Hepatocyte growth factor is produced by blood and alveolar neutrophils in acute respiratory failure

SANDRINE JAFFRÉ, MONIQUE DEHOUX, CATHERINE PAUGAM, ALAIN GRENIER, SYLVIE CHOLLET-MARTIN, JEAN-BAPTISTE STERN, JEAN MANTZ, MICHEL AUBIER, AND BRUNO CRESTANI
Institut National de la Santé et de la Recherche Médicale Unité 408 and 479, Faculté Xavier Bichat, Département d’Anesthésie-Réanimation, Laboratoire de Biochimie A et Service de Pneumologie, Hôpital Bichat, Assistance Publique-Hôpitaux de Paris, 75877 Paris, France

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Jaffré, Sandrine, Monique Dehoux, Catherine Paugam, Alain Grenier, Sylvie Chollet-Martin, Jean-Baptiste Stern, Jean Mantz, Michel Aubier, and Bruno Crestani. Hepatocyte growth factor is produced by blood and alveolar neutrophils in acute respiratory failure. Am J Physiol Lung Cell Mol Physiol 282: L310–L315, 2002; 10.1152/ajplung.00121.2001.—We tested the novel hypothesis that neutrophils in the lung or the airspaces may produce hepatocyte growth factor (HGF) in ventilated patients with acute respiratory failure. Neutrophils were purified from blood and bronchoalveolar lavage (BAL) fluid samples from 16 mechanically ventilated patients who underwent BAL for a diagnostic workup of ventilator-acquired pneumonia. Most of the patients had pneumonia (n = 11). Ten nonventilated patients served as controls. Both blood and BAL neutrophils released HGF in vitro. Basal HGF secretion by blood neutrophils from controls was 823 (666–948) pg·ml⁻¹·10⁻⁷ neutrophils (median, 25th–75th percentile) and doubled to 1,730 (1,684–2,316) pg·ml⁻¹·10⁻⁷ neutrophils (P = 0.001) with lipopolysaccharide (LPS) stimulation. Basal HGF secretion by blood neutrophils from patients was similar to controls. Basal HGF secretion by blood neutrophils from patients was similar to controls (956 (655–1,209) pg·ml⁻¹·10⁻⁷ neutrophils by blood neutrophils from controls was 823 (666–948) pg·ml⁻¹·10⁻⁷ neutrophils). Basal HGF secretion by blood neutrophils from patients with acute respiratory failure can produce HGF, a mitogenic factor that may enhance the alveolar repair process.

alveolar epithelium; alveolar repair; acute respiratory distress syndrome; acute lung injury; growth factors

POLYMORPHONUCLEAR NEUTROPHILS are an essential component of the early response to infection or injury and accumulate in the alveoli in many pathological conditions. Neutrophils are thought to contribute to alveolar injury through the release of reactive oxygen species, potent proteases, and bioactive lipids. However, recent data suggest that neutrophils could also positively influence the tissue repair after injury (10, 11).

We recently observed that bronchoalveolar lavage (BAL) HGF levels were positively correlated with the total BAL neutrophil count in patients with acute alveolar injury (27). Moreover, preliminary data suggested that neutrophils contain immunoreactive HGF (24). Therefore, we hypothesized that neutrophils could secrete HGF in blood or in the alveolar space and therefore might enhance the alveolar repair process. To test this hypothesis, blood and alveolar neutrophils were purified from critically ill ventilated patients so that we could measure their capacity to secrete HGF ex vivo under basal conditions and after lipopolysaccharide (LPS) stimulation.

PATIENTS AND METHODS

Patients with acute alveolar injury. The study was carried out in an intensive care unit (ICU), where BAL is the standard procedure for the diagnosis of ventilator-acquired pneu-
monia. All consecutive mechanically ventilated patients who underwent BAL for the diagnosis of new pulmonary infiltrates, with fever or purulent aspirates, were included in the study. The exclusion criteria were HIV infection, end-stage cancer, age <18 yr, arterial Po$_2$ (Pao$_2$)/fractional rate of inspired O$_2$ (Fio$_2$) <70 mmHg, current pregnancy, septicemia, or inclusion in another protocol. The local ethical committee of Paris-Bichat University Hospital approved the study protocol and waived the need for informed consent. This committee did not approve the performance of repeated sequential BAL without a diagnostic rationale.

