HGF, hepatocyte growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; TNF-α, tumor necrosis factor-α; AM, alveolar macrophage; PAM, polymorphonuclear neutrophil; Ab, antibody; MGG, May-Gruenwald-Giemsa (staining); FBS, fetal bovine serum; FMLP, N-Formyl-Met-Leu-Phe; MMLV-RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSCLC, non-small cell lung cancer.
diagnostic procedures. The subjects were four men and two women ages 61 ± 7 years, and included three smokers. None had a history of cancer, and all had normal BALF findings. AMs accounted for at least 97% of BALF cells in each control sample, as assessed by MGG staining.

Peripheral Blood PMN Isolation. PMNs were isolated from the peripheral blood of patients and healthy controls by density gradient centrifugation (polymorphonuclear cell separation media; Eurobio, Les Ulis, France). PMNs were separated from erythrocytes by hypotonic shock and were washed three times in sterile saline. This method yielded >97% pure PMN, as assessed by MGG staining.

Cell Culture
Alveolar cells and PMNs were resuspended at 10 × 10^6/ml in DMEM (Life Technologies, Invitrogen, Cergy-Pontoise, France) with 5 mM HEPES, 2 mM l-glutamine, 10^5 units/liter penicillin, 100 mg/liter streptomycin, and 2% FBS, hereafter referred to as complete medium. They were cultured in 24-well culture plates (0.5 ml/plate) for 5 min to 24 h at 37°C in humidified 5% CO_2/95% air. PMNs were then centrifuged, and the supernatant was stored at −80°C.

To obtain the PMN lysate used in some experiments, PMNs were cultured at 10 × 10^6/ml in complete medium for 24 h at 37°C in humidified 5% CO_2/95% air. After homogenization by pipetting, PMNs in their conditioned medium were collected and frozen at −80°C for 10 min, then were defrosted at 37°C for 10 min, three times. They were then centrifuged, and the supernatant was stored at −80°C.

In some experiments, potent inducers of neutrophil degranulation such as cytochalasin B (Sigma, Saint-Quentin Fallavier, France), FMLP (Sigma, GM-CSF (Novartis Pharma, Rueil Malmaison, France), and TNF-α (R&D Systems, Abingdon, United Kingdom) were used. PMNs were cultured at 10 × 10^6/ml in complete medium with various degranulating agents, with the following optimal timings and concentrations: (a) cytochalasin B, 5 min at a final concentration of 5 μg/ml; followed by FMLP (10^−7 M) for 15 min; (b) GM-CSF for 20 min at 10 ng/ml; and (c) TNF-α for 20 min at 10 ng/ml. PMN-conditioned media were collected and centrifuged, and the supernatants were stored at −80°C.

RNA Isolation, Reverse Transcription, and PCR Amplification
Total RNA from 10 × 10^6 alveolar cells and from 30 × 10^6 peripheral blood PMNs was extracted using the TRIZOL Reagent (Life Technologies, Inc.) according to the manufacturer’s recommendations. RNA was treated with DNase I (RQ1 RNase-free DNase protocol; Promega, Madison, WI) at 1 unit/μg RNA for 30 min at 37°C, and was then purified using the Cleanup spin column protocol with the RNeasy mini kit (Qiagen SA, Courtaboeuf, France) as indicated by the manufacturer. cDNAs were prepared from 0.75 μg of total RNA using oligo(dT)12-18 primers and MMLV-RT (200 units/μl; Life Technologies, Inc.). cDNA samples were used for HGF-specific PCR amplification with primers designed from the following cDNA sequences (Sigma-Genosys LTD, Cambridge, United Kingdom): HGF-forward (F), 5'-AAATCCTCGAGG-GAAGAAG-3'; HGF-reverse (R), 5'-AGTATGACCATGCTGCTG-3' (20); coamplification of GAPDH cDNA was performed in each experiment, using specific primers (Sigma-Genosys LTD) to compare the amounts of RNA in samples (GAPDH-H, 5'-ACCCAGAGACTTGGTAGTG-3'; GAPDH-R, 5'-AGGGTTCACTAGTGCAACTG-3'). The reaction conditions were as follows: one time at 94°C for 5 min; 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min; and one time at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1.5% agarose gel containing 0.2 μg/ml ethidium bromide. All of the PCR runs included the reverse transcription reaction mixture without MMLV-RT (data not shown), and the PCR mixture without the cDNA template (blank), as negative controls. Band density was scanned on the gels, using an Imager (Appligene, Illkirch, France) and NIH software (version 1.44). The expected sizes of the HGF and GAPDH PCR products were 286 and 598 bases, respectively.

