Expression of Lewis\textsuperscript{X}, Sialylated Lewis\textsuperscript{X}, Lewis\textsuperscript{a}, and Sialylated Lewis\textsuperscript{a} Antigens in Human Lung Carcinoma

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FUKUSHIMA, K. Expression of Lewis\textsuperscript{X}, Sialylated Lewis\textsuperscript{X}, Lewis\textsuperscript{a}, and Sialylated Lewis\textsuperscript{a} Antigens in Human Lung Carcinoma. Tohoku J. Exp. Med., 1991, 163 (1), 17-30 — One hundred and five cases of various human lung neoplasms were studied immunohistochemically using monoclonal antibodies recognizing the antigens, Le\textsuperscript{a}, sialylated Le\textsuperscript{a} (SLEX), Le\textsuperscript{b}, and sialylated Le\textsuperscript{a} (SLEA). Of the 92 lung cancers examined for antigen expression SLEX was detected in 57\% (52 cases), Le\textsuperscript{a} in 42\% (39), SLEA in 36\% (33), and Le\textsuperscript{b} in 23\% (21). None of these antigens were expressed in 9 tumor like lesions of the lung or the 4 other non-epithelial malignant lung tumors examined. Higher expression was seen in the 54 lung adenocarcinomas: SLEX in 72\%, Le\textsuperscript{a} in 48\%, SLEA in 39\%, and Le\textsuperscript{b} in 24\%. The type 2 carbohydrate antigens (SLEX and Le\textsuperscript{a}) were more prevalent than the type 1 antigens (SLEA and Le\textsuperscript{b}) in lung adenocarcinoma tissues. In adenocarcinomas, SLEX was expressed in 71\% (10/14) of the Le\textsuperscript{b} patients, and in 100\% (5/5) of the Le\textsuperscript{a} patients. Unexpectedly, SLEX was not detected in 4 out of 5 Le\textsuperscript{a-b} patients tested. This suggests that the expression of the Le\textsuperscript{b} antigens and the Le\textsuperscript{a} antigens are related in lung adenocarcinoma. These antigens were less expressed in other types of lung cancers. Tissue sections obtained serially showed heterogeneity in the expression of these antigens, as evidenced by the concurrent presence of both SLEX and SLEA. These results indicate that SLEX is a useful tumor-associated marker for lung adenocarcinomas, and that terminal fucosylation and sialylation may be activated heterogeneously in these lung cancers. ——— tumor-associated carbohydrate antigens; lung cancer; Lewis type; immunohistochemistry; heterogeneity

Many attempts have been made to produce MoAb which defines tumor specific antigens. Various tumor-associated cell-surface antigens have been found to be predominantly carbohydrate antigens, e.g., CA19–9 (Koprowski et al. 1979), CA50

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The abbreviations used are: MoAb, monoclonal antibody; Le, Lewis; Le\textsuperscript{a}, Lewis\textsuperscript{a}; Le\textsuperscript{b}, Lewis\textsuperscript{b}; Le\textsuperscript{a}, Lewis\textsuperscript{a}; TBS, Tris-buffered saline (50 mM Tris-HCl 200 mM NaCl, pH 7.6); Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylated neuraminic acid.
CA19-9 is well known as a tumor marker for pancreatic and gastric cancers. Its epitope was determined to be sialylated lacto-N-fucopentaose II also known as sialylated Leα (Magnani et al. 1982). We have reported previously that the sialylated Leα antigen detected by the MoAb CSLEX1 is a tumor-associated antigen (Fukushima et al. 1984) with a chemical structure isomeric to the sialylated Leα antigen defined by the MoAb NS19-9. The CSLEX1 antibody is quite similar to MoAb FH-6 recognizing a sialylated Leα-i epitope (SLX) (Fukushima et al. 1984; Kannagi et al. 1986). The non-sialylated forms of both Leα and Leα are also known to be tumor-associated carbohydrate antigens (Hakomori and Kannagi 1983).

Carbohydrate chains in glycolipids and glycoproteins are frequently altered in association with neoplastic transformation (Hakomori and Kannagi 1983). Expression of certain carbohydrate antigens on the cell surface seems to be closely related to oncogenesis. There are several reports on the immunohistochemical expression of the tumor-associated carbohydrate antigens in human lung cancer (Kasai et al. 1986; Miyake et al. 1988; Zenita et al. 1988). In this study of 105 cases of various human lung neoplasms including lung cancers, tumor-like lesions and non-epithelial malignant lung tumors, the distributions of the following tumor-associated antigens were examined immunohistochemically with the use of MoAbs: sialylated and non-sialylated forms of both Leα (Xhapten) and Leα. It was also investigated whether the Lewis blood group status of the patients would be related to the expression of these antigens in lung adenocarcinoma. Furthermore, the concurrent presence of both sialylated Leα and sialylated Leα was examined using double-staining method.

