Establishment of Human Peripheral Lung Epithelial Cell Lines (HPL1) Retaining Differentiated Characteristics and Responsiveness to Epidermal Growth Factor, Hepatocyte Growth Factor, and Transforming Growth Factor β1

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ABSTRACT

Novel human epithelial cell lines retaining characteristic features of normal peripheral airway cells were established by transfecting the SV40 large T antigen gene into primary in vitro outgrowths from normal peripheral lung specimens. These lines, designated as HPL1A to HPL1E, showed the polygonal shapes typical of epithelial cells and expressed cytokeratin in abundance. Ultrastructural examination revealed the presence of microvilli, multivesicular bodies, and multilamellar body-like structures that are characteristic of type II pneumocytes, but expression of clari transcripts, a highly specific marker for Clara cells, was also observed. Response to transforming growth factor β, epidermal growth factor (EGF), and hepatocyte growth factor, all of which are thought to be important growth-regulatory molecules for cellular proliferation and developmental processes of peripheral lung, was apparent. In the HPL1A case, markedly altered cell morphology and cytoskeletal organization, potent inhibition of cell growth, and increased expression of an extracellular matrix protein were noted with transforming growth factor β. Interestingly, both EGF and hepatocyte growth factor stimulated anchorage-independent growth, whereas only EGF could sustain anchorage-independent proliferation. The HPL1 lines are, to our knowledge, the first series of stable epithelial lines of human peripheral lung to be described. They should be valuable for investigating various aspects of growth regulation and oncogenic processes, including the mechanisms of acquisition of anchorage independence and the interrelationships of genetic changes identified previously in lung cancers. In addition, the HPL1 lines may also prove useful for development of in vitro models for other human lung disorders as well as to elucidate the mechanisms of peripheral lung differentiation.

INTRODUCTION

Lung cancer is one of the most common neoplasms in humans. Recent studies on the molecular pathogenesis of this highly fatal disease have revealed that a complex series of genetic and epigenetic events are involved in the malignant transformation of normal lung cells. There are four major histological subtypes: small cell carcinomas, adenocarcinomas, squamous cell carcinomas, and large cell carcinomas, and the occurrence of genetic changes is known to vary considerably with each, suggesting that certain genetic defects may have differential influence depending on the subtype. Among the four major subtypes, the incidence of adenocarcinomas is the highest in Japan. It is gradually increasing as in the United States and other economically advanced countries. Because adenocarcinomas are thought to arise mostly from the bronchioles and pneumocytes of peripheral lung, studies using NHPL1E cells are of great interest. However, NHPL1E cells can only be maintained in primary culture for a short time before cellular senescence occurs; therefore, their immortalization is necessary for studies of multistage carcinogenic process in vitro. Most squamous cell carcinomas and small cell carcinomas arise from normal human bronchial epithelial cells of the proximal airways, and successful establishment of lines of such cells by the two independent groups of Harris and colleagues (2, 3) and Viallet et al. (4) has made it possible to investigate various aspects of bronchial carcinogenesis, including carcinogen metabolism, cellular physiology, and regulation of differentiation (5–11). Establishment of immortalized epithelial cell lines retaining features of NHPL1E cells has, to our knowledge, not been described thus far.

In the present study, we therefore aimed at immortalizing NHPL1E cells by introduction of the SV40 large T antigen gene. We here report successful establishment and extensive characterization of peripheral lung epithelial cell lines, termed HPL1A to HPL1E, derived from explants in vitro. These lines are nonmutomgeric and retain several characteristics of peripheral lung differentiation. In addition, they respond to EGF, HGF and TGF-β, important growth-regulatory molecules for the peripheral lung in cellular proliferation and developmental processes.