ELISA
HGF concentrations were measured in BALF and cell culture supernatants by using a commercial ELISA kit (R&D systems) as indicated by the manufacturer; the kit consistently detected HGF at concentrations exceeding 40 pg/ml, in linear fashion.

A549 Cell Random Migration Assay and HGF Neutralization
The random migration assay was performed as described by Varani et al. (21) using the A549 cell line (American Type Culture Collection, Rockville, MD); this cell line was originally established in culture from a person with BAC (22) and is known to constitutively express the HGF receptor (23). Briefly, confluent A549 cells were removed from culture dishes with trypsinization, then were washed, pelleted by centrifugation, and resuspended in DMEM containing 10% FBS and 0.3% (w/v) sterile agarose type VII (Sigma) at a final cell density of 40 × 10^6/ml. Before use, the agarose-cell suspension was kept in a water bath at 37°C to prevent solidification. A 2-μl droplet of cell suspension was added to each well of 24-well culture plates precoated with fibronectin (1 μg/well; BD Biosciences, Le Pont de Claix, France). The plates were then cooled for 10 min at 4°C to allow the agarose to solidify. The droplet was then covered with 500 μl of FBS-free complete medium containing either BALF supernatant or 0.9% saline, with or without various concentrations of recombinant human HGF (R&D Systems). After 24 h of incubation at 37°C in humidified 5% CO_2/95% air, the medium was gently aspirated, and cells were stained with HEMACOLOR (VWR International, Fontenay sous Bois, France). Migrating cells were counted from the edge of the agarose droplet to the edge of the migration front, using an inverted tissue culture microscope at x50. Tests were performed in quadruplicate for each condition. In this assay, recombinant HGF (R&D Systems), used at concentrations between 5 and 50 ng/ml, had a concentration-dependent effect on A549 cell migration (data not shown). In some experiments, a mixture of three neutralizing anti-human-HGF antibodies was added to the medium [goat polyclonal (1 μg/ml) and mouse monoclonal clones 24612.111 and 24516.1 (both at 10 μg/ml); R&D Systems]. The percentage neutralization of HGF-related A549 cell migration was determined as follows:

\[
\text{Number of migrating cells in medium} - \text{number of migrating cells in anti-HGF-treated medium} \times 100 = \text{Percentage neutralization} \%
\]

In this assay, anti-HGF cocktail inhibited 87 ± 1% of the promigratory effect of 10 ng/ml recombinant HGF.

Immunohistochemical Studies of HGF and HGF Receptor Expression in BAC
Cytopsin BAL cells were fixed for 10 min in 4% formaldehyde (Sigma), washed with PBS, dried, and stored at −80°C. Tumor tissue and distant normal tissue were immediately frozen in liquid nitrogen and stored at −80°C.

Cytopsin preparations were permeabilized with 0.1% Triton, and tissue sections 4 μm thick were fixed for 10 min in acetone at room temperature (Sigma) before immunostaining with appropriate dilutions of mouse monoclonal anti-human HGF (clone 24612.111, R&D systems) with its isotype-specific antibody.
nonparametric test, for unpaired and paired data, respectively. Spearman’s \( \rho \) (rho) coefficient was determined for correlation studies between quantitative variables. \( P \)s below 0.05 were considered significant.

