Jpn. J. Cancer Res. 83, 1002–1010, September 1992

Pulmonary Large Cell Carcinoma Expressing Neuroendocrine Markers: The Morphological, Biological, and Neuroendocrine Features of Their Cell Lines and Surgical Cases

Kiyoshi Kasai,[1,4] Toru Kameya,[1] Yasuaki Kawakubo,[1] Yuichi Sato,[1] Chieki Wada[2] and Hiroshi Itoh[1,3]

Departments of [1]Pathology, [2]Clinical Pathology, and [3]Neurosurgery, Kitasato University School of Medicine, Kitasato 1-15-1, Sagamihara, Kanagawa 228

A cell line expressing neuroendocrine (NE) markers, designated as KTS9, was established from a human large cell carcinoma of the lung using serum-free medium, ACL-3. KTS9 cells showed morphological characteristics of large cell undifferentiated carcinoma (LCUC) and expressed some general NE markers including neuron-specific enolase (NSE), protein gene product (PGP) 9.5, neural cell adhesion molecule (N-CAM), synaptophysin and neurofilaments (NF) of 200 kd. Some cells of this cell line were positive to chromogranin-A (CG-A), but did not express Leu7 or aromatic L-amino acid decarboxylase (AADC). Such a cell line derived from LCUC with NE properties has not previously been reported. The biological and NE properties of the KTS9 cell line were compared with those of 2 surgical cases of LCUC with NE markers and of the KTA7 cell line previously reported to derive from large cell carcinoma and to possess NE markers such as alpha-hCG, PGP9.5 N-CAM and AADC. Tumor cells of 2 large cell carcinomas expressed NSE, PGP9.5, N-CAM and NF. The KTS9 and KTA7 cell lines and 2 large cell carcinomas were thus considered to be LCUCs with NE differentiation. Both lines had the morphological characteristics of LCUC, relatively short doubling time and discordant expression of NE markers, indicating them to be closely related to the variant type of small cell carcinoma cell lines and thus possibly to represent high-grade malignancy. They may be useful for examining the biological behavior and NE features of large cell-type NE tumors of the lung.

Key words: Pulmonary large cell carcinoma — Neuroendocrine features — N-CAM

Neuroendocrine features in pulmonary tumors including bronchial carcinoid and small cell carcinoma of the lung (SCCL) have been reported. Most such tumors exhibit neuroendocrine (NE) differentiation and can be distinguished from non-small cell carcinomas of the lung (non-SCCL), since their clinical and morphological characteristics have been well documented.[1–3] Various SCCL cell lines have been established *in vitro*.[4,5] The study of these SCCL cell lines has clarified the biological and NE properties of SCCL cells. Recently, NE differentiation in non-SCCL tissues was also observed. Several histopathological features of such NE tumors derived from non-SCCL have been proposed.[6–10] However, the NE features of non-SCCL cell lines have yet to be clarified in detail and the relationship between biological behavior and NE differentiation of such non-SCCL remains unclear. A new cell line derived from non-SCCL, KTS9, was established using a serum-free medium for a non-SCCL cell culture and found to have NE markers. The present study was conducted to elucidate its morphological, biological and NE properties in comparison with those of 2 surgical cases of large cell carcinomas with NE differentiation and of the KTA 7 cell line reported previously.[11]

MATERIALS AND METHODS

Cell lines The original tumor tissue of KTS9 was obtained by surgical operation on a 42-year-old man with lung cancer. The histology of the lung tumor was large cell carcinoma mixed with foci of squamous cell carcinoma but lacking the features of small cell carcinoma. The tumor tissue of the KTA7 consisted of adenocarcinoma and large cell carcinoma as previously reported.[11] The KTZ6 cell line was established from a surgical specimen of the intermediate type of SCCL as described previously.[12] The Lu134 cell line was an SCCL cell line provided by the National Cancer Center Research Institute, Tokyo.[13]

Tissues Cancer tissues of case 1 were obtained at the time of lymph node biopsy from a 76-year-old man with lung tumor. Tumor tissues of case 2 were obtained at the time of lobectomy of the right upper lobe of a 66-year-old man with lung cancer. Histological examination of

[4] To whom correspondence should be addressed.

the tumors of the 2 casses showed the features of large cell carcinoma without morphology of neuroendocrine tumors. Both were selected from our files of surgical materials of non-small cell lung cancers since the tumor cells expressed the neural cell adhesion molecule (N-CAM) by immunohistochemistry as described below.