During the inclusion period, we studied 55 BAL and blood samples from 36 patients. Because of the small volume of BAL recovered from many patients, alveolar neutrophils could be purified from only 20 BAL from 16 patients. There were 15 males and 3 females, age 69 yr (median, 25th–75th percentiles). The patients were hospitalized in a surgical ICU because of cardiac surgery (n = 9), digestive surgery (n = 3), thoracic or vertebral surgery (n = 2), or major trauma (n = 2). None of those patients suffered from extrapulmonary infection at the time of the procedure. Four patients underwent a second BAL procedure 5, 7, 11, and 13 days after the first BAL, and the results were included in the study to increase the total number of samples. The physician in charge of the patients assessed the cause of the lung infiltrates (summarized in Table 1) after reviewing the available clinical and biological data and the results of the bacteriological analysis of BAL. Two patients fulfilled the American-European consensus conference criteria for the acute respiratory distress syndrome (ARDS) at the time of the BAL procedure, and one patient fulfilled the criteria for acute lung injury. One BAL only was studied for each of these patients. The BAL was performed 3.5 (2–6) days after the appearance of the infiltrates.

When BAL was performed, the variables necessary to calculate the simplified acute physiological score II (SAPS II; see Ref. 14), the number of organ system failures (OSF; see Ref. 13), and the lung injury score (LIS; see Ref. 20) were prospectively assessed (Table 2). For the calculation of the LIS, we did not include pulmonary compliance as allowed by Murray in the original paper (20) since compliance was not obtained routinely. The outcome (survival or death) 30 days after the BAL was recorded for all patients. All but three BAL were performed while the patients received positive end-expiratory pressure [median = 5 cmH$_2$O (4–6)].

### Controls

Venous blood samples (5 ml) from 10 healthy volunteers were collected on EDTA to measure plasma HGF concentrations and HGF secretion by blood neutrophils.

We collected BAL fluid from 10 nonventilated patients who underwent a fiberoptic bronchoscopy for the preoperative evaluation of an esophageal cancer (n = 4), for the search of lung cancer in patients with chronic bronchitis (n = 3), and for suspected hemoptysis (n = 3). In all cases, fiberoptic bronchoscopy was normal.

### BAL procedure

The BAL was done as previously described (27). All patients were intubated and mechanically ventilated when the BAL was performed. Briefly, six aliquots of 20 ml of sterile saline solution were injected through the bronchoscope, and the first aliquot, representative of a bronchial lavage, was discarded, and the other aliquots were pooled and filtered on sterile gauze. Ten milliliter of the BAL fluid were immediately processed for a bacteriological direct examination and culture. The diagnosis threshold for lung infection was bacterial growth $\geq 10^4$ colony-forming units/ml (4). The remainder of each BAL (at least 10 ml) was kept on ice and handled rapidly in our laboratory.

BAL fluid was centrifuged (10 min at 180 g). The supernatant was frozen at $-20^\circ$C with 5% aprotinin (vol/vol; Trazylol, Bayer Pharma, Sens, France) until HGF and urea assays. The cell pellet was resuspended in PBS (10$^7$ cells/ml) and used for neutrophil purification. A small aliquot was cytotoxic, air-dried, and stained using May-Grunwald-Giemsa stain for a differential cell count.

Immediately before the BAL procedure, 5 ml of venous blood with EDTA were obtained. One milliliter was immediately centrifuged to recover plasma, which was stored at $-20^\circ$C until HGF and urea assays. Four milliliters were used for neutrophil purification.

### Purification and culture of blood and alveolar neutrophils

Alveolar neutrophils could be purified from 20 BAL samples. Blood neutrophils were available from those patients and were studied in parallel. Blood neutrophils from patients and healthy volunteers were isolated by sedimentation in medium containing 9% Dextran T-500 (Pharmacia, Uppsala, Sweden) and 38% Radioselectan (Serchung, Lys-les-Lannoy, France). The leukocyte-rich suspension was then centrifuged on Ficoll-Paque medium (Pharmacia). The cell pellet was washed with PBS, and erythrocytes were removed by hypotonic lysis.

Blood neutrophils from the leukocyte-rich suspension and alveolar neutrophils from the BAL fluid were then further purified by incubation with pan antihuman HLA class II-coated magnetic beads (Dynal, Oslo, Norway), as previously described (10). The final cell population was $>99\%$ neutrophils, and cell viability was $>98\%$ as assessed by the Trypan blue exclusion test. Neutrophils were then resuspended in RPMI 1640 culture medium (Sigma, St. Louis, MO) supplemented with 2 mM glutamine, antibiotics, and 10% heat-inactivated FCS (Biowittacker, Gagny, France).

Neutrophils were cultured at 37°C with 5% CO$_2$ in 24-well tissue culture plates (Costar, Cambridge, MA) for 20 h (10$^7$ neutrophils·ml$^{-1}$·well$^{-1}$) and were stimulated with 1 µg/ml LPS (from Escherichia coli 055:B5; Sigma). At the end of the culture period, neutrophils and their culture supernatants

| Table 1. Etiology of lung infiltrates in 16 ventilated patients (20 BAL from 16 patients) |
|---------------------------------|------------------|
| Etiology                           | n     |
| Ventilator-associated pneumonia       | 7     |
| ARDS with nosocomial pneumonia       | 2     |
| ALI                               | 1     |
| Cardiogenic pulmonary edema with nosocomial pneumonia | 2 |
| Cardiogenic pulmonary edema         | 3     |
| Atelectasis                        | 3     |
| Resolving bacterial pneumonia      | 1     |
| Lung contusion                     | 1     |

ARDS, acute respiratory distress syndrome; ALI, acute lung injury; BAL, bronchoalveolar lavage; n, no. of subjects.

| Table 2. Summary of the severity of illness scores at the time of the BAL procedure |
|---------------------------------|------------------|
|                                | Means (25th–75th Percentiles) |
| Pao$_2$/Fio$_2$             | 223 (187–254) |
| SAPS II                     | 32 (26–36)     |
| LIS                         | 1.33 (1–1.4)   |
| OSF > 2 (number of patients) | 0               |

Values are for 20 BAL procedures from 16 patients. Pao$_2$, arterial Po$_2$; Fio$_2$, fraction of inspired O$_2$; LIS, lung injury score; OSF, organ system failure.
were collected and centrifuged to separate cell-free supernatants and cell pellets. Supernatants were stored at −20°C until the HGF assays were done. In 10 BAL, a sufficient number of alveolar neutrophils was available, making it possible to evaluate the HGF content of neutrophils through the sum of extracellular and intracellular HGF in neutrophils cultured for 15 min in vitro. Blood neutrophils from those patients were processed similarly. The cell pellets were resuspended in 1 ml of PBS, stored frozen, and sonicated just before HGF assay to determine the intracellular content of HGF.

**HGF and urea assay.** HGF was quantified by using a commercial ELISA kit (Quantikine; R&D Systems, Abington, UK) following the manufacturer’s instructions. Both HGF and pro-HGF were measured in this assay. The detection limit was 40 pg/ml.

BAL and plasma urea concentrations were measured on an Hitachi 911 autoanalyzer (Roche Diagnostics) to estimate the amount of epithelial lining fluid (ELF) according to Rennard et al. (23).

**Immunocytochemical detection of HGF on blood and alveolar polymorphonuclear neutrophils.** Blood smears and BAL cytocentrifuge smears from patients were air-dried for 24 h, fixed in 4% formaldehyde in PBS for 20 min, and permeabilized by incubation in a PBS containing 0.1% Triton X-100 at room temperature for 30 min [according to Sorensen et al. (26)]. Smears were first incubated with normal horse serum for 30 min to block nonspecific binding. Binding of primary antibody was performed during a 1-h incubation at room temperature using anti-human-HGF monoclonal antibodies (12.5 μg/ml; MAb294, clone 24612.111; R&D Systems) diluted in PBS containing 0.3% gelatine. The slides were then consecutively incubated with a biotinylated antibody and alkaline phosphatase-labeled streptavidin following the manufacturer’s instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, UK). Staining was completed by incubation with a substrate chromogen solution (Fast red substrate solution; Dako, Carpinteria, CA). After being washed, the slides were counterstained in Mayer’s hematoxylin and mounted and analyzed with light microscopy. Positive staining developed as a red-colored reaction product.

**RESULTS**

**HGF secretion by blood neutrophils.** The ability of blood neutrophils from 10 control subjects and ventilated patients to produce HGF was tested. The results are summarized in Fig. 1. Unstimulated blood neutrophils from control subjects secreted HGF in vitro. Mean HGF concentration in culture supernatants was 823 (666–948) pg·ml⁻¹·10⁻⁷ neutrophils (median and 25th–75th percentile). With LPS stimulation, the HGF concentration doubled to 1,730 (1,684–2,316) pg·ml⁻¹·10⁻⁷ neutrophils (P = 0.001). Similarly, unstimulated blood neutrophils from patients who secreted HGF in vitro totaled 956 (655–2,140) pg·ml⁻¹·10⁻⁷ neutrophils (P = 0.4 vs. control subjects). With LPS stimulation, HGF release by neutrophils also doubled to 2,767 (2,165–3,688) pg·ml⁻¹·10⁻⁷ neutrophils and was higher than HGF release by LPS-stimulated neutrophils from control subjects (P < 0.0001).

**HGF secretion by alveolar neutrophils.** The analysis of BAL cells from the ventilated critically ill patients showed a prominent neutrophilic alveolitis (Table 3). The ability of alveolar neutrophils to secrete HGF could be measured only for ventilated patients, since the BAL fluid in controls contained virtually no neutrophils. Alveolar neutrophils released HGF in vitro [653 (397–1,209) pg·ml⁻¹·10⁻⁷ neutrophils (Fig. 1)]. Interestingly, LPS stimulation did not significantly increase the HGF release from alveolar neutrophils [762 (434–1,305) pg·ml⁻¹·10⁻⁷ neutrophils]. For each

**Table 3. Total and differential BAL fluid cell counts**

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 20)</th>
<th>Controls (n = 10)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Median 25th–75th Percentiles</td>
<td>Median 25th–75th Percentiles</td>
</tr>
<tr>
<td>Cells/ml (×10⁶)</td>
<td>940  495–1,880</td>
<td>120  107–172</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>92   81–97</td>
<td>4   2–7</td>
</tr>
<tr>
<td>×10⁶/ml</td>
<td>893  384–1,452</td>
<td>6.6  5.5–9</td>
</tr>
<tr>
<td>Macrophage</td>
<td>5    2–17</td>
<td>91   88–95</td>
</tr>
<tr>
<td>×10³/ml</td>
<td>41   19–109</td>
<td>99   87–114</td>
</tr>
</tbody>
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Values are medians and 25th–75th percentiles; n, no. of BAL performed.

Smears incubated with a nonspecific mouse immunoglobulin of the same isotype (10 μg/ml; R&D Systems) served as a negative control.

**Statistical analysis.** All results are expressed as medians with 25th–75th percentiles in parentheses. Statistical analysis was done with Sigmaplot (Jandel Scientifics), with statistical significance defined at P = 0.05. Differences between the data obtained from different cultures of a given patient were analyzed with Wilcoxon’s paired nonparametric test. The significance of differences between patients and healthy volunteers was determined with the Mann-Whitney U-test. For correlation between nonnormally distributed variables, we used the Spearman’s rank order test. For statistical analysis, concentrations of HGF below the detection limit were assigned to the value of the detection limit (40 pg/ml).
patient, HGF release by alveolar neutrophils was significantly lower than that from blood neutrophils, either unstimulated ($P = 0.02$) or LPS stimulated ($P < 0.0001$). HGF release by alveolar neutrophils did not correlate with BAL HGF concentrations nor with HGF secretion by blood neutrophils.

In vitro HGF release by alveolar neutrophils [either unstimulated ($P = 0.3$) or LPS stimulated ($P = 0.4$)] was similar whether there was a lung infection or not. HGF release by alveolar neutrophils did not correlate with any of the severity and illness scores that were tested.

**Alveolar neutrophils contain less HGF than blood neutrophils.** Immunocytochemistry showed a positive granular cytoplasmic staining of blood and BAL neutrophils (Fig. 2). Alveolar macrophages stained weakly positive. Blood lymphocytes were negative (Fig. 2A). We hypothesized that low HGF release by alveolar neutrophils and hyporesponsiveness to LPS could be the result of prior degranulation (17). To test this hypothesis, we estimated the HGF contents of neutrophils through the sum of extracellular and intracellular HGF in neutrophils cultured for 15 min in basal conditions. Total HGF was higher in blood neutrophils [3,370 (2,826–5,239) pg·ml$^{-1}$·10$^{-7}$ neutrophils, $n = 10$ samples] than in alveolar neutrophils [1,401 (895–1,969) pg·ml$^{-1}$·10$^{-7}$ neutrophils, $n = 10$ samples, $P = 0.02$].

**Plasma and alveolar HGF concentrations.** HGF was detected in the plasma of both normal volunteers and patients. The plasma HGF level in patients [2,267 (1,568–3,981) pg/ml] was significantly higher than that of controls [464 (410–544) pg/ml; $P < 0.0001$]. HGF was detected in all of the BAL supernatants from patients [1,209 (516–2,605) pg/ml], whereas it was undetectable in all of the BAL from nonventilated controls. BAL HGF concentration was not different in patients with or without lung infection.

To compare lung and plasma HGF levels, we estimated the volume of ELF by using the urea dilution method, according to Rennard et al. (23), and we calculated the HGF concentration in the ELF. HGF concentration in ELF [13,773 (7,033–25,151) pg/ml] was higher than in plasma [2,793 (1,682–4,671) pg/ml, $P < 0.0001$], thus suggesting some intra-alveolar production of HGF.

**Correlation between HGF concentration and biological or clinical parameters.** Plasma HGF and BAL HGF concentration did not correlate with any of the clinical scores (LIS, SAPS II, OSF, PaO$_2$/FiO$_2$). Interestingly, however, HGF concentration in BAL supernatant was positively correlated with the absolute number of neutrophils in BAL fluid ($P = 0.01$, $R = 0.58$, Spearman’s rank correlation test; Fig. 3).

**DISCUSSION**

This study demonstrates for the first time that blood and alveolar neutrophils from ventilated critically ill patients with acute respiratory failure participate in the local and systemic production of HGF, a growth factor involved in the regulation of type 2 pneumocyte proliferation and alveolar repair. Moreover, the alveolar neutrophils obtained from the ventilated patients are hyporesponsive to LPS in terms of HGF release, compared with blood neutrophils.

The results indicate some local production of HGF in the alveolar space, since HGF concentration in the ELF was higher than in plasma. Yamanouchi et al. (32)
found that alveolar HGF concentrations were higher than plasma HGF concentrations in patients with pulmonary fibrosis. These results are in agreement with those of Verghese et al. (29), who also reported that HGF concentrations in the pulmonary edema fluid were seven times higher than plasma HGF concentrations in patients with acute alveolar injury.

Activated neutrophils can damage the lung through several potential mechanisms, including the release of proteases, cytokines, and reactive oxygen species, but recent data also suggest a role for neutrophils in the modulation of inflammation and tissue repair. In particular, we found that blood neutrophils release oncostatin M (10), a potent inducer of several proteases, including α1-antitrypsin (3). Blood neutrophils have been shown to secrete vascular endothelial growth factor, a mediator of vascular repair (9). In the current study, the results support a role for neutrophils in the production of HGF in the alveolus during acute respiratory failure from pneumonia, atelectasis, contusion, or even severe hydrostatic edema (Table 1).

First, we found for the first time that alveolar and blood neutrophils contain immunoreactive HGF and have the capacity to secrete immunoreactive HGF in vitro. Second, BAL neutrophils stained positive with an anti-HGF antibody, as previously shown for neutrophils in the skin (17) and the liver (24). Third, there was a positive correlation between the absolute number of BAL neutrophils and BAL HGF concentration in patients with acute lung injury; this is a consistent result, since we obtained similar results in a different series of patients in a previous study (27). These results suggest that human alveolar macrophages likely contribute to the alveolar HGF burden, since alveolar macrophages were weakly immunostained with an anti-HGF antibody. This conclusion is supported by Sakai et al. (25), who found that human alveolar macrophages contained immunoreactive HGF. Moreover, LPS-stimulated human blood monocytes have been shown to secrete HGF in vitro (8).

We observed that blood neutrophils from ventilated patients and from control subjects spontaneously secrete HGF in vitro and that blood neutrophils from ventilated patients are more reactive to LPS than neutrophils from control subjects, thus suggesting a preactivation of blood neutrophils from these patients. Chollet-Martin et al. (5) previously found that, in the basal state, both whole blood neutrophils and alveolar neutrophils obtained by BAL from ARDS patients were activated, as shown by decreased L-selectin CD62L, increased β2-integrin CD11b expression, and decreased F-actin content. We also observed that HGF secretion by alveolar neutrophils was not increased with LPS stimulation, whereas blood neutrophils could be further stimulated to secrete HGF. Our group has previously described the local LPS hyporesponsiveness of alveolar macrophages during bacterial pneumonia (6). However, in the current study, pulmonary infection is not relevant to alveolar neutrophil hyporesponsiveness since HGF secretion by alveolar neutrophils was not different in patients with or without pneumonia.

Immunohistochemistry results, as well as the evaluation of the sum of intracellular and extracellular HGF, suggest that the hyporesponsiveness of alveolar neutrophils could be because of their previous degranulation in vivo, as suggested originally by Martin et al. (15).

At this time, we do not know if the HGF secreted by neutrophils is biologically active since the assay that we used recognized both pro-HGF (inactive) and HGF, which is the biologically active form of the protein obtained by cleavage at a specific site. However, it should be noted that alveolar HGF has been shown by Verghese et al. (29) to be biologically active in patients with acute alveolar injury. We believe that HGF secretion by neutrophils is biologically significant. Indeed, during acute lung injury and pneumonia, there is a massive influx of neutrophils into the alveoli, and neutrophils will release HGF in the immediate vicinity of epithelial cells, the main cellular target of HGF. There may be local deleterious effects of HGF. Indeed, HGF inhibits the rate-limiting enzyme in de novo phosphatidylycholine synthesis and is capable of significantly inhibiting the synthesis and secretion of the phosphatidylycholines of pulmonary surfactant (30). However, HGF release by neutrophils in the airspaces of the lung may be beneficial in terms of alveolar repair, as has been reported for liver repair (28, 33). In different animal models of lung injury, intratracheal or intravenous HGF administration can decrease the extent of pulmonary lesions and improve the survival of the animals (21, 22, 31), even when given after the insult (31). HGF may exert its protective action through the following different pathways: stimulation of proliferation (16) and migration (12) of type 2 pneumocytes, inhibition of their apoptosis (7), and stimulation of angiogenesis (1).

In conclusion, these results demonstrate that both blood and alveolar neutrophils contribute to the production of HGF in the lung in ventilated critically ill patients with acute respiratory failure from infectious or noninfectious causes. We speculate that, in addition to their potent proinflammatory effects, neutrophils may have a beneficial effect during alveolar repair.

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