**Materials and Methods**

**Tissue specimens**

Tissue from various lung neoplasms was obtained from 105 patients undergoing surgical resection at Nagasaki University Hospital and affiliated hospital. There were 92 primary lung cancers i.e., 54 adenocarcinomas, 25 squamous cell carcinomas, 8 large cell carcinomas, 5 small cell carcinomas, 9 tumor-like lesions of the lung including 3 hamartomas and 6 sclerosing hemangiomas, and 4 other malignant lung tumors including a pulmonary involvement of malignant lymphoma and 3 diffuse mesotheliomas. The ABO blood group and Lewis type for 24 cases of lung adenocarcinoma were determined by means of hemagglutination tests.

All tissue samples were fixed in formalin, embedded in paraffin, and cut into serial sections for immunoperoxidase staining.

**Antibodies**

Table 1 lists the MoAbs employed in this study along with their antigenic determinants. MoAbs CLEX1, CSLEX1, CLE1, and CSLEA1 recognize Leα, sialylated Leα (SLEX), Leα, and sialylated Leα (SLEA), respectively (Fukushima et al. 1984; Chia et al. 1985) and were kindly provided by Professor P.I. Terasaki (UCLA Tissue Typing Laboratory, Los Angeles, CA, USA).
Peroxidase-conjugated goat F(ab')₂ anti-mouse IgG and IgM were purchased from Cappel (West Chester, PA, USA).

**Immunoperoxidase staining**

Indirect immunoperoxidase staining was accomplished as follows: Paraffin embedded sections were heated to 60°C for 30 min, treated for 5 min with 3% hydrogen peroxide in 0.05 M TBS (to block endogenous peroxidase), then rinsed three times with TBS. The sections were incubated for one hour at room temperature with the appropriately diluted MoAbs listed in Table 1. After three washings in TBS, peroxidase-conjugated goat F(ab')₂ anti-mouse IgG diluted 1:100 in TBS containing 3% normal goat serum was added to the previously labeled sections and incubated for 45 min at room temperature. After being rinsed with TBS, slides were flooded for 5 min with a freshly made mixture of 0.05% 3,3′-diaminobenzidine and 0.03% hydrogen peroxide. Slides were washed again with TBS and counter stained with hematoxylin. Sections were evaluated using a light microscope with results expressed as a score based on the percentage of the total field staining positively with the various MoAbs. Scores were based on the following scale: * for over 50% of the field showing positive staining; 1 for 30% to 50%; + for 5% to 30% and − for less than 5%.

**Double staining**

SLEX and SLEA were stained simultaneously using a modified form of Nakane’s double staining method (Nakane 1968). The deparaffinized sections were incubated with CSLEA1 MoAb followed by the addition of peroxidase-conjugated anti-mouse IgG antibody. 0.05% 3,3′-diaminobenzidine and 0.03% hydrogen peroxide were added to complete the staining of SLEA antigen. After rinsing 3 times with TBS, sections were then incubat-
ed with CSLEX1 MoAb followed by the addition of peroxidase-conjugated goat anti-mouse IgM antibody. (Mouse IgG cross-reactivity was removed by preincubating the reagent with normal mouse IgG followed by centrifugation.) The sections were then flooded with 20 mg% of 4-chloro-1-naphthol and 0.001% hydrogenperoxide. This procedure resulted in simultaneous staining of SLEA (yellowish brown) and SLEX (bluish gray).

Controls

Normal mouse sera were used as a negative control for the mouse monoclonal antibodies. Results were analyzed by the $\chi^2$ test or used for calculation of Spearman rank correlation coefficient. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Non-neoplastic lung tissues

Table 2 shows the expression of the various carbohydrate antigens in non-neoplastic tissues of the lung studied. SLEX and SLEA were expressed strongly in bronchial glands, in which non-sialylated forms were weakly positive. These antigens were weakly stained in bronchial surface epithelium, goblet cells of bronchial epithelium and bronchiolar epithelium, but negative in alveolar cell. CSLEX1 and CLEX1 reacted weakly with alveolar macrophages, but CSLEA1 and CLEA1 did not.