Cell culture procedure and transplantation of cells in nude mice Cell culture was performed as described previously.[11] Cells of the lung tumor were cultured in ACL-3 medium.[14] The established line was designated as KTS9. The tumor cells were then transplanted subcutaneously into BALB/c female nude (*nu/nu*) mice (Clea Japan Inc., Tokyo) as described previously.[11] The KTA7 cell line was cultured in ACL-3 medium. The KTZ6 and Lu134 cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum.

Cell growth study Growth curves were made based on protein measurements according to the Oyama-Eagle method.[15] Doubling times of the KTS9 cell line were determined from the points of most rapid growth on the growth curves.

Morphological and immunohistochemical study Cultured cells were cytocentrifuged and fixed immediately for 5 min in cold acetone or 10% formalin. The transplanted tumors were fixed in 10% formalin and embedded in paraffin. Cytocentrifuged KTS9 cells and paraffin-embedded sections were stained with hematoxylin and eosin and immunostained by the avidin-biotin-complex method as described previously.[11] The following antibodies were used for immunohistochemistry: antibodies reactive with epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA), secretory component (SC), keratin (polyclonal antibody), alpha-fetoprotein (AFP) and neuron specific enolase (NSE) were purchased from DAKOpatts, Copenhagen, Denmark. Anti-cytokeratin monoclonal antibody reacting with low-molecular-weight keratin was purchased from Becton-Dickinson, Mountain View, CA. Monoclonal antibodies (MAbs) reacting with sialosylated Lewis^x (SLEX) or sialosylated Lewis^a (SLEA) were supplied by UCLA Tissue Typing Laboratory, Los Angeles, CA.[16,17] A MAb against a surface antigen of neuroendocrine tumors and tissues, 6H7, was developed at our laboratory.[18] An anti-synaptophysin (SYN) monoclonal antibody was purchased from Boehringer, Mannheim, Germany.[19] An antibody reactive with the protein gene product (PGP) 9.5 was purchased from Ultra Clone, Isle of Wight, England.[20] An anti-chromogranin A monoclonal antibody (CG-A) was purchased from Lipshaw, Detroit, MI.[21] An anti-neurofilament (NF) monoclonal antibody reactive with NF of 200 kd was purchased from Labsystems, Helsinki, Finland. An anti-aromatic L-amino acid decarboxylase (AADC) antibody was purchased from Eugene Tech International Inc., Ramsey, NJ. A pro-gastrin releasing peptide (pro-GRP) antibody was kindly donated by Dr. K. Yamaguchi, National Cancer Center Research Institute, Tokyo.

Immunofluorescence (IF) staining and flow cytometry In the indirect IF method, 1×10^6 cells were incubated with biotinylated 6H7 antibody at 4°C for 30 min. After being washed twice with phosphate-buffered saline (PBS), the cells were then incubated with fluorescein-conjugated avidin (Tago Inc., Burlingame, CA) for 30 min at 4°C and washed twice in PBS. In the direct IF method, the cells were stained with phycoerythin-labeled anti-Leu19 antibody (Becton-Dickinson) for 30 min at 4°C and washed twice in PBS. Negative controls consisted of cells incubated with no primary antibody or cells incubated with biotinylated mouse IgG1 as the primary antibody. After being fixed in 1% formaldehyde in PBS, ten thousand cells from each sample were analyzed on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Fluorescence intensity was indicated on the x-axis (logarithmic scale) and cell number on the y-axis in the histograms.

Electron microscopic study Centrifuged KTS9 cells and nude mouse-transplanted KTS9 tumors were fixed in 2.5% glutaraldehyde solution followed by 1% osmium tetroxide and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate and lead citrate and observed under an electron microscope.

Gel electrophoresis and immunoblotting assay Cultured cells were sonicated for 3 min at 4°C in PBS containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF: Merck, Darmstadt, Germany). After centrifugation (5,000*g*) for 10 min, the supernatants were collected and boiled for 5 min in sodium dodecyl sulphate (SDS) sample buffer. Samples were then electrophoresed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight markers were purchased from Amersham, Little Chalsont, Buckinghamshire, England.

For immunoblotting, electrophoretically separated polypeptides were transferred to nitrocellulose sheets by overnight blotting at 30 V, as described by Towbin *et al.*[22] Each sheet was immunostained by the ABC method and colored by using bromochloroindolyl phosphate/nitroblue tetrazolium substrate. Dilution of the primary antibodies was as follows: anti-NSE antibody was diluted 1:400 and anti-PGP 9.5 antibody was diluted 1:400. Normal rabbit serum diluted 1:400 was used as the negative control.

