Transferrin Receptor Expression in Adenocarcinoma of the Lung as a Histopathologic Indicator of Prognosis

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Immunohistochemical reactivity with OKT9 (antitransferrin receptor) monoclonal antibody was studied in 58 surgically resected adenocarcinomas of the lung. The cell surface and cytoplasm of the tumor cells were stained in 44 cases (76 percent). The expression of the transferrin receptor (TrfR) in tumor cells was compared with histopathologic prognostic factors, such as TNM factors, degree of histologic differentiation, degree of nuclear atypia, and frequency of mitotic figures. The expression of TrfR showed significant correlation with the degree of histopathologic differentiation (p<0.025), degree of nuclear atypia (p<0.025), and frequency of mitotic figures (p<0.001). However, there was no definite correlation with TNM factors. These results indicate that the expression of the TrfR in pulmonary adenocarcinoma corresponds to the elevated proliferative activity of the tumor cells, and that immunohistochemical reactivity with OKT9 can be used as one of the histopathologic indicators of prognosis of pulmonary adenocarcinoma.

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A denocarcinoma of the lung is the most common histologic type of lung carcinoma in Japan, and even in tumors classified as stage I, postoperative recurrence and distant metastasis are seen occasionally. Approximately 30 percent of patients with stage I adenocarcinoma die because of spread of the cancer within five years after surgical resection.1,2 Although the TNM system is the best method for arriving at a prognosis, several authors have studied other prognostic indicators for pulmonary adenocarcinoma, such as histopathologic characteristics, immunohistochemical reactivity with certain antibodies,3,4 and nuclear DNA content.5 Among these indicators, proliferative activity has been regarded as one of the most important.

The proliferative activity of many malignant tumors has been roughly estimated from the number of mitotic figures observed in histologic preparations. In addition to radionuclide uptake assay and cytofluorometric evaluation of the DNA content, immunohistochemical methods using several monoclonal antibodies that recognize proliferating cells have been used to evaluate the dynamics of cellular proliferation.6-10 Particularly, immunohistochemical staining for transferrin receptor (TrfR) has been used most extensively for measuring the proliferation rate of cells and is thought to be of some prognostic value in several types of malignant tumor.11-12 Sato et al14 studied the immunohistochemical stainability of OKT9 in 101 cases of primary lung carcinoma and showed that 11 (19 percent) of 57 adenocarcinomas were positively stained. To estimate the significance of TrfR expression as one of the prognostic indicators of adenocarcinoma, we studied the OKT9 immunohistochemical reactivity of 58 cases of surgically resected pulmonary adenocarcinoma.

Materials and Methods

Patients

Fifty-eight cases of primary adenocarcinoma of the lung were selected randomly for the study. The tumors were obtained at surgery at the National Cancer Center Hospital, Tokyo, Japan, during the period from April 1987 to August 1988. The patients' medical records were reviewed to obtain clinical information and follow-up data. Assignment to T and N categories, which was made using the Union Internationale Contre le Cancer (UICC) TNM staging system,15 is summarized in Table 1. Pathologic stages were assigned as follows: 20 cases in stage I, six cases in stage II, 17 cases in stage IIIA, two cases in stage IIIB, and 13 cases in stage IV.

Pathologic Features

After observation of routine histologic preparations of each tumor, various histocytologic parameters were evaluated as indicated below. The tumors were sub grouped into 19 cases of well-differentiated adenocarcinoma, 21 cases of moderately differentiated adenocarcinoma, and 18 cases of poorly differentiated adenocarcinoma according to the World Health Organization (WHO) classification of lung tumors (1981).16

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The 58 adenocarcinomas were divided into three groups according to the degree of nuclear atypia (mild, moderate, severe). Typical examples from each of the groups are illustrated in Figure 1.

The mitotic index (MI) was expressed as a percentage after counting 2,000 tumor cell nuclei; low mitotic index, MI≤0.25 percent; moderate mitotic index, 0.25 percent<MI≤0.70 percent; and high mitotic index, 0.70 percent<MI.

