**Objective:** Neutrophil elastase (NE) is the only neutral protease that is able to degrade insoluble elastin and other extracellular matrix constituents, and thus, may be involved in tumor invasion and metastasis. Using a highly specific and sensitive enzyme immunoassay (EIA), we recently demonstrated that immunoreactive (ir)-NE is produced by non-small cell lung cancer cell lines. We have measured the ir-NE concentration in non-small cell lung cancer tumor extracts and have evaluated its association with disease stage.

**Methods:** We measured the ir-NE concentration in 144 non-small cell lung cancer tumor extracts using EIA, which permits the rapid measurement of both the free and α₁-protease inhibitor (α₁-PI) complexed form of ir-NE. In 15 clinical T4 (cT4) patients, we also determined the concentration of free ir-NE in tumor extracts using a kit that detects only NE complexed with α₁-PI and subtracting that value from the total NE concentration.

**Results:** ir-NE was detected in tumor extracts from 115 of 144 patients, ranging from 0.21 to 23.35 μg/100 mg protein. When the 144 specimens were grouped according to the clinical stage of disease, the ir-NE concentration (mean±SE) was significantly higher in those with cT4 disease (n=15; 7.90±1.88 μg/100 mg protein) than in those with cT1 (n=29; 1.27±0.27; p<0.001), cT2 (n=64; 1.18±0.17; p<0.001), or cT3 disease (n=26; 1.99±0.38; p<0.003). There was no significant association between the ir-NE concentration and cN-factor or any other clinical features. When the ir-NE concentration in the tumor extracts of the cT4 patients was compared with respect to the tumor invasion sites, the ir-NE level was significantly higher in those with surgical T4 (sT4) disease with aortic invasion (n=4; 17.4±3.10) than in those who were down-staged postoperatively (n=5; 4.9±1.33; p=0.005) or those with sT4 disease with involvement of other sites (n=6; 4.07±1.83; p=0.004). Similar results were observed for the free form of ir-NE.

**Conclusions:** These data suggest that NE may be involved in tumor progression of non-small cell lung cancer. Since the aorta is one of the richest sources of polymeric and insoluble elastin, this enzyme may play an active role in the direct extension of the tumor into the aorta.

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**Abbreviations:** α₁-PI=α₁-protease inhibitor; cT-factor=clinical T-factor; CV=coefficient of variation; ECM=extracellular matrix; EIA=enzyme immunoassay; ir-NE=immunoreactive neutrophil elastase; NE=neutrophil elastase; sT-factor=surgical T-factor

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During the invasion and metastasis formation process, tumor cells confront a variety of natural tissue barriers *in vivo*, such as basement membranes and surrounding tissue stromal matrices composed of elastins, collagens, and proteoglycans. It is thus necessary for tumor cells to elaborate a battery of extracellular matrix (ECM) degradative enzymes to achieve metastatic invasion. Many different types of ECM degradative enzymes have been implicated in the invasive growth and metastasis of cancer cells.1-3
Neutrophil elastase (NE) is the only neutral protease that is able to degrade insoluble elastin.\(^4\) NE can also hydrolyze other ECM proteins, including type IV collagens,\(^5\) fibronectins,\(^6\) and proteoglycans,\(^7\) and has been reported to potentiate the conversion of plasminogen to plasmin by urokinase-type plasminogen activator,\(^8\) an enzyme that has been postulated to play a role in cancer spread.\(^9\) Thus, tumor NE may play a pathologic role in facilitating cancer cell invasion and metastasis either directly by the dissolution of the tumor matrix or indirectly through such a protease cascade.

We recently have reported that NE is produced by human non-small cell lung cancer cell lines,\(^10\) using a recently developed highly specific and sensitive enzyme immunoassay (EIA).\(^11\) In addition, a preliminary study has suggested that the concentration of immunoreactive NE (ir-NE) in tumor extracts may be associated with tumor invasiveness in non-small cell lung cancer.\(^10\) Accordingly, we have now extended this study and determined the ir-NE concentrations in tissue extracts from another group of patients with non-small cell lung cancer, and evaluated the association between clinical stage and the enzyme concentration in this tissue. We have found that the ir-NE concentration was closely associated with the clinically evaluated T stage, especially in those with the direct extension of tumor into the aorta.

**Materials and Methods**

**Subjects**

One hundred forty-four patients with non-small cell lung cancer, 112 men and 32 women aged 40 to 78 years (mean, 59 years), were studied. Ninety-seven had adenocarcinomas, 38 had squamous cell carcinomas, and nine had large cell carcinomas. All patients had their tumors staged preoperatively according to the classification of the International Union Against Cancer (UICC),\(^12\) and they subsequently underwent curative or noncurative surgery to remove the main tumors. Although surgery for unresectable tumors is not a common practice in Japan, we performed it on patient request. This study was approved by our Institutional Ethics Committee, and informed consent was obtained from each patient’s family. The resected tumors were immediately stored at −80°C until use.