Human lung neoplasms

Table 3 shows the distributions of the sialylated and non-sialylated forms of both Le$^x$ and Le$^a$ for 105 cases of human lung neoplasms. SLEX (57%), Le$^x$ (42%), SLEA (36%) and Le$^a$ (23%) were expressed in 92 cases of lung cancers studied. In 54 cases of lung adenocarcinomas, antigens were expressed; SLEX in 72%, Le$^x$ in 48%, SLEA in 39%, and Le$^a$ in 24%. Antigens were positive for 32% to 44% of 25 cases of lung squamous cell carcinomas. Only one or two cases of 8 large cell carcinomas and 5 small cell carcinomas were found to be positive.

Table 2. Expression of carbohydrate antigens in non-neoplastic lung tissue

<table>
<thead>
<tr>
<th></th>
<th>Le$^x$</th>
<th>SLEX</th>
<th>Le$^a$</th>
<th>SLEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial surface epithelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bronchial goblet cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Bronchial gland</td>
<td>+</td>
<td>#</td>
<td>+</td>
<td>#</td>
</tr>
<tr>
<td>Bronchiolar surface epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ciliated</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>non-ciliated</td>
<td>-/+(^1)</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
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<tr>
<td>Alveolar cell</td>
<td></td>
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<td></td>
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<tr>
<td>Alveolar macrophage</td>
<td>+</td>
<td>+</td>
<td>-</td>
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\(^{1}\)The antigens were expressed in a small number of the non-ciliated bronchiolar epithelium.
for SLEX, Le\textsuperscript{x} and SLEA. None of the antigens examined was detected in four other malignant tumors (a malignant lymphoma and 3 diffuse mesotheliomas) nor 9 lung tumor-like lesions including 3 hamartomas and 6 sclerosing hemangiomas.

Fig. 1 shows the results of the expressions of the various carbohydrate antigens studied grouped according to the degree of differentiation of the lung adenocarcinomas (Fig. 1A) and squamous cell carcinomas (Fig. 1B). Out of the 31 cases of well-differentiated lung adenocarcinomas studied, SLEX was expressed in 25 cases (81%), Le\textsuperscript{x} and SLEA were positive in 15 cases (48%), and Le\textsuperscript{a} was present in only nine cases (29%). Of the 17 cases of moderately-differentiated cancers tested, SLEX was detected in 59% (10 of 17), Le\textsuperscript{x} in 47% (8/17), SLEA in 29% (5), and Le\textsuperscript{a} in 23% (4). There were only 6 cases of poorly-differentiated adenocarcinomas included in this study with SLEX found in 4 of the 6 cases, Le\textsuperscript{x} found in 3 cases, SLEA found in 1 case, and Le\textsuperscript{a} found in none of the 6 cases. Positive cases of which over 50% of the tumor tissue section was stained for SLEX were observed in 42% of the 31 cases of well-differentiated adenocarcinomas. However, none of the 6 cases of poorly-differentiated adenocarcinomas showed positive staining over 50% of the tumor tissue section with any 4 antigens studied here.

On the other hand, there were 4 well-differentiated, 13 moderately-differentiated, and 8 poorly-differentiated squamous cell carcinomas studied. Antigens were expressed in the 25 cases of squamous cell cancers; SLEX in 40%, Le\textsuperscript{x} in 44%, SLEA in 36%, and Le\textsuperscript{a} in 32%. None of the cases had greater than 50% of the tumor tissue section showing positive staining for any antigens.
examined. These antigens were expressed positively in only 5% to 30% of the tumor tissue section in almost all of the positive cases in the squamous cell lung cancers. The type 2 antigens (sialylated and non-sialylated Le\textsuperscript{a}) were expressed more often than the type 1 antigens (sialylated and non-sialylated Le\textsuperscript{a}). The relationship between the degree of differentiation of the lung adenocarcinomas and the expression of the carbohydrate antigens studied was not statistically significant ($\chi^2$ test).

In lung adenocarcinoma the carbohydrate antigens were localized in the cell membrane and the cytoplasm of the cancer cells as well as, in mucous-like secretions (Fig. 2A). The antigens in squamous cell lung cancer were localized in the cell membrane and the cytoplasm of a small number of cancer cells around keratinizing cells (Fig. 2B).