Survival rates were calculated with the Kaplan-Meier method, and survival curves were compared using the log-rank test. Qualitative and quantitative variables were coded as dichotomous (present or absent, and high or low level), and the cutoff values were the medians of distributions. The threshold of significance was set at \( P < 0.05 \). Multivariate analysis was performed with Cox’s multiple regression model. The lack of collinearity between variables was verified using the \( \chi^2 \) test. Variables with \( P < 0.1 \) in univariate analysis were tested in the Cox model. Data were processed with StatView and Survival Tools 5.0 software (Abacus Concepts, Berkeley, CA).

RESULTS

BALF Cell Counts

Total BALF cell counts were higher in patients with BAC than in controls (805 ± 177 versus 242 ± 101 cells/µl; \( P = 0.038 \)). This resulted mainly from a marked increase in total (330 ± 116 versus 4 ± 2 cells/µl; \( P = 0.007 \)) and differential (30 ± 5 versus 1 ± 0.2%; \( P = 0.004 \)) neutrophil counts. Macrophage and lymphocyte counts did not differ between patients and controls.

BALF HGF Levels

HGF was detected in BALF supernatants from all but two of the patients (mean, 311 ± 69 pg/ml; range, 0–1723 pg/ml), and none of the controls (\( P < 0.001; \) Fig. 1).

To determine whether BALF HGF was locally produced, we recovered fresh alveolar cells from seven patients and three controls and tested them for HGF mRNA expression and protein release. BAL samples from patients were selected on the basis of availability, with no knowledge of BALF HGF content. Cytological results were not significantly different from those in the overall group, with 7–98% neutrophils, and tumor cells in samples from four of the seven patients. As shown in Fig. 2A, HGF mRNA was expressed by fresh alveolar cells from patients but not from controls. Extracellular HGF protein was detected in the supernatants of 24-h alveolar cell cultures as described in “Patients and Methods.” Results (pg/ml) are means ± SE.

Table 2. HGF and c-Met expression in BAL cells from BAC patients and controls

<table>
<thead>
<tr>
<th>Case/control</th>
<th>AM (%)</th>
<th>Ly(^a) (%)</th>
<th>PMN (%)</th>
<th>Tumor cells</th>
<th>AM</th>
<th>Ly</th>
<th>PMN</th>
<th>Tumor cells</th>
<th>AM</th>
<th>Ly</th>
<th>PMN</th>
<th>Tumor cells</th>
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<td>50</td>
<td>+</td>
<td>–</td>
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<td>–</td>
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<td>Case 2</td>
<td>76</td>
<td>21</td>
<td>3</td>
<td>+</td>
<td>+/–</td>
<td>–</td>
<td>2+</td>
<td>+/–</td>
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<td>+/–</td>
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<td>2+</td>
<td>+/–</td>
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<td>2+</td>
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<td>2+</td>
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<td>12</td>
<td>+/–</td>
<td>–</td>
<td>–</td>
<td>2+</td>
<td>–</td>
<td>–</td>
<td>2+</td>
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<tr>
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<td>2</td>
<td>91</td>
<td>+/–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>Case 8</td>
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<td>8</td>
<td>3</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>2+</td>
<td>–</td>
<td>–</td>
<td>2+</td>
<td>–</td>
<td>+</td>
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<tr>
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<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2+</td>
<td>–</td>
<td>–</td>
<td>2+</td>
<td>–</td>
<td>+</td>
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<td>–</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>Control 3</td>
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<td>–</td>
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<td>2+</td>
<td>–</td>
<td>–</td>
<td>2+</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

\( ^a \) Ly, lymphocytes; UD, undeterminable because of technical problems; NA, not applicable.

Statistical Analysis

Data are presented as means ± SE. For quantitative variables, comparisons were made using the Mann-Whitney nonparametric test and the Wilcoxon

Fig. 1. HGF levels in BALF supernatants from BAC patients and controls. HGF was measured in BALF supernatants from patients with BAC \( (n = 36) \) and controls \( (n = 6) \) by ELISA, as described in “Patients and Methods.” Results (pg/ml) are means ± SE.