RT-PCR of N-CAM expression Extraction of total cellular RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) were carried out as previously described.[23]

Oligoprimers of the N-CAM used in PCR were chosen within N-CAM cDNA and synthesized on an Applied Biosystems Model 381A DNA synthesizer (Applied Bio-

systems Inc., Foster City, CA). Their sequences were as follows: for the first and the second primers and probe, 5 > TCCTTGTTCAAGCAGACACC < 3′ (residues 1478–1497), 5′ > TGATCTCACCCAGCCCTTTG < 3′ (residues 1743–1762) and 5′ > GGCCCACGATGG-TGACGATG < 3′ (residues 1677–1696) were synthesized.[24, 25] The N-CAM primers and probe were located in extracellular non-immunoglobulin-like region, so that RT-PCR with these primers amplified all N-CAM isoforms. PCR was performed using the Program Temp. Control System PC-700 (ASTEC, Tokyo) as described previously.[23] The amplification was carried out for 25 cycles, each consisting of annealing for 1 min at 56°C, extension for 3 min at 72°C, and denaturation for 1 min at 97°C. The PCR products were electrophoresed in 2% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham) and hybridized with the 5′-end labeled probe using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham) as previously described.[23]

RESULTS

The established KTS9 cell line continued to grow in 25 cm^2 flasks containing ACL-3 medium; the population doubling time was 43 h. Although KTS9 cells formed monolayers of epithelioid cells in loose contact with each other, single cells and small cell clusters detached spontaneously from the monolayers. KTS9 cells had a single round or polygonal nucleus with one prominent nucleolus and round cytoplasm (Fig. 1a). Nude mouse xenografts exhibited undifferentiated large cell carcinoma without formation of acini or keratinization (Fig. 1b).

Immunohistochemical study Immunohistochemical study on both cytocentrifuged KTS9 cells and nude mouse xenografts indicated KTS9 to express neuronal and neuroendocrine cell markers including NSE, NF of 200 kd, SYN and PGP9.5. The last is a 27,000-molecular-weight soluble protein isolated from brain and recently identified as a neuron specific isozyme of ubiquitin hydroxylase.[26] In the KTS9 transplanted tumor, some cells were positive to CG-A, but negative to Leu7 and AADC. KTS9 cells expressed SLEX, EMA and cytokeratin but not CEA, SC, SLEA or AFP.

Results of flow cytometry KTS9 and KTA7 cells were stained by direct or indirect immunofluorescence with anti-Leu19 or 6H7 antibodies and expression of the cell surface antigens was also analyzed by flow cytometry. As shown in Fig. 2, KTS9 cells were positive for 6H7 and Leu19. Ninety-five % of KTS9 cells reacted with 6H7 and 87% with Leu19 antigen. Positive fluorescence was characterized by a bimodal distribution. In contrast, 24% of KTA7 cells reacted with 6H7 and 10% with Leu19. Fluorescence intensity of the positive cells was low.

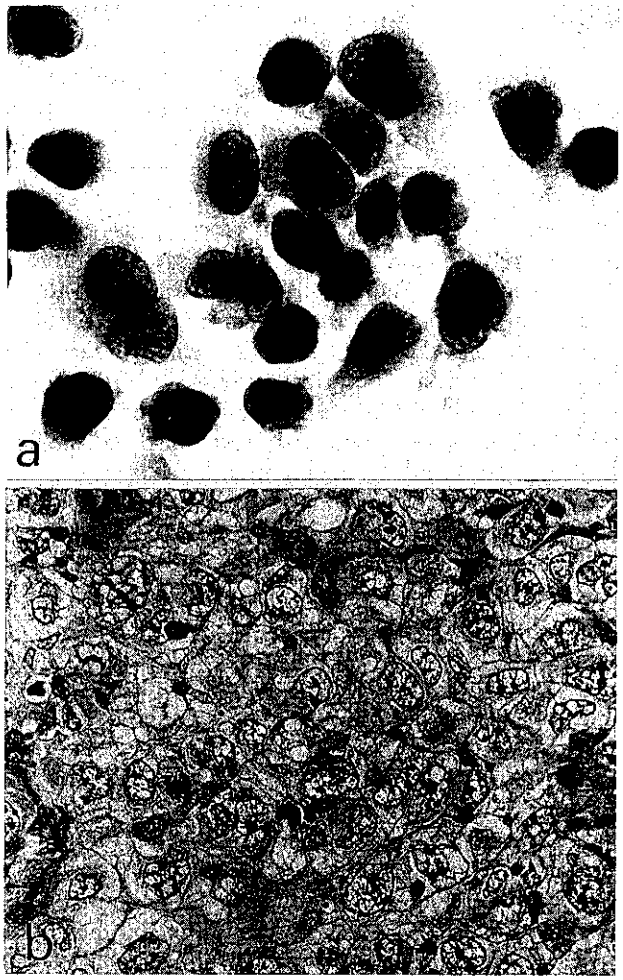

Fig. 1. Hematoxylin and eosin staining of KTS9 cultured cells (a) and nude mouse transplanted tumor (b). a, b; ×200