**Lewis type and antigen expression**

Twenty four cases of lung adenocarcinomas were retrospectively determined Lewis type of peripheral blood cells by means of hemagglutination tests with the
Fig. 2. Immunoperoxidase staining of well-differentiated adenocarcinoma from a patient of blood type ALe\(^a\) (2A) and moderately differentiated squamous cell carcinoma (2B) with CSLEX1 antibody. Almost all tumor cells and mucous-like secretions are positively stained with CSLEX1 antibody \((\times 140)\) (2A). A small number of cancer cells around keratinizing cells are stained with CSLEX1 \((\times 140)\) (2B). Staining procedures are described in the Materials and Methods section.
following results: Le\textsuperscript{a\textendash}b\textsuperscript{+}, 14 cases; Le\textsuperscript{a+b\textendash}, 5 cases; Le\textsuperscript{a\textendash}b\textsuperscript{−}, 5 cases. Table 4 shows the antigen expressions in 24 lung adenocarcinomas are shown according to the Lewis type. Among the 14 Le\textsuperscript{b} cases tested, SLEA was expressed in 7 and SLEX was present in 10. Of the 5 Le\textsuperscript{a} cases, SLEA was found in 3 cases and SLEX in 5 cases. In the five Le\textsuperscript{a\textendash}b\textsuperscript{−} patients with lung adenocarcinomas, neither SLEA, Le\textsuperscript{a} nor Le\textsuperscript{x} were expressed, while SLEX was detected in only one case with weak reactivity. Le\textsuperscript{x} and SLEX were weakly expressed, but neither Le\textsuperscript{a} or SLEA were detected in the bronchiolar epithelium of the these Le\textsuperscript{a\textendash}b\textsuperscript{−} cases.

Sialylated and non-sialylated antigens

Fig. 3A shows the relationship between expression of the sialylated and non-sialylated forms of Le\textsuperscript{x} in lung adenocarcinomas. Twenty-five of the 26 (96\%) cases positive for Le\textsuperscript{x} were also positive for SLEX, while 18 of the 39 (46\%)
cases positive for SLEX were also positive for Le\textsuperscript{x} (Fig. 3A). Namely, the positive cases for Le\textsuperscript{x} were included in the positive cases for SLEX except in one case. Similarly for the type 1 antigens, the cases positive for Le\textsuperscript{a} were all included in the cases of that for SLEA (data not shown).

Sialylated forms of Le\textsuperscript{a} and Le\textsuperscript{x} were observed more frequently than their respective non-sialylated forms. The correlations of the expressions of SLEX vs. Le\textsuperscript{x} and SLEA vs. Le\textsuperscript{a} were statistically significant, with Spearman correlation coefficient $r_s$ of 0.649 ($p < 0.01$) and 0.866 ($p < 0.01$), respectively.

**Expression of sialylated Le\textsuperscript{x} vs. sialylated Le\textsuperscript{a}**

The expression of SLEX was compared with the expression of SLEA in lung adenocarcinomas as shown in Fig. 3B. Nineteen of the 21 (90\%) cases positive for SLEA were also positive for SLEX, while 19 (49\%) of the 39 cases positive for SLEX were positive for SLEA (Fig. 3B). Twenty-four percent (13 of 54) of the lung adenocarcinomas studied were negative for SLEX and SLEA. The correlation between SLEX and SLEA was statistically significant ($r_s$ of 0.578 ($p < 0.01$)).

**Double staining of sialylated Le\textsuperscript{x} and sialylated Le\textsuperscript{a}**

A double staining method was used to observe simultaneous localization of SLEX and SLEA in adenocarcinoma of the lung. Thirty such cases positive for both of these sialylated antigens were studied. In cancer cells and secretions, some stained positively for either SLEA or SLEX while others were positive for...
both. Almost all of these doubly-stained samples exhibited dual staining within an individual histologic section (Fig. 4). The degree of heterogeneity in the double staining varied from case to case.

**Discussion**

The expression of various tumor-associated carbohydrate antigens was studied immunohistochemically in human lung neoplasms. These antigens were found in lung cancer, especially often in lung adenocarcinoma. However, these were not present in sclerosing hemangiomas and diffuse mesotheliomas examined which are not always easily distinguished from lung adenocarcinomas microscopically. Although it is difficult to obtain the appropriate controls for human lung cancer, in the non-neoplastic lung tissues, these antigens were expressed weakly in the surface epithelium of bronchi and bronchioles and both SLEX and SLEA were strongly positive in the bronchial glands. Our previous report of no expression of SLEX in bronchi (Fukushima et al. 1984) should be corrected. The antigens of SLEX and SLEA may not represent "neo"-antigens in the cases of adenocarcinomas arising from bronchial glands since normal bronchial glands and adenocarcinomas were strongly positive for these antigens. The expression of
carbohydrate antigens in lung adenocarcinoma was mainly further investigated because of its highly frequent expression.