Fig. 2. HGF production by alveolar cells from patients and controls. A, HGF mRNA expression by alveolar cells from patients and controls. HGF expression was measured in alveolar cells from patients and controls after RNA isolation, reverse transcription, and PCR amplification as described in “Patients and Methods.” Co-amplification of GAPDH cDNA was performed in each experiment to compare the total amounts of RNA in samples. \( \text{H}_2\text{O} \), negative control. M, molecular weight marker. The sizes of HGF and GAPDH PCR products were 286 and 598 bases, respectively. HGF mRNA was expressed by alveolar cells from patients with BAC but not from controls. B, extracellular release of HGF protein by alveolar cells from patients \( (n = 7) \) and controls \( (n = 3) \). Extracellular HGF protein was measured in the supernatants of 24-h alveolar cell cultures as described in “Patients and Methods.” Results (pg/ml) are means ± SE.
was five times higher in patients with neutrophil alveolitis (>5%) than in other patients (424 ± 95 pg/ml versus 83 ± 25 pg/ml; P < 0.05).

All of the examined neutrophils in cytospin specimens from the patients tested (n = 8) were HGF positive, including those from patients with low alveolar neutrophil counts (Table 2). As shown in Fig. 3A, the staining was cytoplasmic and strong (2+/3+). The few neutrophils present in cytospin preparations from controls (n = 3) were also HGF positive (Table 2). Alveolar lymphocytes were always negative, but results obtained for AMs and tumor cells were inconsistent (Table 2). Tumor cells and AMs were positive in 3 patients (Table 2). This positivity was less intense than in neutrophils and was inconsistent from cell to cell in a given specimen (Fig. 3, A and B).

We also performed an immunohistochemical study of both tumor tissue and adjacent normal lung tissue (Table 3). The results obtained with five different specimens were similar and confirmed those of BALF cytospin immunocytochemical analysis. They also showed that HGF was not expressed by interstitial mononuclear cells or by resident cells (Fig. 3F). There was no HGF expression in normal lung tissue distant from the tumor (Table 3).

**Biological Activity of Alveolar Neutrophil-derived HGF**

To ensure that the alveolar neutrophil-derived HGF produced in vivo was biologically active, we first tested the ability of BALF from patients and controls to induce the migration of the BAC-derived...
tumor cell line A549 in vitro. As shown in Fig. 4A, BALF from patients (n = 9) and from controls (n = 3) induced the migration of tumor cells, with a scattering pattern, contrary to migration with NaCl (n = 6). This pattern of migration was similar to that observed with recombinant HGF. However, the ability to induce tumor-cell migration was three times higher with BALF from patients than from controls (648 ± 86 versus 193 ± 38 migrating cells; P < 0.002) and 10-fold higher than from NaCl (648 ± 86 versus 69 ± 9 migrating cells; P < 0.001; Fig. 4B). Furthermore, the concentration of HGF measured in BALF from patients correlated with the ability of the same BALF samples to promote the migration of tumor cells in vitro (ρ = 0.765, P < 0.005; Fig. 4C). We then performed HGF neutralization experiments (Figs. 4, A, lower left panel, and D) and found that 40 ± 6% of the migratory effect of patients’ BALF was attributable to the alveolar neutrophil-derived HGF contained in BALF. By contrast, the migratory effect observed with BALF from controls was not inhibited by HGF neutralization, in keeping with the lack of HGF in these samples (see “BALF HGF levels”).

HGF Production by Peripheral Blood Neutrophils from BAC Patients and Controls

Because we have previously demonstrated that several tumor-derived cytokines (IL-8, GM-CSF, G-CSF, and TNF-α) contribute to the onset of neutrophil alveolitis observed in BAC patients in vivo (7, 8), we compared the capacity of these cytokines to induce HGF release by peripheral blood PMN from BAC patients and healthy volunteers. As shown in Fig. 5, HGF levels increased significantly in PMN cell culture supernatants after exposure to GM-CSF and TNF-α, but not after exposure to IL-8 or G-CSF (data not shown). This effect was similar on PMNs from BAC patients (n = 6) and healthy volunteers (n = 6) but less intense than that observed with cytochalasin B and FMLP (used as positive controls of neutrophil degranulation).