Electron microscopic examination Electron microscopic examination of KTS9 cells showed some of their nuclei to possess moderate amounts of euchromatin and prominent nucleoli. The cytoplasm of the KTS9 cells contained scattered vesicular rough endoplasmic reticulum, a moderate amount of mitochondria and free ribosomes and a small number of microfilaments (Fig. 3). Adjacent cell membranes were in close contact with each other, with scattered desmosomes and cell interdigitations (photograph not shown). Some cells contained annulate lamellae in the cytoplasm. No dense-core secretory granules could be found in any cell. No basement membrane separating the parenchyma from connective tissue was present in transplants of KTS9 (photograph not shown).

Results of SDS-PAGE and immunoblotting PGP9.5 and NSE antigens in two cell lines were analyzed by SDS-

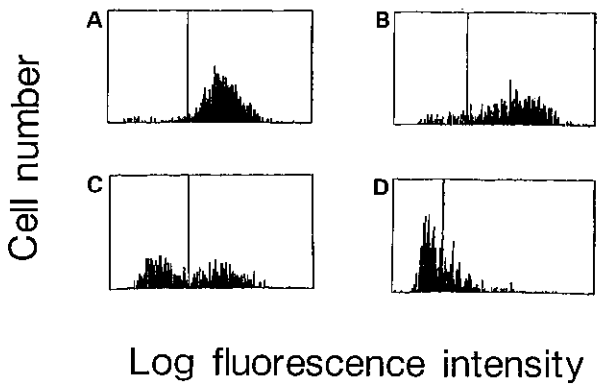

Fig. 2. Histograms showing log fluorescence intensity (x axis) versus cell number (y axis). Fluorescence intensities for KTS9 cells labeled with each antibody by biotin-avidin staining or direct immunofluorescence, as described in "Materials and Method," are indicated for (A) 6H7; (B) Leu19. Fluorescence intensity for KTA7 cells is shown for (C) 6H7; (D) Leu19. Negative gating, indicated by a line, was determined by using negative control cells.

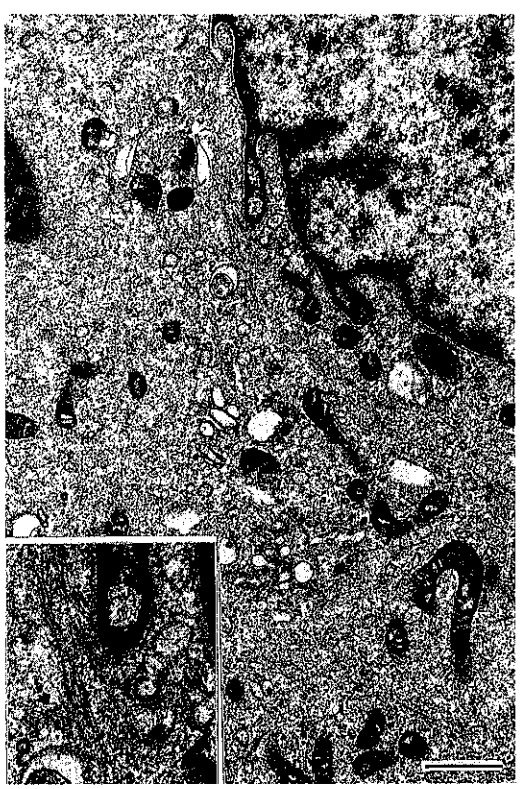

Fig. 3. Electron microscopic examination of cultured KTS9 cells. The cytoplasm contains abundant free ribosomes, scattered vesicular rough endoplasmic membranes and a moderate number of mitochondria. The inset shows a small portion of a filamentous structure in the cytoplasm. ×10,000; inset, ×33,000. Bar: 1 μm