MATERIALS AND METHODS

Chemicals and Culture Medium. Bovine insulin, human transferrin, ECGS, hydrocortisone, triiodothyronine, and N-2-hydroxypyridinone-2-N'-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human EGF and recombinant human HGF were obtained from Austral Biologicals (San Ramon, CA) and Becton Dickinson Labware (Bedford, MA), respectively. Suppliers of other chemicals were as follows: Life Technologies, Inc. (Gaithersburg, MD) for ham's F-12 powder; List Biological Laboratories, Inc. (Campbell, CA) for cholera toxin; and Irvine Scientific (Santa Ana, CA) for FCS. The medium used for primary culture of explants was essentially the same as that described by Wu et al. (12). Ham's F-12 was buffered with 15 mM HEPES (pH 7.3) and supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 10−7 M hydrocortisone, 2 x 10−16 M triiodothyronine, 10 ng/ml cholera toxin, 20 ng/ml EGF, 10 μg/ml ECGS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone (hereafter referred to as the primary culture medium). FCS was added at the concentration of 5% until transfection of SV40 large T antigen gene, and then FCS was reduced to 1% during the course of further propagation. The established cell lines were maintained in Ham's F-12 medium buffered with 15 mM HEPES (pH 7.3) and supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 10−7 M hydrocortisone, 2 x 10−16 M triiodothyronine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 1% FCS (hereafter referred to as the standard medium).

Received 3/17/97; accepted 9/3/97.

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1 This work was supported in part by a Grant-in-Aid for the Second Term Comprehensive Ten-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan, as well as a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, Japan.

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3 The abbreviations used are: NHPL1E, normal human peripheral lung epithelial; EGF, epidermal growth factor; HGF, hepatocyte growth factor; TGF, transforming growth factor; ECGS, endothelial cell growth supplement; CMV, cytomegalovirus; RT-PCR, reverse transcription-PCR.
Establishment of Cell Lines. Human normal lung tissues were obtained at surgery for lung cancer. Small pieces of parenchyma, remote from cancerous areas, were cut into approximately 0.3 × 0.3 × 0.3-cm fragments using scissors, vigorously washed with Ham's F-12, and placed onto 35-mm culture dishes at sites where approximately 0.5 × 0.5-cm areas of the surface had been scratched with a scalpel blade. Typically, six dishes containing five explants each were prepared from a donor. The explants were allowed to adhere to the dish surface by incubation at 37°C for 30 min and then supplied with 1.5 ml of primary culture medium and incubated at 37°C in a humidified atmosphere with 5% CO₂. The medium was changed twice a week.

Outgrowths of epithelial cells obtained by further culture for 25 days were then transfected with 2 μg of a CMV promoter-driven expression vector of SV40 large T antigen gene (Ref. 13; CMV-SLT, a gift from Dr. T. Kiyono, Aichi Cancer Center) using 5 μl of lipofectin reagent (Life Technologies, Inc.) as described in the manufacturer's protocol. Transfected cells were maintained for 2 weeks in the same dish and then trypsinized and transferred to new dishes. After secondary culture, the cells were cultured in medium supplemented with 1% FCS to prevent cellular senescence and passed every 2 weeks. After 2 months, colonies that continued to grow were selected and replated at low cell density on mitomycin C-treated Swiss 3T3 feeder layers for further isolation with cloning cylinders.

Soft Agar Culture. Colony formation in soft agar was assayed essentially as described previously (14). Briefly, aliquots of 2.5 × 10⁴ cells were suspended in 1.5 ml of the standard medium containing 0.33% Noble agar (Difco, Detroit, MI) at 43°C and plated on 35-mm culture dishes layered with 1 ml of solidified 0.5% Noble agar in the standard medium. EGF and HGF were supplied to both layers. Dishes were incubated at 37°C for 2 or 3 weeks. The viable colonies were stained with p-iodonitrotetrazolium violet (Sigma) (15), and those consisting of more than 10 cells were counted.

Cell Growth Analysis. Cells were dissociated with 0.1% trypsin and 0.05% EDTA in PBS for 5 min at 37°C, plated into 35-mm plastic dishes (5 × 10⁴ cells/dish), and cultured in the standard medium supplemented with 1% FCS. After 24 h, cells were refed with fresh medium containing the indicated amounts of EGF or HGF. Medium was changed every other day. Cells were dissociated by trypsinization and counted with a hemocytometer on day 3 (Figs. 2A and 7) or every other day (Fig. 3). TGF-β treatment was also initiated 24 h after inoculation of aliquots of 5 × 10⁴ cells into 35-mm dishes. Cells were fed every other day until they were counted at day 7.