Because alveolar PMNs originate from blood, we also compared the capacity of peripheral blood PMNs from patients and healthy donors to synthesize and release HGF in vitro. HGF mRNA was expressed to the same extent by PMNs from patients and controls, as shown by band densitometry of HGF PCR electrophoresis gels (Fig. 6A). In addition, neither secreted nor cell-associated HGF (PMN lysate) measured in 24-h peripheral blood neutrophil cultures differed between patients (n = 6) and controls (n = 6; Fig. 6B). HGF levels released by PMN after FMLP stimulation (Fig. 5) were similar to levels of cell-associated HGF (PMN lysate) measured in 24-h PMN cultures (Fig. 6A). This suggested that most of the released HGF derived from preformed intracellular stocks rather than from de novo synthesis.

Influence of Alveolar HGF on BAC Tumor Progression

BAC Tumor Cells Express HGF Receptor in Vivo. To investigate whether local HGF production impacts on BAC progression in vivo, we performed immunocytochemical studies of its receptor, the proto-oncogene c-Met, in BALF cytotypic preparations and tumor sections. The results obtained with the eight different BALF cytotypic specimens tested were similar and showed that HGF receptor-bearing cells were exclusively tumor cells and hyperplastic atypical alveolar cells surrounding the tumor (Table 2; Fig. 3C); c-Met expression was absent from AMs, neutrophils and lymphocytes (Table 2). Tumor cell expression was strong (2+/3+) and localized to the cell surface, as expected for a membrane receptor.

Results obtained with the five tumor specimens and adjacent nor-

<table>
<thead>
<tr>
<th>Lung tissue</th>
<th>HGF</th>
<th>c-Met</th>
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<tbody>
<tr>
<td>Tumor area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor cells</td>
<td>4+/−</td>
<td>3+</td>
</tr>
<tr>
<td>Hyperplastic atypical alveolar cells</td>
<td>−</td>
<td>2+</td>
</tr>
<tr>
<td>Macrophages</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Polymorphonuclear cells</td>
<td>2+</td>
<td>−</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>−</td>
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<tr>
<td>Smooth muscle cells</td>
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<td>Alveolar, bronchiolar and bronchial cells</td>
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<td>Fibroblasts</td>
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Fig. 4. Biological activity of alveolar neutrophil-derived HGF. A, tumor cell migration pattern observed in the A549 cell random migration assay performed as described in “Patients and Methods” using BALF from a patient (upper left panel) and a control (upper right panel), a patient, after sample treatment with anti-HGF antibodies (lower left panel), and recombinant HGF (5 ng/ml; lower right panel) instead of culture medium. B, effect on tumor cell migration in the A549 random migration assay. of BALF from patients (n = 9; □) and controls (n = 3; □) in comparison with NaCl (n = 3; ◇). C, correlation between the BALF HGF concentration in patients (n = 9) and the capacity of the same BALF samples to promote migration (ρ = 0.765; P < 0.005). D, inhibition by the preincubation by a cocktail of three specific anti-HGF Abs of tumor cell migration obtained with recombinant HGF (10 ng/ml; n = 3; ◇) and BALF from patients (n = 3; □). Results are means ± SE.

Fig. 5. HGF release by patients’ and controls’ blood neutrophils exposed to various degranulating agents. Extracellular HGF release by neutrophils from controls (n = 6, □) and patients (n = 6, □), after exposure for 20 min to (a) cytochalasin B (5 μg/ml) and FMLP (10−7 M; a); (b) TNF-α (10 ng/ml); and (c) GM-CSF (10 ng/ml), as described in “Patients and Methods.” Results (pg/ml) are means ± SE.
mal lung tissue specimens were similar and confirmed those obtained with BALF cytospin specimens (Fig. 3G; Table 3). However, HGF receptor was also expressed by endothelial cells (2+), albeit less intensely than by tumor cells (3+). Intersitial mononuclear cells and AMs did not express c-Met. In normal lung tissue distant from the tumor, HGF receptor was expressed by normal epithelial cells (2+) and endothelial cells (2+; Table 3). Taken together, these data suggested a possible interaction between HGF-derived tumor-infiltrating neutrophils and c-Met-expressing tumor epithelial cells in vivo.

Prognostic Significance of BALF HGF Levels in Univariate and Multivariate Analysis. None of the patients was lost to follow-up. Twenty-seven patients who died by the cutoff date for this analysis. The median survival time after diagnosis was 11.3 months (range, 1–61 months), and the actuarial survival rates at 1, 2, and 5 years were 51, 36, and 10%, respectively. To investigate whether the BALF supernatant HGF level had prognostic significance in patients with BAC, it was included with other prognostic factors in a univariate survival analysis (Table 4). Increased total BAL cell and BAL neutrophil numbers, the presence of tumor cells in BALF, and high BALF supernatant HGF levels were significantly associated with shorter survival (P < 0.05). A trend toward significance was also observed for performance status and pulmonary extension on computed tomography. When the variables were submitted to stepwise forward Cox regression analysis, high BALF supernatant HGF levels were independently associated with shorter survival (Table 5). The risk of death was substantially increased among patients with HGF levels >117 pg/ml as compared with other patients (hazards ratio, 2.9; 95% confidence interval, 1.0–8.3).

DISCUSSION

HGF was detected in BALF supernatants from nearly all of the patients with BAC and was related to local production, because fresh alveolar cells, recovered by BAL and cultured for 24 h, expressed HGF mRNA and released significant amounts of HGF protein. By contrast, HGF was never detected in BALF supernatants from healthy controls, whose alveolar cells did not express HGF mRNA or release HGF protein. Initial studies showed that HGF is a stromal-derived factor affecting the proliferation and motility of normal and tumoral epithelial cells, especially in NSCLC (24). It was also shown that tumor cells were a potential source of HGF in NSCLC (25–28). We show, for the first time, that tumor-infiltrating neutrophils are a potential source of HGF in patients with BAC-subtype ADC. Indeed, HGF levels in BALF supernatants correlated with neutrophil numbers in alveolar spaces but not with counts of other inflammatory cells (lymphocytes and macrophages). Second, immunostaining of BALF cytospin specimens and tumor sections showed strong cytoplasmic HGF positivity of neutrophils in all of the patients tested. Third, purified peripheral blood neutrophils obtained from the same patients also released HGF when stimulated in vitro. Tumor cells and AMs could also contribute to alveolar HGF levels, because they were also HGF immunopositive. However, this positivity was less intense than for neutrophils, was not observed in all of the patients, and was inconsistent from cell to cell in specimens from a given patient. This is in agreement with the report by Yamashita et al. (26) that HGF expression by tumor cells is restricted to a subgroup of patients with BAC. Similarly, faint HGF expression was reported in AMs from patients with inflammatory lung disorders associated with neutrophil alveolitis (29, 30); this might result from AM activation during phagocytosis of apoptotic neutrophils, as recently suggested in an animal model (31). The absence or very weak expression of HGF in resident cells observed in our patients with BAC is in keeping with the results of other HGF-immunostaining studies in lung cancer (25, 26, 28) but conflicts with the strong capacity of these cells to produce

![Fig. 6. HGF production by blood neutrophils from patients and controls. A, HGF mRNA expression in blood PMNs from patients and controls. HGF expression was measured after RNA isolation, reverse transcription, and PCR amplification, as described in “Patients and Methods.” Coamplification of GAPDH cDNA was performed in each experiment to compare the total amounts of RNA in samples. H2O, negative control. M, molecular weight marker. The sizes of the PCR products for HGF and GAPDH were 286 and 598 bases, respectively. B, extracellular HGF release by PMNs from patients with BAC and from controls. B, extracellular HGF release by PMNs from patients (n = 6) and controls (n = 6). Extracellular (B) and PMN-associated HGF concentrations (□) were measured in 24-h PMN cultures as described in “Patients and Methods.” Results (pg/ml) are means ± SE.](https://cancerres.aacrjournals.org)
biologically active HGF in primary culture (24, 32). It is conceivable that the in vivo microenvironment does not permit constitutive HGF release by resident cells because of the presence of regulatory factors that are lacking in vitro.

