Increased collagenase activity in macrophages from bronchial lavage as a diagnostic marker of non-small cell lung cancer

Y Hakoda, Y Ito, A Nagate, K Minemura, K Utsumi, M Aoshima, K Ohyashiki

Background: The roles of matrix metalloproteinases (MMPs) in cancer metastasis have been studied. Macrophages are considered to release MMPs in the tissues of patients with lung cancer.

Methods: Intracellular collagenase activity was measured in CD14+ CD45+ cells from bronchial lavage fluid to establish a new diagnostic tool for lung cancer. Between August 2000 and November 2001 bronchoscopy and bronchial lavage were performed in 45 patients with abnormal shadows on the chest radiograph; 21 had lung cancer and 24 had non-malignant disease.

Results: Collagenase activity in patients with primary lung cancer (5.54 [0.65]) or non-small cell lung cancer (NSCLC) (5.62 [0.71]) was significantly higher than in those with non-malignant disease (3.63 [0.78], p=0.006 and p=0.008, respectively). Only three of 18 patients in the low activity group were diagnosed as having cancer compared with 18 of 27 in the high activity group (p=0.001). This significance was not seen in non-smokers but it was apparent in smokers/ex-smokers. Excluding non-smokers improved the specificity of collagenase activity in differentiating cancer and non-malignant disease from 62.5% to 80.0%. The sensitivity of the test was 85.7% in all patients and 88.2% in smokers/ex-smokers.

Conclusions: Measurement of intracellular collagenase activity in macrophages in bronchial lavage fluid is a useful diagnostic tool for distinguishing between cancer and non-malignant diseases, especially in smokers and ex-smokers.
The fibreoptic bronchoscope was wedged at the point of division of the bronchus that showed abnormal shadows on the CT scan. 50 ml of saline was flushed into the bronchus and the cells in the lavage fluid were collected by bronchoscopy. In six patients with lung cancer, bronchial lavage was also performed on the healthy bronchus on the mirror side of that affected by cancer.

In our analysis of collagenase activity in patients according to their smoking status, smokers were defined as those with a current habitual experience of smoking or experience within the previous 6 months. Patients with past habitual experience of smoking more than 6 months previously were defined as ex-smokers, and those with no experience of habitual smoking were defined as non-smokers.

**Agents**

Phycoerythrin cyanin 5 (PC5) conjugated CD45 monoclonal antibody and RD1 conjugated CD14 monoclonal antibody were purchased from Beckman Coulter. GFGA-Collagenase (CellProbe) was also obtained from Beckman Coulter.

**Macrophage intracellular enzyme activity**

Measurement of intracellular enzyme activity was performed with a CellProbe. It included enzyme substrates labelled with fluorescent dyes. Fluorescence does not occur until the dyes are released from the substrates by these enzymes. The fluorescence intensity is related to the amount of products converted from the substrates.

Cell suspensions of about $1 \times 10^6 / ml$ were prepared from bronchial fluid. Each sample of 100 ml was mixed with 5 µl of anti-CD14 monoclonal antibody. After the samples had been kept for 10–15 minutes at room temperature, they were washed twice with phosphate buffered saline (PBS). Cells were kept for 10–15 minutes at room temperature again after addition of 5 µl anti-CD45 monoclonal antibody. The cell suspension was adjusted to $3 \times 10^6 / ml$ after the cells had been washed twice with PBS. A 50 µl sample was incubated for 7 minutes at 37°C; 25 µl of CellProbe was added before incubation for an additional 10 minutes at 37°C. The reaction was stopped on ice for 5 minutes and samples were treated with Q-Prep (Beckman Coulter) to lyse the erythrocytes. The emitted fluorescence was measured with an EPICS XL flow cytometer (Beckman Coulter). Samples were analysed by three-colour patterns in the flow cytometer. CD14 positive and CD45 strongly positive cells were considered to be macrophages. The settings of the flow cytometer were adjusted by Flow-Check (Beckman Coulter) and the fluorescence intensity was standardised for each assay with Flow-Set (Beckman Coulter) to maintain reproducibility from day to day. Enzyme activity was quantitated as mean fluorescence intensity. Values were expressed as the ratio of fluorescence intensity between samples with and without CellProbe, as previously described.

**Statistical analysis**

Data were expressed as mean (SE) values. Wilcoxon’s rank sum test and Fisher’s exact probability test were used, and a $p$ value of 0.05 or less was considered significant.

**RESULTS**

The collagenase activity inside macrophages from the bronchial lavage fluid of patients with lung cancer or non-malignant respiratory disease was measured, and was significantly higher in patients with primary lung cancer than in those with non-malignant disease (5.54 (0.65) vs 3.63 (0.78); $p=0.006$, fig 1A). Patients with NSCLC had significantly higher levels of collagenase activity than those from the mirror sides in the same patients with lung cancer, including metastatic cancer (n=6); **p=0.035**.