Immunohistochemistry. Following serial fixation of cells cultured on coverslips (24 × 24 mm) with 70% and 100% ethanol for 15 min each at −20°C, they were incubated with anti-cytokeratin monoclonal antibody (K8.13 from Sigma) at a 1:100 dilution for 1 h at 37°C, followed by incubation with fluorescein-conjugated goat anti-mouse IgG antiserum (Cappel Laboratories, Shizuoka, Japan), which were then monitored for visual and palpable tumors for 100 days before sacrifice for further examination. Each cell line was tested with uranyl acetate and lead citrate and examined with a JEOL-1200EX transmission electron microscope (Nikon Co., Tokyo, Japan).

Rhodamine-conjugated phallolidin (Molecular Probes, Inc., Eugene, OR) was used as a direct probe to detect actin filaments. Cells on plastic culture dishes were rinsed with PBS three times, fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 50 mM phosphate buffer (pH 7.4), postfixed in 1% OsO₄, dehydrated in graded ethanol, and embedded in Epon 812. Ultrathin sections prepared using an ultramicrotome (Reichert-Jung, Wien, Austria; Ultratc E type) were stained with uranyl acetate and lead citrate and examined with a JEOL-1200EX (JEOL, Tokyo, Japan) electron microscope.

Tumorigenicity Assay in Nude Mice. Aliquots of 5 × 10⁶ cells were injected s.c. into the flanks of 5-week-old KSN-nu/nu mice (Japan SLC Inc., Shizuoka, Japan), which were then monitored for visual and palpable tumors for 100 days before sacrifice for further examination. Each cell line was tested in three separate mice. The number inoculated (5 × 10⁶) is the same as that used for the tumorigenicity assay of BEAS2B. A representative human bronchial epithelial cell line immortalized with SV40 large T antigen, as well as for its derivatives transfected with oncogenes (2, 7, 9).

Western Blot Analysis. An anti-tropomyosin monoclonal antibody (TM311), specific for high molecular weight tropomyosin isoforms, was purchased from Sigma. Anti-chicken cellular fibronectin polyclonal antibody, cross-reacting with human cellular fibronectin but not serum fibronectin, was kindly supplied by Dr. K. Kimata (Aichi Medical University). After treatment with TGF-β at the indicated concentrations for 6 days, cells were lysed in Laemmli's sample buffer. Two-μg samples of the solubilized proteins were electrophoresed on 12.5% SDS-polyacrylamide gels for tropomyosins and transferred to Clear Blot Membrane-P filters (Atto, Tokyo, Japan). The filters were incubated with the primary antibodies and then stained by the ABC method using Vectastain (Vector Laboratories, Inc., Burlingame, CA) and Konica Immunostaining HPR (Konica, Tokyo, Japan) kits. Experimental conditions for the detection of human cellular fibronectin were the same except for the use of 10 μg of proteins and 7% SDS-polyacrylamide gels.

Southern Blot Analysis. Five-μg aliquots of genomic DNA were digested with EcoRI or NcoI and subjected to electrophoresis on 1.0% agarose gels. DNA was then transferred to Hybond N+ nylon membranes (Amersham, Buckinghamshire, United Kingdom) in 0.1 M NaOH/0.1 M NaCl. Hybridization, washing, and autoradiography were performed as described previously (16). A 2.6-kb EcoRI and BamH1 fragment excised from the CMV-SLT expression construct was used as a probe.

RT-PCR Analysis. RT-PCR analysis was performed with randomly primed cDNAs synthesized from 5 μg of total RNA using SuperScript II (Life Technologies, Inc.). After 35 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C, PCR products were separated on 2% agarose gels and stained with ethidium bromide. Primers used and molecular sizes of the resulting PCR products were as follows: human CC10, 5'-TCAGAGACGGAAAC-GAGAC-3' and 5'-GATCTTCCAGCTTTCAATTGTC-3' (340 bp); human SP-A (exons 3 and 6), 5'-CTCATCGGTGGAAGGGAAGAAGG-3' and 5'-CCAGGTTGTTGATTTACAG-3' (606 bp); human SP-B (exons 4 and 9), 5'-GAACAAAGTGCGTGTGACAG-3' and 5'-CTGTGCCCAAAACCTGTG-3' (689 bp); human SP-C (exons 1 and 4), 5'-AAAGAGGGTCCTGATGGAAAGG-3' and 5'-TGGACTCTTTTCTAGTGAGGC-3' (410 bp); and human β-actin (exons 4 and 6), 5'-GACCTGCATGAAGATC-3' and 5'-GATCCACATCTGCTGGA-3' (517 bp).