The four types of terminal carbohydrate antigenic determinants examined here consisted of tri- or tetra-saccharides including Fuc, Gal, GlcNAc or NeuAc backbones. The carbohydrate sequences are formed by the sequential activation of the appropriate sugar transferases (Morgan and Watkins 1969). These carbohydrate chains are attached to glycoproteins or glycolipids on the cell surface. The backbone regions of the oligosaccharides usually consist of alternating Gal and GlcNAc residues in one of two types of disaccharide units known as type 1 (Gal 1→3 GlcNAc) and type 2 (Gal 1→4 GlcNAc) (Watkins 1980). Le\(^a\) and SLEA (CA19-9) are of type 1 structure, while Le\(^x\) and SLEX are of type 2 structure. Since Lewis antigens are genetically inherited, the presence of the type 1 chains will depend on the Lewis type of the patients. The previous suggestion that Le\(\text{a}^-\text{b}^+\) cancer patients cannot synthesize CA19-9 (Koprowski et al. 1982) is consistent with our present data on expression of SLEA. SLEX has been suggested to be expressed in the tumors irrespective of Lewis phenotype since \(\alpha\ 1→3\) fucosyltransferase coded by X gene (X enzyme) has been considered to be no genetic polymorphism regulating its expression. Unexpectedly, SLEX was negative in 4 of 5 cases of Le\(\text{a}^-\text{b}^+\) lung adenocarcinoma patients tested, even though SLEX was weakly expressed in the bronchiolar epithelium of these Le\(\text{a}^-\text{b}^+\) individuals. This suggests that the expression of the X and Le antigens might be related in lung adenocarcinomas, as described with regard to normal saliva and some normal tissues previously (Sakamoto et al. 1984; Oriol et al. 1986). There is also assumed the existence of the epistatic interaction between Le gene and X gene in lung adenocarcinomas.

In the current study, the sialylated forms of both Le\(^a\) and Le\(^x\) were found to be more prevalent than their respective non-sialylated forms. There have been many reports of sialic acid in neoplasma (Warren et al. 1972; Bosmann and Hall 1974; Ganzinger and Deutsch 1980). Increases in sialyltransferase activity in both cancer tissue (Bosmann and Hall 1974) and sera of cancer patients (Ganzinger and Deutsch 1980) have been reported. The frequent expression of fucosylated type 2 antigens in lung adenocarcinomas has described previously (Miyake et al. 1988; Zenita et al. 1988). The followings might also operate: activation of both 2→3 sialyltransferase and \(\alpha\ 1→3\) fucosyltransferase (coded by the X gene), increased availability of substrates between some enzymes or decreased activity of sugar nucleotide antiporters. Our current results suggest possible activation of both fucosylation and sialylation of terminal sugar sequences in patients with lung adenocarcinomas, resulting in the detection of SLEX in 72% of the patients studied.

We reported previously that SLEX was detected respectively in 46% of sera from 74 patients with lung adenocarcinomas (Hirot a et al. 1985). The SLEX antigen should be detected less frequently in the sera than in the tissues because
it may not pass easily into the bloodstream and it could be degenerated easily by some enzymes in the serum.

The summary of the relationship between the expression of tumor-associated carbohydrate antigens and the degree of differentiation in the lung adenocarcinomas studied showed no correlation statistically. Significant correlations in the expressions of SLEX vs. Le\(^x\), SLEA vs. Le\(^a\), and SLEX vs. SLEA in lung cancer tissues were observed.

Staining for the various antigens in serial tissue sections showed heterogeneity in their localization patterns. The localization of SLEX and SLEA antigens in lung cancers reported here is the same as that previously reported (Kasai et al. 1986) each histological type of lung cancer showing a different localization pattern. Although ninety percent of the SLEA positive cases were also positive for SLEX in this study, double staining for SLEA and SLEX with in an individual tissue section showed clear heterogeneity in their expression (Fig. 4). This had not been studied previously.

There have recently been structural studies of carbohydrate sequences in carcinoembryonic antigen (CEA) (Yamashita et al. 1987). The complex type sugar chains of CEA contain primarily type 2 chains, X-, Y-, and type 2 H-determinants on its major outer chain moieties. Small amounts of Le\(^a\) antigen and type 1 chains have also been found in some CEA samples (Yamashita et al. 1987). These findings are consistent with the current data. Occurrence of the Y-antigenic determinant of human-seminoprotein (Van Halbeek et al. 1985) and the presence of the sialyl Le\(^a\) and Y-determinant tumor-associated antigens in epidermal growth factor receptor (Basu et al. 1987) have been reported. Furthermore, investigation of glycolipid antigen expression in human lung cancer performed by means of immunological techniques has suggested that enhanced c-myc expression may influence the types of glycolipids expressed on the surface of lung tumor cells (Spitalnik et al. 1986).

Though the biological function of these sugar chains in cancer is unclear, they may play important roles in cell proliferation, escape from immunological surveillance, or metastasis of cancer cells. Further investigation of the abnormal activation of glycosyltransferase in cancer could be of great interest.

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