Immunohistochemistry

One block of carcinoma tissue was fixed in cold acetone and embedded in paraffin by the AMeX method. The avidin-biotin-peroxidase complex (ABC) method was applied to tissues processed by the AMeX method as described previously. Sections 5 μm thick were dewaxed in xylene and acetone, rinsed in 0.0075 M of phosphate-buffered saline solution (PBS), and treated with 10 percent normal swine serum for 15 minutes to minimize any nonspecific absorption of antibodies. The primary antibody was a monoclonal antibody OKT9 (Ortho Pharmaceutical Corp., Raritan, NJ) used at 1:100 dilution in PBS with 1 percent normal swine serum. Incubation with the primary antibody was done for 3 h at room temperature. Then the sections were incubated with the second antibody, biotinylated anti-mouse IgG immunoglobulin (Vector Laboratories, Burlingame, Calif) for 30 minutes, followed by further incubation with streptavidin biotinylated horseradish peroxidase complex (Amersham International, England). The site of antigen localization was visualized by incubation in 3,3'-diaminobenzidine solution containing 0.03 percent H2O2 and 1 percent NaN3. Then the sections were counterstained lightly with Mayer’s hematoxylin or methyl green. Extensive washing (three times, each with 0.0075 M of PBS) was done between each incubation.

The immunohistologic staining of cancer cells in each case was graded as follows: 2+, more than one half of all tumor cells were stained; +, less than one half of all tumor cells were stained; and −, none of the tumor cells were stained.

Statistical Analysis

To analyze the correlation between the immunoreactivity of OKT9 and TNM factors or other prognostic indicators, χ2 analysis for more than two categories was used. Correlation was considered to be statistically significant at a p value of less than 0.05.

RESULTS

In normal lung tissue, bronchoalveolar epithelia and bronchial glands were negative for TrR, but alveolar macrophages were strongly positive. Of 58 cases of adenocarcinoma, 44 (76 percent) showed a positive reaction with OKT9 (Table 2). The cell membrane of the tumor cell was stained predominantly, but the cytoplasm was also stained in some cases (Fig 2 and 3).

The correlation between adenocarcinoma stainability by OKT9 and various pathologic factors was evaluated.

Table 2—Transferrin Receptor Expression and Degree of Histologic Differentiation*

<table>
<thead>
<tr>
<th>OKT9 Reactivity</th>
<th>Diff</th>
<th>−</th>
<th>+</th>
<th>2+</th>
<th>Total No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well</td>
<td>9</td>
<td>7</td>
<td>3</td>
<td>19</td>
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</tr>
<tr>
<td>Moderate</td>
<td>2</td>
<td>9</td>
<td>10</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>3</td>
<td>5</td>
<td>10</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>21</td>
<td>23</td>
<td>58</td>
<td></td>
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</tbody>
</table>

*The correlation between immunoreactivity with OKT9 and the degree of histologic differentiation was significant (p<0.025): χ2(4, 0.025) = 11.14; χ2(4, 0.025) = 11.33; −, none of tumor cells with positive reactivity; +, less than half of tumor cells with positive reactivity; 2+, more than half of tumor cells with positive reactivity; and Diff, degree of histologic differentiation.
Table 3—Transferrin Receptor Expression and Nuclear Atypia*

<table>
<thead>
<tr>
<th>OKT9 Reactivity</th>
<th>No. of Cases with</th>
<th>Total No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mild</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
<td>9</td>
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</table>

*The correlation between immunoreactivity with OKT9 and the degree of nuclear atypia was significant (p<0.025); χ²(2, 0.025) = 7.378 < χ² = 7.5435; a, the mild and moderate nuclear atypia groups put together; and NA, degree of nuclear atypia.

with OKT9 was compared with TNM factors. Eleven (58 percent) of 19 cases in the T1 group and 14 (93 percent) of 15 cases in the T3 and T4 groups showed positive reactions. Eighteen (64 percent) of 28 cases in the N0 group and 15 (88 percent) of 17 cases in the N2 and N3 groups demonstrated positive reactions. However, there was no significant correlation between the immunoreactivity with OKT9 and T or N factor.

The immunoreactivity of adenocarcinoma with OKT9 was not strongly correlated with tumor stage. Eleven (55 percent) of 20 cases in stage I showed positive reaction. Eighteen (95 percent) of 19 cases in stage III A B and ten (77 percent) of 13 cases in stage IV were positive. These results indicated that tumor cells in advanced cases tended to have increased immunoreactivity with OKT9.

Table 3 illustrates the relationship between nuclear atypia of the tumor cells and immunoreactivity with OKT9. In the mild nuclear atypia group, only 12.5 percent of cases showed a positive reaction, whereas in the severe nuclear atypia group, 95 percent showed a positive reaction. The percentage of cases positive with OKT9 increased significantly as the degree of nuclear atypia became pronounced (p<0.025). In well-differentiated adenocarcinomas, seven (88 percent) of eight cases showing negative immunoreactivity with OKT9 belonged to the mild nuclear atypia group and ten (91 percent) of 11 positive cases belonged to the moderate and severe groups. In poorly differentiated

Table 4—Transferrin Receptor Expression and Mitotic Index*

<table>
<thead>
<tr>
<th>OKT9 Reactivity</th>
<th>No. of Cases with</th>
<th>Total No. of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Low</td>
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</tr>
<tr>
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<td></td>
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<td>21</td>
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</table>

*The correlation between immunoreactivity with OKT9 and the mitotic index was significant (p<0.001); χ²(2, 0.001) = 13.816 < χ² = 22.230; a, the moderate and high mitotic index groups put together; MI, mitotic index; low, MI≤0.25 percent; moderate, 0.25 percent<MI≤0.70 percent; and high, 0.70 percent<MI.
adenocarcinomas, there was no difference between the degree of nuclear atypia and the immunoreactivity with OKT9. Therefore, in well-differentiated adenocarcinomas the degree of nuclear atypia was more pronounced in cases showing positive immunoreactivity with OKT9 than in negative cases (Fig 2A and B).

The relationship between mitotic index and immunoreactivity with OKT9 is summarized in Table 4. In the low mitotic index group, 58 percent of cases showed a positive reaction. On the other hand, all cases (100 percent) in the moderate group and 92 percent in the high group showed strongly positive reactions. The immunoreactivity with OKT9 revealed a significant correlation with the mitotic index (p<0.001). In well-differentiated adenocarcinomas, none of nine cases showing negative immunoreactivity with OKT9 belonged to the moderate and high mitotic index groups, and three (30 percent) of ten positive cases belonged to the moderate and high groups. In poorly differentiated adenocarcinomas, one (33 percent) of three cases showing negative immunoreactivity with OKT9 belonged to the moderate and high mitotic index group and 13 (87 percent) of 15 positive cases belonged to the moderate and high groups. Therefore, irrespective of histologic differentiation, the mitotic index was higher in cases showing positive immunoreactivity with OKT9 than in negative cases. Most of the mitotic tumor cells in each case were strongly stained by OKT9. However, in one case in the high mitotic index group, tumor cells undergoing mitosis were not stained.

**DISCUSSION**

The TrfR<sup>18-20</sup> is a glycoprotein expressed on the cytoplasmic membrane and it mediates iron uptake via internalization of an iron-carrying serum protein, transferrin. OKT9, BK19.9, B3/25, T56/14, and T51/8<sup>22</sup> are monoclonal antibodies against TrfR. OKT9, which was used in this study, is a murine monoclonal antibody raised against human leukemic cells,<sup>23</sup> and it has been shown to react with TrfR in various cells.<sup>24</sup> In normal tissues, cells positive for TrfR are found in a limited number of sites, notably the basal layer of the epidermis, the endocrine pancreas, hepatocytes, Kupffer cells, seminiferous tubules of the testis, and the anterior pituitary.<sup>25</sup> Since Faulk et al<sup>11</sup> reported that TrfR was observed in breast cancer, the expression of TrfR has been identified in many types of malignant tumor, including malignant lymphoma,<sup>12</sup> gastric cancer,<sup>13</sup> uterine cancer,<sup>14</sup> and lung cancer.<sup>14</sup> In malignant lymphomas<sup>15</sup> and breast cancer,<sup>11,13</sup> it has been demonstrated that the expression of TrfR correlates with tumor differentiation, possibly implying some prognostic value.

The present study demonstrated that TrfR was expressed in three quarters (76 percent) of all the primary pulmonary adenocarcinomas examined. In addition to adenocarcinoma, we studied small-cell carcinomas (seven cases) and large-cell carcinomas (six cases) for OKT9 reactivity. In small-cell carcinomas, six (86 percent) of seven tumors showed a positive reaction and in large-cell carcinomas, all six tumors were positive (unpublished data). Sato et al<sup>16</sup> previously reported that positive immunoreactivity for OKT9 was seen in 11 (19 percent) of 57 adenocarcinomas, none of four small-cell carcinomas, four (67 percent) of six large-cell carcinomas, and all of 26 squamous cell carcinomas. The OKT9 immunoreactivity was thus lower than that in our present study. This discrepancy may have been due to the different fixation methods used. Sato et al used frozen sections of fresh tissues fixed in periodate-lysine-paraformaldehyde (PLP) solution for immunostaining. In the present study, fresh tissue was fixed in acetone and processed with the AMeX method. In a similar study, Doria et al<sup>17</sup> used frozen sections fixed in acetone and demonstrated that all of nine small-cell carcinomas were labeled by OKT9. Therefore, it is possible that tissues fixed in PLP solution may show less immunoreactivity for OKT9 than those fixed in acetone.

Most cases of small-cell, large-cell, and squamous cell carcinoma showed positive immunoreactivity with OKT9. On the other hand, adenocarcinoma showed more variable staining results with OKT9 ranging from strongly positive immunoreactivity to negative immunoreactivity, indicating quite variable proliferative activity from case to case.

In this study, TrfR expression did not show any significant correlation with T, N, and M factors, although there was a tendency for increased OKT9 immunoreactivity to be related to more advanced stage. In contrast, a significant correlation was found between TrfR expression and the degree of histologic differentiation, the degree of nuclear atypia, and mitotic index. These results were in agreement with the report by Wrba et al<sup>15</sup> on primary breast cancers. In general, degree of the nuclear atypia and mitotic index tend to be low in well-differentiated adenocarcinomas. Our results demonstrated that in well-differentiated adenocarcinomas, the degree of nuclear atypia was more pronounced and mitotic index was higher in cases showing positive immunoreactivity with OKT9 than in negative cases. In poorly differentiated adenocarcinomas, the mitotic index was lower in negative cases than in positive cases. These results indicated that, particularly in well-differentiated adenocarcinomas, TrfR expression was correlated with cytologic but not architectural tumor differentiation, and that mitotic index was probably the factor most strongly related to OKT9 immunoreactivity.

The important prognostic factors in pulmonary adenocarcinoma are lymph node metastasis,<sup>2,20-30</sup> tu-
mor size, pleural involvement, \textsuperscript{2,20,30} and distant metastasis.\textsuperscript{31} Takise et al.\textsuperscript{3} examined 75 pulmonary adenocarcinomas measuring less than 2 cm in diameter to assess the prognostic significance of the histopathologic features. They reported that when stage factors were excluded, the important prognostic factors were the SD of nuclear area (roughly corresponding to the degree of nuclear atypia) and mitotic index. Also Asamura et al.\textsuperscript{4} further investigated the nuclear features of stage I adenocarcinoma of the lung. They reported that the nuclear DNA content was higher in the recurrent group than in the nonrecurrent group, and this was a good indicator of prognosis. As the TrFR expression in pulmonary adenocarcinomas revealed a significant correlation with the degree of nuclear atypia and the number of mitotic figures, TrFR expression may well be another important prognostic indicator.

In conclusion, our data indicate that the expression of TrFR by pulmonary adenocarcinoma is correlated with the elevated proliferative activity of tumor cells, and therefore it has prognostic value. Immunohistochemical labeling with OKT9 antibody is thus a reliable procedure for obtaining kinetic data helpful for both an understanding of tumor biology and choosing an appropriate treatment for pulmonary adenocarcinoma.

REFERENCES

19. Trowbridge IS, Omary MB. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 1981; 78:3039-43