RESULTS

Establishment of Cell Lines. Epithelial cells emerged around explants approximately 10 days after initiation of primary culture. When cultured in the primary culture medium supplemented with 1% FCS, most of the outgrowing cells showed an oval shape and were loosely attached to neighboring cells (Fig. 1A), whereas those in the medium supplemented with 5% FCS were polygonal in shape with a typical cobblestone appearance (Fig. 1B). Although these cells actively proliferated in the primary culture, this generally ceased after a single passage.

We, therefore, transfected SV40 large T antigen gene at day 25 to immortalize the primary culture cells. Transfected cells were transferred to 60-mm dishes 2 weeks later. Seven independent experiments were conducted with normal lung specimens from seven separate donors. Finally, five stable clones were obtained from the normal lung of a 74-year-old female donor, who had never smoked and underwent surgery for a small well-demarcated squamous cell carcinoma of the earliest disease stage (ptN₂M₀, stage I). Precancerous regions of peripheral lung such as atypical adenomatous hyperplasia were not noted by the conventional pathological examination. Because the resulting clonal lines appeared to have arisen from a single immortalized cell (see below), they were designated as HPLI1 to HPLI16 to reflect this. The HPLI1 lines have continued to proliferate for more than 15 months and appear to have acquired an infinite life span. In contrast, all other 46 clones isolated from the same donor eventually underwent a marked slowing in proliferation and entered cellular senescence after several passages.

Because ECGS and cholera toxin did not significantly affect the growth and morphology of the cell lines, they were omitted from the standard medium to maintain the HPLI1 lines (Fig. 1, C and D). In addition, we also noted that withdrawal of EGF from the standard...
Fig. 1. Phase-contrast and immunofluorescence microphotographs of primary NHPLE cell cultures and the HPL1 series of immortalized peripheral lung cell lines. A and B, primary cultured cells at day 25 in a medium containing 1 and 5% FCS, respectively. C, HPL1A in the standard medium. D and E, HPL1D cultured in the standard medium in the presence and absence of 20 ng/ml of EGF, respectively. Bar, 100 \( \mu \text{m} \). Photographs from A to E were taken at the same magnification. F, immunofluorescence staining of HPL1D for cytokeratins. Every cell shows abundant cytokeratin expression. Other HPL1 lines were also stained with the cytokeratin antibody (data not shown). Bar, 50 \( \mu \text{m} \).

medium was required to maintain their epithelial polygonal morphology, because in the continued presence of EGF, they tended to become elongated, and the paving stone-like architecture was disrupted (Fig. 1, compare D and E).

Morphological Characteristics. Under phase-contrast microscopy, all five cell lines predominantly demonstrated the polygonal shape typical of epithelial cells, although slight inter-line variations were noted. HPL1A was slightly smaller and loosely contacted to the neighboring cells (Fig. 1C), whereas HPL1B and HPL1C had flatter shapes. HPL1D and HPL1E formed more tightly packed cell sheets of typical polygonal shape after reaching confluence (HPL1D in Fig. 1D).

The epithelial nature of the HPL1 lines was supported by uniform positive staining with the anti-keratin monoclonal antibody K8.13 (Fig. 1F). In addition, electron microscopy revealed microvilli and abundant vesicular structures (Fig. 2A), the latter including multivesicular bodies and incomplete multilamellar body-like structures, which are specific to type II pneumocytes (Ref. 17; Fig. 2B). Junctional complexes such as desmosomes or tight junctions were not evident, even in regions where cells were in close contact with each other (Fig. 2A), although adherence junction-like structures were occasionally observed. All of the established cell lines showed similar ultrastructural characteristics.

Chromosome numbers in metaphase were near-diploid in HPL1A, near-triploid in HPL1B, HPL1D, and HPL1E, and near-pentaploid in HPL1C. No clonal chromosomal translocations or deletions of regions frequently occurring in human lung cancers were observed. Such considerable variability in chromosome number has been suggested to be characteristic of SV40-induced immortalization (18). