Sensitive and Specific New Enzyme-Linked Immunosorbent Assay for N-ERC/Mesothelin Increases its Potential as a Useful Serum Tumor Marker for Mesothelioma

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Abstract

Background: Because mesothelioma initially progresses on the surface of the pleura and peritoneum without forming masses, it has been difficult to diagnose at an early stage. It would be very useful to identify a tumor marker that could be used for screening to enable more diagnoses to be made at an early, treatable stage.

Materials and Methods: We had previously identified N-ERC/mesothelin as a potential biomarker for mesothelioma. In the current work, we used a newly developed ELISA system to gain data on N-ERC/mesothelin levels in various clinical settings. A total of 102 healthy volunteers were recruited. In addition, 39 patients were diagnosed with mesothelioma, 53 patients were diagnosed with diseases that should be distinguished from mesothelioma, and 201 subjects were diagnosed with asbestos-related nonmalignant diseases (including simple exposure to asbestos) who were treated at any of the cooperating hospitals were enrolled.

Results: Serum N-ERC/mesothelin levels measured by a new ELISA system showed that the median values from patients with mesothelioma were extremely high compared with levels obtained from other patients. Analysis in terms of histologic type showed that serum levels of N-ERC/mesothelin were elevated in epithelioid type mesothelioma, especially. In four important models of clinical settings, the sensitivity and specificity of N-ERC/mesothelin were about 71% to 90% and 88% to 93%, respectively.

Conclusion: N-ERC/mesothelin is a very promising tumor marker for mesothelioma, especially epithelioid mesothelioma.

Mesothelioma initially progresses along the surfaces of the pleura and peritoneum without forming masses; it is anatomically difficult to diagnose at an early stage and to completely remove with surgery. Moreover, mesothelioma typically has a long incubation period before it becomes clinically evident among high-risk individuals with severe exposure to asbestos. Sugarbaker et al. (1) has reported a groundbreaking result: for patients with early stage disease, 5-year survival after multimodality therapy exceeded 40%. This finding that early disease may be effectively treated emphasizes the importance of identifying a tumor marker that is practical for screening and can allow physicians to make an early diagnosis.

Recently, osteopontin, soluble mesothelin-related protein, and serum mesothelin have been reported as candidates for a mesothelioma tumor marker (2–7). We have postulated (8) that another product may be useful as a tumor marker: N-ERC/mesothelin, a NH2 terminal 31-kDa fragment of mesothelin gene products that was first cloned as a megakaryocyte-potentiating factor in humans and that is physiologically secreted into blood. Since the time of that report, we have established a new ELISA system that detects the NH2 terminal fragments of ERC/mesothelin products at a higher sensitivity and specificity. The current work was done to obtain data for
N-ERC/mesothelin with use of the new ELISA system in various clinical settings and to compare its data with that of the previous ELISA system.

Materials and Methods

Preparation of novel anti-ERC/mesothelin antibodies. The anti-N-ERC/mesothelin monoclonal antibody (MoAb) clone 7E7 has been previously reported (8). We established a novel MoAb clone in the same way. Briefly, N-ERC/mesothelin, expressed in Escherichia coli as glutathione S-transferase–tagged and histidine-tagged fusion proteins, was purified and used as an immunogen. Splenocytes from immunized mice were fused with myeloma cell line X63-Ag8.653. Supernatants of the hybridoma cells were screened for reactivity to immunogen using ELISA, and several positive clones were selected by limiting dilution method. One novel clone, 16K16, was chosen for use with the new ELISA system in this study.

Cell culture, protein expression, and Western blot analysis. CHO-K1 cells were cultured in DMEM supplemented with 10% FCS. Full-length cDNA of the ERC/mesothelin coding region was inserted into the pcDNA3.1(+) vector (Invitrogen) to enable expression in CHO-K1 cells. Transfection was done using FuGENE6 transfection reagent (Roche-Diagnostics). A stable transfectant of CHO-K1 cells that expressed ERC/mesothelin protein was screened by G418 resistance and established as the cell line for further study.

Culture supernatants and ERC/mesothelin/CHO-K1 transfectant cells were harvested. CHO-K1 cells containing expressed ERC/mesothelin protein were lysed in a solution containing 2% SDS, 10% glycerol, 50 mmol/L Tris-HCl (pH 6.8), and 100 mmol/L DTT, and then boiled. The cell lysates and culture supernatants were electrophoresed on 12.5% Laemmli gels and transferred to polyvinylidene fluoride membranes. Two identical membranes were blocked in 1% skim milk in PBS with 0.1% Tween 20 (PBS-T) for 1 h at room temperature. Next, one of the two membranes was incubated with 1 μg/mL 7E7 MoAb; the other was incubated with 16K16 MoAb in 1% skim milk with PBS-T for an additional 1 h at room temperature. Secondary antibodies in the form of rabbit anti-mouse immunoglobulin conjugated to peroxidase (IBL) were then added and allowed to react with the membranes for 1 h longer at room temperature. ERC/mesothelin on the membranes was visualized by the enhanced chemiluminescence detection system (Amersham Biosciences).

Epitope mapping of MoAbs against N-ERC/mesothelin. The epitopes of MoAb-7E7 and MoAb-16K16 were searched against a series of deletion mutants of recombinant N-ERC/mesothelin/CHO-K1 transfectant cells. The cDNA of N-ERC/mesothelin was inserted into pEF1 vector (CellFree Sciences) as a glutathione S-transferase–tagged protein. A series of cDNA coding deletion mutants of recombinant N-ERC/mesothelin protein was amplified by PCR reactions with a series of antisense primers, each of which was displaced by 18 nucleotides from the COOH terminal region of N-ERC/mesothelin, then transcribed and translated in an in vitro protein expression system (ENDEXT Wheat Germ Expression Premium Kit, CellFree Sciences), according to the manufacturer's protocol. A series of recombinant proteins of deletion mutant N-ERC/mesothelin was applied to Dot blotting analysis with MoAb-7E7 and MoAb-16K16 to determine epitopes of each MoAb, as described in the paragraph of Western blotting.

Novel sandwich ELISA using MoAb-16K16. A novel sandwich ELISA system using clone 16K16 was established in the same manner as described previously (8). Microtiter plates (96 wells) were coated with 100 μL/well 100 mmol/L carbonate buffer (pH 9.5) containing purified 7E7 MoAb and allowed to adhere overnight at 4°C. Plates were washed with PBS-T and blocked for 1 h at room temperature with 200 μL/well 1% (w/v) bovine serum albumin in PBS containing 0.05% NaN3. After three washes with PBS-T, 100-μL aliquots of test samples or recombinant N-ERC/mesothelin as a standard, serially diluted in 1% bovine serum albumin in PBS-T, were added in duplicate to wells and incubated at 37°C for 1 h. After seven washes with PBS-T, 100 μL horseradish peroxidase–conjugated MoAb-16K16 mouse IgG was added to each well and incubated for 30 min at 4°C. Wells were washed nine times with PBS-T, then 100 μL freshly prepared tetramethyl benzidine solution was added to each well as a substrate and incubated in the dark for 30 min at room temperature. The reaction was terminated by addition of 100 μL of 1 N H2SO4. Absorbance of the solution at 450 nm was measured in an ELISA reader (E-Max, Molecular Devices Co.). Recombinant N-ERC/mesothelin used as the standard protein in the ELISA system was purified from culture supernatants of
CHO-K1 cells transfected with ERC/mesothelin cDNA using a formylcellulofine affinity column coupled with anti-ERC/mesothelin PoAb-282. The concentration of affinity-purified N-ERC/mesothelin was determined by protein assay using Bradford methods (Bio-Rad).

282. The concentration of affinity-purified N-ERC/mesothelin was determined by protein assay using Bradford methods (Bio-Rad).