In patients with NSCLC we failed to show any significant differences in collagenase activity with clinical stage according to TNM criteria. Although there were no significant differences among clinical stages, collagenase activity was relatively high in earlier stage cancers (7.02 (2.27) in stage I, 6.26 (1.07) in stage III, 3.97 (0.76) in stage IV; fig 2A). Among three patients in stage I disease, collagenase activities in the two patients with stage IA were both relatively higher (9.95 and 8.54) than that of a patient in stage IB (2.56). No significant difference was observed in collagenase activity between patients with adenocarcinoma and squamous cell carcinoma (4.80 (0.74) vs 6.74 (1.60); $p=0.221$, fig 2B).

We then analysed the correlations between collagenase activity and other factors such as smoking status (fig 3A), age (fig 3B), sex (fig 3C), and C reactive protein (CRP) positivity (fig 3D). These values were calculated in patients with non-malignant disease (n=26) and in samples from the healthy mirror side bronchi of cancer patients (n=6). There were no significant differences between the groups in relation to either age or sex (figs 3B and 3C). Subjects with positive CRP activity (n=13) did not have significantly different levels...
of collagenase activity from those with negative CRP (n=15) (3.73 (1.28) vs 2.40 (0.38), p=0.695, fig 3D). There were no differences between patients under 60 years of age (n=12) and those aged 60 years or more (n=16) (2.21 (0.42) vs 3.51 (1.03), p=0.210, fig 3B). Female patients (n=9) had a mean collagenase activity of 4.50 (1.76) compared with 2.44 (0.37) in male subjects (n=20; p=0.322, fig 3C). Although we failed to demonstrate significant differences, the collagenase activity value of 3.59 (1.28) in non-smokers (n=13) was higher than the value of 2.87 (0.58) in smokers (n=11; p=0.582, fig 3A). The value of 2.20 (0.33) in ex-smokers (n=5) was lower (but not significantly so) than the value in non-smokers (p=0.313, fig 3A). The standard error was larger in non-smokers than in smokers or ex-smokers.

Among non-smokers, the mean collagenase activity value of 6.06 (1.42) in patients with NSCLC (n=5) was not significantly different from the value of 4.35 (1.25) in patients with non-malignant disease (n=14; p=0.139, fig 4A). In contrast, there was a significant difference between the value of 5.45 (0.86) in patients with NSCLC (n=13) and the value of 2.64 (0.59) in patients with non-malignant disease (n=10) when smokers and ex-smokers were analysed together (p=0.016, fig 4B).

We also analysed these data using the Fisher’s exact probability test. Patients were divided according to their collagenase activity values into two groups: a “high” group with values of ≥3.0 and a “low” group with values of <3.0. Only three of 18 patients in the low group were diagnosed with cancer, whereas 18 of 27 patients in the high group had cancer (p=0.001, table 1). When only patients with NSCLC were analysed, the results were also significant with 15 of 24 NSCLC patients in the high group but only three of 18 NSCLC patients in the low group (p=0.003). These trends were also analysed in non-smokers and smokers/ex-smokers. Among non-smokers, three cancer patients out of 10 were noted in the high group and one of eight in the low group (not significant: p=0.3824, table 1). In contrast, among the group of smokers/ex-smokers 15 of 17 in the high group and two of 10 in the low group were diagnosed as having cancer (p=0.0007, table 1).

We also analysed the sensitivities and specificities of collagenase activity detection in distinguishing cancer from non-malignant disease.

Table 2 Sensitivities and specificities of collagenase activity detection in distinguishing cancer from non-malignant disease

<table>
<thead>
<tr>
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<th>Sensitivity</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>All patients</td>
<td>85.7%</td>
<td>62.5%</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>75.0%</td>
<td>50.0%</td>
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<tr>
<td>Smokers/ex-smokers</td>
<td>88.2%</td>
<td>80.0%</td>
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DISCUSSION

Lung cancer is difficult to detect in the early stages and many cases are inoperable at diagnosis. Coin lesions, infiltrative shadows, and consolidation are commonly seen clinically on radiographs. A diagnosis of cancer requires pathological evidence, but an appropriate pathological diagnosis cannot always be obtained. We attempted to establish a new diagnostic method that could be used to verify the malignancy or
non-malignancy of an abnormal shadow in the lung field by examination of bronchial lavage fluid. We also attempted to measure collagenase activity inside macrophages.

CD45 is weakly expressed on granulocytes. CD14+ and CD45++ have various roles.

Collagenase activity in macrophages in bronchial lavage fluids from patients with primary lung cancer was significantly higher than in those from patients without malignant disease (fig 1A). This result was strengthened by the significantly higher collagenase activity in bronchial lavage fluids from cancerous bronchi than in those from the presumably healthy mirror sides (fig 1B). These results suggest that macrophages around cancerous tissue are somehow activated to have increased potential to release collagenase. Although our result was not significant, the macrophages of patients in advanced clinical stages of cancer had lower intracellular collagenase activity than those of patients in less advanced stages (fig 2A). From these results, the hypothesis arises that collagenase is consumed as the stage of cancer progresses. This hypothesis is supported by studies that have used immunohistochemical and reverse transcriptase-polymerase chain reaction techniques to detect enhanced expression of MMP-2 and MMP-9 in lung cancer tissues.11–13 Serum levels of MMP-2 and MMP-9 are also increased in patients with NSCLC.11–12 Further, increased serum levels of MMP-2 are associated with metastasis and therapeutic response of lung cancer.11 In our study the relatively high activity of macrophage collagenase in the earlier stages increases the possibility that our technique may be of diagnostic use in the earlier stages of lung cancer.

We investigated factors other than cancer that may affect collagenase activity (fig 3). In the literature, high levels of plasma MMP-9 have been described in cigarette smokers.10 Production of collagenase by alveolar macrophages from patients with emphysema has also been reported as higher than in those from control subjects.11 We demonstrated that cigarette smoking might influence collagenase activity, although the result was not significant. Both current smokers and ex-smokers had relatively low intracellular collagenase activity compared with non-smokers (fig 3A). The reason for this is unclear. A plausible explanation is that consumption of collagenase after its release may cause the intracellular activity to be low. Since collagenase activity was low in smokers and ex-smokers (fig 3A), the power to distinguish patients with cancer would be enhanced in these groups. The specificity of collagenase activity values in differentiating malignancies in smokers and ex-smokers was 80% compared with 62.5% in all patients (table 2). The sensitivity was more than 80% in all patients and in smokers and ex-smokers (table 2). In contrast, the higher collagenase activity values and larger deviations in these values in non-smokers (fig 3A) make it more difficult to distinguish cancer and non-malignant disease in this group, as indicated by the significance values (fig 4A) and the marked reduction in sensitivity and specificity (table 2). These findings suggest that measurement of intracellular collagenase activity in macrophages in bronchial lavage fluid is a useful supportive diagnostic tool for distinguishing cancer and benign diseases, especially in smokers and ex-smokers. Since we have only two patients with small cell lung cancer, further investigation will be necessary for this type.

The method we usually use to distinguish between malignant and non-malignant diseases is cytolysis. Cytology generally has quite high specificity but the sensitivity is not always high. Among the patients we investigated, higher specificity and lower sensitivity were noted when we used cytology in bronchial lavage fluid (data not shown). The cases in which our method would be useful would be those in which there is a high index of suspicion of cancer but a negative cytological study. If intracellular collagenase activity was high in such cases, we would recommend a repeat bronchoscopic study. It is also important to point out that the results of intracellular collagenase activity measurements can be obtained a couple of hours after bronchoscopy, thus providing a clinical advantage.

Much attention has been focused on MMPs as contributing factors to metastasis or progression of malignant disease. The presence of an increased level of collagenase activity increases the possibility that lung cancer will be diagnosed. This report provides evidence that the measurement of collagenase activity in macrophages from patients with abnormal findings on chest radiography may have a clinical role and may be a useful diagnostic marker.

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Authors’ affiliations
Y Hakoda, Y Ito, A Nagata, K Minemura, K Utsumi, M Aoshima, K Ohyashiki, First Department of Internal Medicine, Tokyo Medical University, Tokyo, Japan

REFERENCES
Hyperbaric oxygen decreases the incidence of neurological sequelae in acute carbon monoxide poisoning


This double blind, randomised trial compared treatment of acute carbon monoxide poisoning with three sessions of either hyperbaric oxygen (100% oxygen at 3 atmospheres during the first session and then 2 atmospheres absolute in the subsequent two sessions) or normobaric oxygen (air at 1 atmosphere absolute during all three sessions). The incidence of neurological sequelae at 6 weeks was used as the primary outcome and assessment included a neurological examination, neuropsychological tests, and a questionnaire based on symptoms. Cognitive sequelae occurred in 25% of patients in the hyperbaric oxygen group compared with 46% in the normobaric oxygen group (unadjusted odds ratio 0.39; p=0.007). There was also a reduction in cognitive sequelae at 6 and 12 months in the hyperbaric oxygen group.

The study shows that hyperbaric oxygen therapy reduces the risk of neurological sequelae at 6 weeks and 12 months after acute carbon monoxide poisoning.

S Shah
sapna.shah1@bartsandthelondon.nhs.uk
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