**Assay for NE**

A frozen tissue specimen from each subject was quickly thawed at room temperature, minced, and rinsed with ice-cold 3 mmol/L Tris-HCl buffer, at a pH of 7.5, containing 250 mmol/L sucrose. The minced pieces were suspended in 1 mL of an ice-cold solution containing 3 mmol/L Tris-HCl buffer, at a pH of 7.5, and 250 mmol/L sucrose, followed by homogenization in an ice bath, using a glass homogenizer (ANEX 30420; Teraoka Co, Ltd; Osaka, Japan). The homogenate was further homogenized with an ultrasonic homogenizer (USH-20220S; Tokyo Rikakikai Co, Ltd; Tokyo, Japan) for 2 min at 5°C, and centrifuged at 3,000 rpm for 20 min at 5°C. The resulting supernatant was assayed for ir-NE enzyme concentration.

The ir-NE concentration in the tissue extracts was determined using a recently developed EIA kit (Mochida Pharmaceutical Co; Tokyo, Japan). In this assay, the labeled antibody recognizes NE itself, thus allowing the rapid measurement of NE complexed to \(\alpha_1\)-proteinase inhibitor (\(\alpha_1\)-PI) and of any free NE that may be present in the tissue samples. This procedure is superior to conventional kits that detect only NE complexed with \(\alpha_1\)-PI. The detection limit of ir-NE with this new assay is 0.25 µg/L. The intra-assay coefficients of variation (CV) for the high, middle, and low sample levels were 6.2%, 6.4%, and 7.8%, respectively. The interassay CV for the three sample levels were 5.5%, 7.3%, and 7.7%, respectively. The data from the new EIA were highly correlated (correlation coefficients = 0.94) with those obtained using a conventional kit (E. Merck; Darmstadt, Germany) in the presence of an excess amount of \(\alpha_1\)-antitrypsin.\(^11\)

**Statistics**

Data are expressed as the mean ± SE. Kruskal-Wallis tests were used for the analysis of the ir-NE concentration in relation to the clinicopathologic factors. Two-sided values below 0.05 were regarded as statistically significant.

**RESULTS**

**ir-NE in Tumor Extracts**

By using the newly established EIA kit that detects both free and \(\alpha_1\)-PI-bound forms of NE, ir-NE was detected in the extracts from 115 of 144 specimens at concentrations that ranged from 0.21 to 23.35 µg/100 mg protein. The mean ir-NE concentration in patients with clinical T1 (cT1) non-small cell lung cancer was 1.27±0.27 µg/100 mg protein (± SE), 1.18±0.17 in patients with cT2 lung cancer, 1.99±0.38 in patients with cT3 lung cancer, and 7.90±1.88 in patients with cT4 lung cancer (Fig 1). There was a significant difference in the tissue level of ir-NE between those with cT4 vs cT1 disease (p<0.001), cT4 vs cT2 disease (p<0.001), cT4 vs cT3 disease (p<0.003), and cT3 vs cT2 disease (p=0.0276). In contrast, there was no significant difference in the tissue level of ir-NE among the patients with clinical N1 (cN1: 2.14±0.49 µg/100 mg protein), cN2 (1.16±0.31), or cN3 (2.29±0.42) lung cancer (Fig 2). Similarly, when the ir-NE level was compared with respect to sex, age, smoking history, and histologic type, no significant association was found between the ir-NE level and any of these features (data not shown).

**Clinical Characteristics of T4 Patients**

Table 1 shows the clinical characteristics of the patients with cT4 non-small cell lung cancer. These include 15 patients whose tumors were preoperatively staged as T4 based on radiographic studies.
The suspicious invasion sites were the aorta (n=9), heart (n=1), esophagus (n=1), vertebrae (n=2), and malignant effusion (n=2). Table 1 also shows the surgical T (sT)-factor that was reassessed postoperatively. Among the nine patients diagnosed as having cT4 disease based on aortic invasion (Fig 3 shows the CT scan of these cases), four (cases 2, 4, 7, and 9) were sT4, three (cases 1, 3, and 8) were sT3, and two (cases 5 and 6) were sT2. The six cT4 cases with involvement of other sites (heart, esophagus, vertebrae, and effusion) were all sT4.

**Immunoreactive-NE in T4 Tumor Extracts**

As shown in Table 1 and Figure 4, the total ir-NE concentration was higher in those with cT4 disease with aortic invasion (n=9; 10.46±2.63 µg/100 mg protein) compared to those with cT4 disease and involvement of other sites (n=6; 4.07±1.83), although it did not reach statistical significance (p=0.097). However, the ir-NE concentration was significantly higher in those with sT4 disease with aortic invasion (n=4; 17.4±3.10) compared to those who were down-staged postoperatively (cases 1, 3, 5, 6, and 8) (n=5; 4.9±1.33; p=0.005) or with sT4 disease but other sites of involvement (n=6; 4.07±1.83; p=0.004).

In the cT4 patients, to evaluate the ratio of free ir-NE to the total amount of ir-NE in the tissue extracts, we determined the ir-NE concentration in each sample in the presence and absence of an excess amount (100 µg/mL) of α₁-antitrypsin (Sigma; St. Louis) using the conventional kit (Merck) according to the method of Neumann et al. Since this assay kit detects only NE complexed with α₁-PI, the difference between these levels represents the amount of free-form ir-NE. The ratio of the concentration of the free-form of ir-NE to the total concentration of ir-NE in tissue extracts varied over a wide range, between 0% (case 14) and 96.3% (case 2) of the total ir-NE concentration. The tissue extracts of those with sT4 disease with aortic invasion contained significantly higher concentrations of free ir-NE (12.6±2.71 µg/100 mg protein) compared to those who were down-staged postoperatively (1.32±0.35; p=0.002) or with sT4 disease and involvement of other sites (2.33±1.13; p=0.004).

**Figure 1.** The ir-NE concentration in non-small cell lung cancer tumor extracts. One hundred forty-four patients were grouped according to their cT-factor. Columns=mean; bar=SE.
DISCUSSION

An understanding of the processes that are involved in metastasis and its regulation is crucial to the development of new strategies for the treatment and prevention of human cancers. ECM degradative enzymes produced by tumor cells have been implicated in tumor cell invasion into adjacent tissues and subsequent metastasis.\textsuperscript{14,15} Several investigators have reported that elastinolytic enzymes are produced by human and rodent tumor cells.\textsuperscript{16-18} However, these enzymes have not been isolated or characterized. There are three types of mammalian elastases, including pancreatic elastase I, a serine protease se-

![Figure 2. The ir-NE concentration in non-small cell lung cancer tumor extracts. One hundred forty-four patients were grouped according to their cN-factor. Columns=mean; bar=SE.](image)

Table 1—Clinical Characteristics of 15 Patients With cT4 Non-Small Cell Lung Cancer

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, yr</th>
<th>Major Site of cT4(^*)</th>
<th>sT-factor(^1)</th>
<th>ir-NE, (\mu g/100) mg protein</th>
<th>Free-Form/Total, %</th>
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\(^*\)cT4: clinical T-factor was preoperatively evaluated by radiographic studies.

\(^1\)sT-factor: surgical T-factor was reassessed postoperatively.
creted by pancreatic acinar cells; NE, which is found in the granules of human polymorphonuclear leukocytes; and a metalloprotease that is secreted by inflammatory macrophages. Of these elastases, NE exhibits the most proteolytic activity under physiologic conditions.

Very recently, we have demonstrated that ir-NE is produced by non-small cell lung cancer cell lines. NE is the only protease that is able to degrade insoluble elastin, a structural component of elastic tissues such as blood vessels, skin, lung, and breast tissues. NE also can hydrolyze other proteins, including type IV collagens, fibronectins, and proteoglycans. Since the structure of the penetrated tissues consists mainly of these proteins, the production of NE by the tumor could increase its ability to invade into surrounding tissues. Furthermore, NE has been reported to potentiate the conversion of plasminogen to plasmin by urokinase-type plasminogen activator, which is also synthesized by lung cancer cells. Thus, the NE produced by lung cancer cells may play a pathologic role in facilitating cancer cell invasion and metastasis either directly by dissolution of the tumor matrix or indirectly through such a protease cascade. Our data indicate that tissue extracts of tumors that invade the aorta contain the largest amounts of free (active form) NE and may support this hypothesis. Interestingly, the aorta and ligamentum nuchae are the two richest sources of elastin in humans that form a three-dimensional reticulated fibrous network.

The interactions between tumor and normal cells are complex events that occur continuously throughout the entire invasion process. Wide variability in the relative proportions of tumor and host cells has been observed at the zone of tumor invasion. Lung tumors also are heterogeneous with varying tumor cellularity and varying amounts of stroma. It is therefore possible that some of the NE protein detected in this study was extracted from the infiltrating inflammatory cells and that this inflammatory cell involvement correlates with the present results. Normal cells such as neutrophils, fibroblasts, macrophages, and lymphocytes, all of which appear in the tumor invasion zone, may cooperate in the destruction of the host ECM. In fact, inflammatory cell infiltration has been reported to be associated with a poor prognosis in human cancer.
Figures 4. The concentration of total and free ir-NE in the tumor extracts of those with cT4 non-small cell lung cancer. Columns = mean; bar = SE. The differences were statistically significant between a and c (p = 0.005), a and e (p = 0.004), b and d (p = 0.002), and b and f (p = 0.004).

In conclusion, this is the first report (to our knowledge) demonstrating that the concentration of ir-NE is closely associated with the progression of non-small cell lung cancer. The results also suggest that this enzyme may contribute to the direct extension of the tumor into the aorta. Tumor NE, whatever its cellular origin, may play an active role in the invasive growth of non-small cell lung cancer. Recently, specific NE inhibitors have been developed and are under laboratory investigation. Our present study suggests that these inhibitors may have a clinical role in the treatment of non-small cell lung cancer.

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References