Estimation of tumor marker effectiveness in clinical settings. Study design for the evaluation of N-ERC/mesothelin as a tumor marker of mesothelioma was approved by the Institutional Review Board of Juntendo University School of Medicine, National Organization Tokyo Hospital, Hyogo Prefectural Tsukaguchi Hospital, Japan Antitubercolosis Association Fukujuji Hospital, Yokusuka Kyoai Hospital, St. Marianna University School of Medicine, Tohoku University School of Medicine, Hirano Kameido Himavari Clinic, and Immunobiological Laboratories. Patients and healthy volunteers gave signed informed consent before enrollment.

To study the diagnostic value of N-ERC/mesothelin, 102 healthy volunteers were recruited, coming close to the goal of 10 men and 10 women in each 10-year age category from 30 to 70 years (e.g., 30-39, 40-49, 50-59, 60-70 years). Patients treated at any of the above-mentioned hospitals from August 2005 through October 2006 who had mesothelioma, asbestos-related disease, or significant pleural effusion or chest wall mass were prospectively enrolled. This study included both consecutive patients attending the above hospitals who were clinically suspected of the target condition because of presenting symptoms or referred by another health care professional because of diagnostic suspicions and a small number of patients who had already been diagnosed with the target condition. Blood sampling to determine the diagnostic value of N-ERC/mesothelin as a tumor marker was conducted in daily clinical practice, before and independent of final diagnosis. Furthermore, sample tubes were sent to Juntendo University School of Medicine; measurement of serum N-ERC/mesothelin was done there by one specialist in a blinded fashion. Tissue sections were obtained from archival paraffin-embedded tumor blocks from thoracoscopic biopsies or surgical resection and sent to Juntendo University School of Medicine before enrollment.

Mesothelioma was diagnosed by immunohistochemistry using antibodies against the following molecules: calretinin, Wilm’s tumor 1, mesothelin, cytokeratin5/6, D2-40, vimentin, AE1/AE3, epithelial membrane antigen, carcinoembryonic antigen, Ber-EP4, and thyroid transcription factor-1. Other diseases were diagnosed comprehensively, including both pathologic and clinical information.

Statistical analysis. We analyzed ELISA data using JMP and SAS version 8.1.3 (SAS institute) and GraphPad Prism 4.0 (GraphPad Software). To compare serum levels between groups, the Mann-Whitney test was used. To analyze the trend of increasing serum level with increasing age, we used the linear trend test using a general linear model with linear contrast. P < 0.05 was considered statistically significant. Area under the curve of the receiving operating characteristics curve was calculated by the trapezoidal method. To examine the cutoff values of serum levels, we first calculated the total value of the specificity and sensitivity for each cutoff value and then chose the best cutoff values (e.g., the values that maximized total value).

Results

Characterization of anti-ERC/mesothelin antibodies. The novel MoAb-16K16 detected N-ERC/mesothelin in culture supernatants and N-ERC and full-length ERC/mesothelin in the cell lysates of CHO-K1 cells transfected with full-length cDNA of ERC/mesothelin (Fig. 1A).

Epitope mapping of MoAbs against N-ERC/mesothelin. MoAb-7E7 was able to detect the deletion mutant N-ERC/mesothelin protein that consisted of 139 amino acids, but not the mutant with 133 amino acids, suggesting that the epitope of MoAb-7E7 was in the region of 134 to 139 amino acids of N-ERC/mesothelin (data not shown). Similarly, we found that MoAb-16K16 recognized the sequence from 68 to 73 amino acids of N-ERC/mesothelin protein (Fig. 1B).

Establishment of ELISA system using novel MoAb-16K16 for N-ERC/mesothelin. To detect N-ERC/mesothelin in clinical samples, we developed ELISA combinations using MoAb-7E7 and PoAb-282, as described previously (8). The new ELISA system using MoAb-7E7 and the novel MoAb-16K16 for N-ERC/mesothelin was established and evaluated by measuring N-ERC/mesothelin against the standard ELISA system (MoAb-7E7 and PoAb-282). The new ELISA system was designated as N-ERC/mesothelin ELISA system (7-16) to distinguish it from the previous ELISA system. The standard dose-response curve of ELISA system (7-16) exhibited a linear shape when plotted on a log/log scale over a range from 0.081 to 5.2 ng/mL or 2.62 to 168 pmol/L calculated with N-ERC/mesothelin as a standard protein expressed in CHO-K1 cells transfected with ERC/mesothelin cDNA (Fig. 1C).

Serum levels of N-ERC/mesothelin in various clinical populations. We recruited a total of 293 patients. Of them, 39 had confirmed mesothelioma, 98 had pleural plaque, 83 had

| Table 1. Characteristics of patients and volunteers |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Mesothelioma (n=39) | Healthy volunteers (n=102) | ARD/E (n=201) | Lung cancer (±pleuritis; n=45) | Others (n=8) |
| Age            | 65±18 (47-81)     | 52±13 (30-79)     | 62±11 (29-80) | 65±10 (35-83)     | 62±13 (33-62) |
| Sex            | Male 29           | Female 10         | Male 171      | Female 30         | Male 6       |
|                | 52               | 50              | 30            | 15               | 2           |
| Smoker (%)     | 68               | 46              | 73            | 67               | 100         |
| Histology (n)  | Epithelioid 21    | Biphasic 9       | Sarcomatous 9 | Stages I 7       | Others 11    |
|                |                  |                 |               | II 5             |             |
|                |                  |                 |               | III 7            |             |
|                |                  |                 |               | IV (recurrence) 20|             |

Abbreviations: ARD/E, asbestos-related disease (pleural plaque, benign asbestos pleuritis, asbestosis) and exposure.
exposure to asbestos, 14 had benign asbestos-related pleuritis, 6 had asbestosis, 45 had lung cancer (including eight patients who had carcinomatous pleuritis), and 8 patients whose disease could be distinguished from mesothelioma by chest computed tomography, with diagnoses including tubercular pleuritis, metastatic malignant melanoma, empyema, cardiac failure, and postmediastinal tumors. In addition, there were 102 healthy volunteers. Characteristics of both groups are reported in Table 1.

When serum N-ERC/mesothelin levels were measured with ELISA system (7-16), there were no significant differences among healthy volunteers based on sex or smoking status ($P = 0.96$ and $P = 0.87$, respectively; Fig. 2A and B). However, serum level did increase with increasing age ($P = 0.013$; Fig. 2C).

Scatter plots of serum N-ERC/mesothelin level measured by ELISA system (7-16) showed that the median values from patients with mesothelioma were extremely high compared with levels obtained from other patients (Fig. 3A). Furthermore, analysis in terms of histologic type showed that serum levels of N-ERC/mesothelin were significantly elevated in epithelioid-type mesothelioma than other types ($P = 0.039$; Fig. 3B). Moreover, the scatter plots of the N-ERC/mesothelin in epithelioid

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**Fig. 2.** Scatter plots of values for serum N-ERC/mesothelin (7-16) in healthy volunteers analyzing for possible effects of sex (A), smoking status (B), and age (C).

**Fig. 3.** A, scatter plots of values for serum N-ERC/mesothelin (7-16). MM, mesothelioma; As, asbestosis; PP, pleural plaque; BAP/DPT, benign asbestos pleuritis/diffuse pleurithickening; LC, lung cancer; Ex, exposure. B, scatter plots of serum N-ERC/mesothelin (7-16) by histologic type. C, scatter plots of serum N-ERC/mesothelin (7-16) by stage in epithelioid type.
type showed the tendencies for the N-ERC/mesothelin value to increase as stage went up (Fig. 3C).