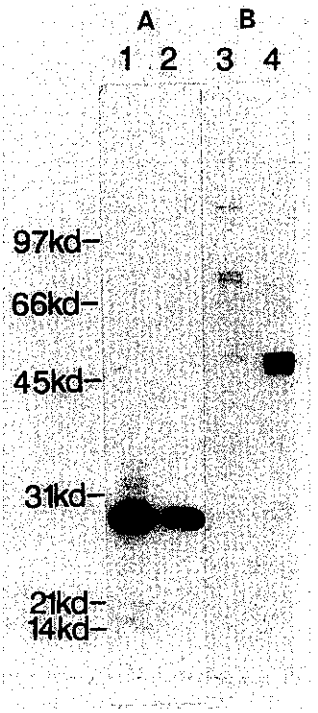

Fig. 4. Immunoblotting of KTA7 and KTS9 cells with anti-PGP9.5 and anti-NSE sera. Immunostaining of 2 μg of KTA7 and KTS9 cell protein following SDS-PAGE and western blotting using anti-PGP9.5 serum (A). Lane 1, KTA7; lane 2, KTS9. Western blots of KTA7 and KTS9 cells using anti-NSE serum (B). Lane 3, KTA7; lane 4, KTS9.

PAGE and immunoblotting using anti-PGP9.5 and anti-NSE sera. When analyzing samples of KTA7 and KTS9 cells by immunoblotting, a PGP9.5 polypeptide of molecular weight (MW) 27 kd was specifically immunostained in KTA7 and KTS9 cells. KTS9 cells were positive for NSE with a band of MW 48 kd, while KTA7 was not immunostained (Fig. 4). Cells of these two cell lines were not immunostained by normal rabbit serum (data not shown).

Results of RT-PCR The RT-PCR procedure was conducted on total cellular RNA to detect N-CAM gene expression in pulmonary neuroendocrine tumor cell lines including Lu134, KTZ6 and KTS9 cells, human lung and cerebral cortex. The results are shown in Fig. 5. N-CAM transcripts were found in the cerebral cortex, fetal lung tissue, Lu134, KTZ6 and KTS9 cell lines. However, no transcripts of the N-CAM gene could be detected in adult lung.

Comparison among KTA7 and KTS9 cell lines and 2 surgical cases of large cell carcinoma with NE features Table I summarizes the results of a comparison among

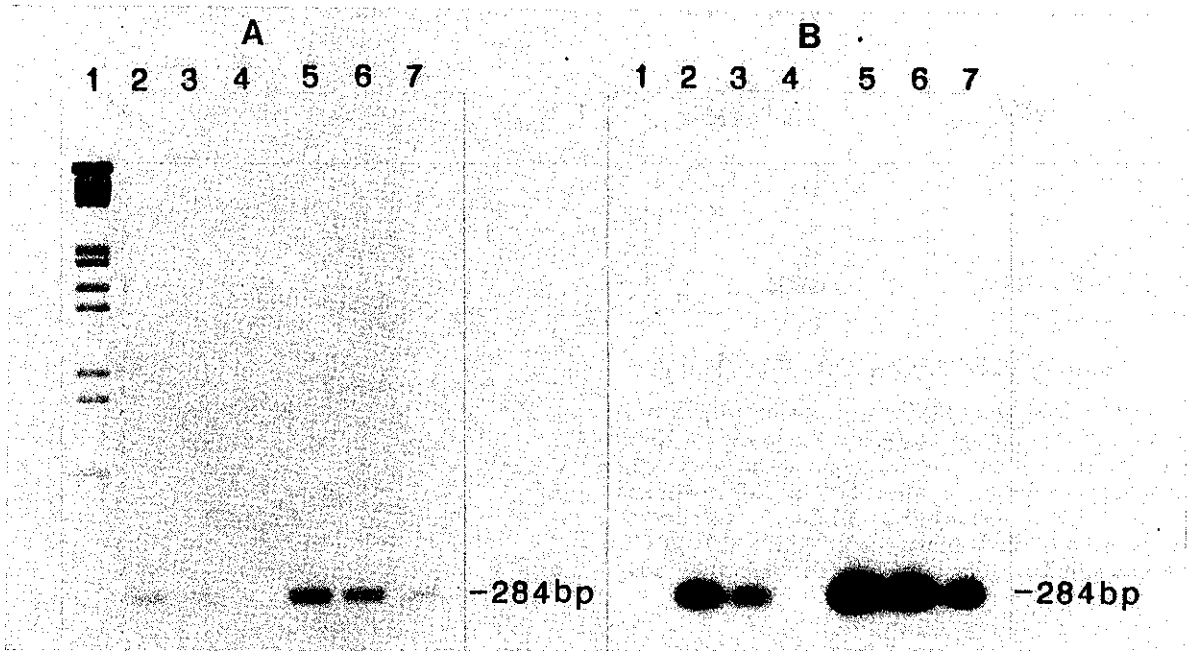

Fig. 5. RT-PCR analysis. cDNA was synthesized with total cellular RNA from cell lines and tissues. Twenty-five cycles of PCR were coducted. (A) shows ethidium bromide staining after 2% agarose electrophoresis of PCR products. The bar indicates 284 bp of N-CAM transcripts to be specifically amplified. (B) Southern blot analysis of PCR products with N-CAM probe. Lane 1, phage DNA following digestion by *Hind* III as molecular size markers; lane 2, cerebral cortex; lane 3, fetal lung tissue; lane 4, adult lung tissue; lane 5, Lu134 cell line; lane 6, KTZ6 cell line; lane 7, KTS9 cell line.

Table I. Comparison among KTA7 and KTS9 Cell Lines, and 2 Surgical Cases of Large Cell Carcinoma with NE Differentiation

	KTA7	KTS9	Case 1	Case 2
Histology of original tumor	Large cell ca +adenocarcinoma	Large cell ca +squamous cell ca	Large cell ca	Large cell ca
Histology of cultured cell and nude mouse transplanted tumor	Large cell ca	Large cell ca		
Growth patten of cultured cells	Grew in floating cell clusters	Formed monolayers of epithelioid cells and detached spontaneously		
Doubling time	28 h	43 h		
EM	Neurosecretory granules absent	Neurosecretory granules absent	Neurosecretory granules absent	A few neurosecretory granules present
NE markers	PGP9.5, N-CAM, alpha-hCG, AADC	NSE, PGP9.5, SYN, N-CAM, NF, CG-A	NSE, PGP9.5 N-CAM, NF	NSE, PGP9.5, NF, N-CAM, proGRP
Epithelial markers	Cytokeratin	EMA, SLEX,	EMA, CA19-9 Cytokeratin	EMA, Cytokeratin
Stage	I	II	IV	I
Survival[a)]	dead, 10 mo[b)]	dead, 11 mo	dead, 12 mo	alive, 9 mo

a) The survival of KTA7 or KTS9 is that of patients with the original tumors of both cell lines.
b) Months.

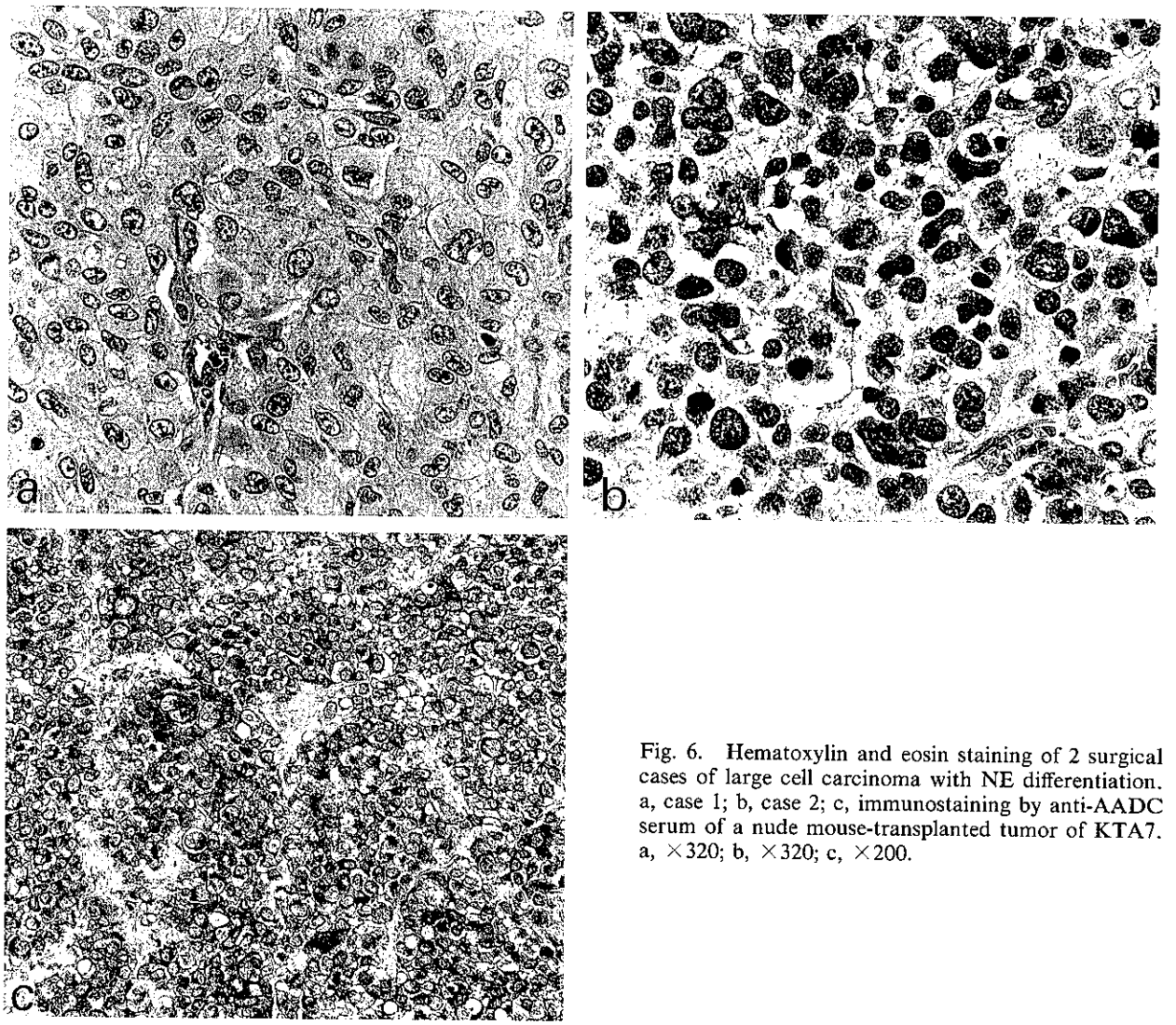

Fig. 6. Hematoxylin and eosin staining of 2 surgical cases of large cell carcinoma with NE differentiation. a, case 1; b, case 2; c, immunostaining by anti-AADC serum of a nude mouse-transplanted tumor of KTA7. a, ×320; b, ×320; c, ×200.

KTA7 and KTS9 cell lines and 2 large cell carcinomas with NE features. The original tumors of KTA7 and KTS9 cell lines consisted mainly of large cell undifferentiated carcinomas and lacked the morphological features of small cell carcinoma, as shown in Table I. The histological features of cultured cells and nude mouse transplanted tumors and 2 surgical cases of large cell carcinomas were the same as those of large cell carcinomas (Fig. 6a, 6b) but without the morphological features of neuroendocrine tumors, such as organoid, palisading, and trabecular or rosette-like histological patterns. KTA7 cultured cells grew as floating cell clusters, while KTS9 formed monolayers of epithelioid cells and detached spontaneously from the flask floor, giving rise to single cells and small cell clusters. The population doubling times of KTA7 and KTS9 were relatively short, 28 h and 43 h, respectively. On electron microscopic examination of KTA7 and KTS9, no consistent or specific features were seen but the cytoplasm possessed free ribosomes and scattered organelles and lacked neurosecretory granules. KTA7 expressed PGP9.5, N-CAM, alpha-hCG and AADC (Fig. 6c) and KTS9 had NSE, PGP9.5, N-CAM, NF, SYN and CG-A. The tumor cells of 2 large cell carcinomas with NE features were positive for Grimelius staining and expressed NSE, PGP9.5, N-CAM, and NF but not CG-A, alpha-hCG, SYN or AADC. The cancer cells of case 1 had no neurosecretory granules, while some tumor cells of case 2 showed a few scattered neurosecretory granules and pro-GRP, a peptide closely related to bombesin.

The stage and survival of patients with the original tumors of KTA7 or KTS9 cells and 2 surgical cases are summarized in Table I. Two patients with the original tumors of KTA7 and KTS9 had stage I and II tumors and died of their disease at 10 months and 11 months following the initial diagnosis. One patient (case 1) of the 2 surgical cases had stage IV tumor and was dead within 12 months. The remaining patient (case 2) had stage I tumor and was alive without evidence of disease at 9 months.

DISCUSSION

A new pulmonary large cell carcinoma cell line, KTS9, established in a serum-free medium, ACL-3, was found to express neural and neuroendocrine markers including NSE, PGP9.5, NF, N-CAM, SYN and CG-A, but not Leu7 or AADC. Such a cell line derived from large cell carcinoma with NE markres has not previously been described. A large cell carcinoma cell line, KTA7, was found to express PGP 9.5, N-CAM, alpha-hCG and AADC but not NSE, NF, SYN, CG-A or Leu7. The original tumors of the KTS9 and KTA7 cell lines consisted mainly of large cell undifferentiated carcinoma (LCUC) components and lacked a small cell carcinoma component. These cell lines were thus considered to have been derived from large cell components having various NE markers. Some LCUCs express neuroendocrine properties and have been subdivided into large cell undifferentiated carcinomas with neuroendocrine differentiation (LCUC with NE) and large cell neuroendocrine carcinomas (LCNEC).[6-10] LCNECs have light microscopic (LM) features in common with NE tumor and express NE markers as detected by immunohistochemistry (IHS) or electron microscopy (EM).[10] In contrast to LCNEC, LCUC with NE has NE properties as demonstrated by IHS or EM but lacks the LM morphological features seen in NE tumors.[10] The present study indicates KTS9 and KTA7 cell lines to derive from LCUC-NE since both have some general NE markers but lack LM morphological features. This was also noted for 2 surgical cases of large cell carcinoma with NE differentiation.

Recently, various SCCL-derived cell lines have been established. Examination of SCCL cell lines has clarified the biological and NE features of SCCL cells, which are of classic and variant types.[4,5] SCCL morphological variant cell lines were established from patients with mixed small cell / large cell carcinoma, a subtype of SCCL.[4] Certain SCCL cell lines of classic type have undergone conversion to variant types during *in vitro* cultivation or xenotransplantation.[5] Variant cell lines have altered morphology resembling mixed small cell/large cell carcinomas or LCUC and relatively short doubling times.[5] KTA7 and KTS9 cell lines were derived from LCUC-NEs, which did not contain a small cell component and were thus diagnosed as non-SCCL by LM and showed NE properties by IHS, while variant cell lines are believed to be an *in vitro* correlate of the mixed small cell/large cell carcinoma, a subtype of SCCL.[5] SCCL variant types lack certain NE markers such as dense core granules, AADC and GRP. The feature is referred to as the discordant expression of NE markers in SCCL variant cell lines.[4,5] KTA7 and KTS9 had some but not all general NE markers, indicating their discordant expression of NE markers. They had large cell carcinoma morphology and relatively short doubling times, indicating their close relation to morphological SCCL variant cell lines. Patients with SCCL variant morphology have a poor prognosis and response to therapy compared to those with pure type of SCCL.[4] Large cell neuroendocrine carcinomas predictably take a more aggressive clinical course than most large cell carcinomas.[6] Two patients with LCUC-NE had stage I and II tumors and died of their malignancy between 10 and 11 months following the initial diagnosis in this study. Large cell carcinoma morphology of pulmonary carcinomas with NE features may thus represent high-grade malignancy.

Patients having non-SCCL with NE tumors have been suggested to respond more favorably to chemotherapy than patients having other non-SCCL tumors.[27] KTA7 and KTS9 cell lines are expected to be useful for the study of the *in vitro* response to chemotherapeutic drugs and radiation of LCUC-NE, since clinical response of the tumors was correlated with the *in vitro* sensitivity of the cell lines to chemotherapeutic drugs and irradiation. Thus, such responses should be tested by a soft agar clonogenic assay in future.

Leu19 and 6H7 antigens were shown to be expressed in large cell carcinomas with NE markers by flow cytometry or immunohistochemistory. The 6H7 antigen is a polypeptide of 128 kd[18] and may thus be considered to belong to the cluster I antigen of small cell carcinoma.[11,28] Recently, the antigenic specificity of the cluster I antigen was found to be quite similar to that of the N-CAM.[29,30] Leu19 was also found to recognize the N-CAM molecule of 140 kd.[31] N-CAM polypeptides were noted to be present in various human NE cells and tumors by immunohistochemistry, flow cytometry or western blotting.[32-35] Recently, the distribution of N-CAM mRNA in NE cells and tumors has been demonstrated by *in situ* hybridization.[33] RT-PCR using oligonucleotide primers and a probe demonstrated its messages to be expressed in pulmonary NE cell lines and neural tissues in the present study. Thus, N-CAM mRNA as well as its polypeptide are broad-spectrum markers for NE cells and tumors.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research (I-5) from the Ministry of Health and Welfare of Japan, and a Grant-in-Aid for Scientific Research (02770166) from the Ministry of Education, Science and Culture of Japan. We are grateful to Drs. Yukio Shimosato and Takeshi Terasaki, the National Cancer Center Research Institute, Tokyo, for kindly providing the Lu134 cell line and Mrs. Tomoko Tsuruta, Kuniko Kadoya and Benio Sato for their technical assistance.

(Received April 22, 1992/Accepted June 26, 1992)

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