Neutrophils are not usually present in lung tissue, and HGF production is not a characteristic of tissue-infiltrating neutrophils. Grenier et al. (18) recently reported that HGF synthesis likely occurs during neutrophil maturation in bone marrow and is stored as pro-HGF in secretory vesicles and gelatinase-specific granules of mature neutrophils. Thus, pulmonary malignancies probably generate a local environment that favors neutrophil recruitment from peripheral blood and HGF release on contact with tumor cells. We have previously reported that tumor cells produce the neutrophil-specific chemokine IL-8 (8) and the neutrophil antiapoptotic cytokines G-CSF and GM-CSF (7); these phenomena are probably amplified by pro-inflammatory cytokines such as IL-1β and TNF-α released into the tumor microenvironment (7). In the present study, we found that GM-CSF and TNF-α, but not G-CSF or IL-8, were also able to provoke HGF release from intracellular preformed stocks of alveolar neutrophils in vitro.

HGF mRNA expression and cell-associated and secreted HGF protein levels measured in 24-h cultures of peripheral blood neutrophils did not differ between patients and healthy subjects. Likewise, HGF release by neutrophils after exposure to various degranulating agents was similar in patients and controls. These findings argue against “priming” of blood neutrophils from BAC patients before their transfer to the lung, contrary to the report by Jaffre et al. (29) in patients with acute lung injury, in which a systemic inflammatory response syndrome is well documented (33).

The biological effects of HGF are mediated by its interaction with its high-affinity binding site known as the tyrosine kinase receptor, c-Met proto-oncogene, which is expressed at the surface of target cells (epithelial cells and endothelial cells; Ref. 34). Such an interaction is highly probable in the alveolar spaces of patients with BAC; (a) HGF is synthesized and locally released by tumor-infiltrating neutrophils; (b) HGF is released by blood neutrophils in bioactive form (18); and (c) HGF receptor expression by BAC tumor cells was consistently observed in tissue specimens from our patients (confirming previous studies of NSCLC that included some cases of BAC subtype ADC; Refs. 32, 35, and 36). HGF may exert its biological effects on tumor cells by stimulating their proliferation, inhibiting their apoptotic death, and, especially through its mitogenic and scattering properties, favoring tumor cell migration along the alveolar basal membrane (37, 38). This hypothesis is clearly supported by our results showing that alveolar neutrophil-derived HGF induces significant, concentration-dependent migration of BAC tumor cells in vitro. However, the fact that anti-HGF antibodies did not completely abrogate this tumor cell migration suggests that other soluble motility factors (39–41) are also involved. BAC being a well-differentiated ADC characterized by basal-membrane integrity, HGF might interact preferentially with c-Met-expressing cells present in the alveolar lumen (normal epithelial cells, atypical hyperplastic cells, and tumor cells), rather than with cells of the vascular bed (endothelial cells) and could thereby favor aerogenous spread rather than metastatic propagation. This could explain why high BALF HGF concentrations were associated with poor outcome in our BAC patients.

In conclusion, our results support the notion that BAC-subtype ADC generates a local environment that attracts functionally normal neutrophils from peripheral blood (7, 8), as well as their release of biologically active HGF on contact with tumor cells. This would result in the intrapulmonary spread of HGF-receptor-expressing tumor cells and thereby contribute to the poorer outcome of patients who have larger numbers of tumor-infiltrating neutrophils. These findings also provide a rationale for the effect of HGF antagonists such as HGF/NK4, and that of potent inhibitors of Trk family receptor tyrosine kinases (42–44) in patients with BAC-subtype ADC.

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Hepatocyte Growth Factor Production by Neutrophils Infiltrating Bronchioloalveolar Subtype Pulmonary Adenocarcinoma: Role in Tumor Progression and Death

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