Receiving operating characteristics analysis, comparing ELISA system (7-16) with the previous ELISA system, was done in four models of clinical settings (Fig. 4A-D). The first setting (A) was the context of screening of at-risk individuals (mesothelioma versus other patient groups; excluding volunteers from the above groups). The second setting (B) tested differentiation (mesothelioma versus patient groups excluding asbestos exposure, asbestosis, and volunteers). The same comparisons were done with epithelioid mesothelioma instead of all mesothelioma [screening (C) and differentiation (D)]. Unfortunately, the only type which we can get the benefits from treatments is epithelioid mesothelioma, at present. So, to know the data focused on epithelioid mesothelioma is very important for its effective treatment strategy. In all clinical settings, the area under the curve values of the new ELISA system (7-16) were higher than those of the previous one. Furthermore, we compared not only the area under the curve but also statistical best sensitivities and specificities. In all settings, the new ELISA system (7-16) was superior to the previous one.

We were able to gather only a few samples to assess the value of N-ERC/mesothelin for monitoring patients with mesothelioma; there seemed to be a pattern that serum levels of N-ERC/mesothelin varied sensitively with tumor volume (Fig. 5).

Discussion

Sugarbaker and colleagues have shown that patients with early-stage mesothelioma have the potential for prolonged survival if treated with multimodality therapies (1, 9, 10). Increased use of thoracoscopy has permitted earlier diagnosis for more patients with mesothelioma. However, only a very small percentage of patients with mesothelioma can currently benefit from aggressive, potentially curative intervention because the vast majority has locally advanced disease, advanced age, or significant comorbid illnesses at time of diagnosis. Furthermore, extrapleural pneumonectomy, which is the center of trimodality therapy, is a high-risk procedure (11). Therefore,
Fig. 5. Monitoring of N-ERC/mesothelin in some patients with confirmed mesothelioma.

efforts toward diagnosing mesothelioma at early stages (stages IA and IB for IMIG staging) are important to give more patients the option to be treated with not only less invasive therapies, but also locally effective therapeutic options; the combination of which may result in improved prognosis.

Several potential tumor markers for mesothelioma have previously been described, including Cyfra, tissue polypeptide antigen, carcinoembryonic antigen, CA-125, and hyaluronic acid (12–20), but none currently provides satisfactory reliability. These studies included small numbers of patients and insufficient analysis about diagnostic values for the potential serum tumor markers. Recently, osteopontin, soluble mesothelin-related protein, and serum mesothelin have been reported to have potential utility for early diagnosis and monitoring of mesothelioma (3–7). Among these markers, soluble mesothelin-related protein and serum mesothelin are particularly interesting because, like N-ERC/mesothelin, they are mesothelium-specific.

On the other hand, we identified the ERC gene, the homologue of the human mesothelin gene, in the course of the research on carcinogenesis in Eker rats in 1995 (21–26). Very recently, we reported initial data on serum N-ERC/mesothelin measured by a previous ELISA system (8, 27). Now, we have developed a more sensitive and stable ELISA system (7-16) for use in measuring serum N-ERC/mesothelin levels. The data about N-ERC/mesothelin measured with the ELISA system (7-16) that we presented in this paper are exciting compared with previous reports. In contrast with COOH terminal fragments, such as soluble mesothelin-related protein and serum mesothelin, most of which remain on the cell surface, N-ERC/mesothelin is found mainly in blood. Therefore, we think the new system can detect our target proteins with high reliability and identify the inflection point more easily.

Moreover, we noticed in this research that the affinity of antibodies used in the ELISA system and the stability of the molecule itself in the blood are very important. Therefore, we will continue to develop more precise ELISA systems. As a next step, we are examining the combination and comparison of other tumor markers for mesothelioma to see whether there is a possibility of early diagnosis using not only tumor markers but also other diagnostic modalities and development of a new, more sensitive ELISA system.

Conclusion

We have developed a new sandwich ELISA system (7-16), which is more sensitive than the previous system. Using this new system, we showed that N-ERC/mesothelin shows promise as a tumor marker for mesothelioma, especially for epithelioid type.

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References

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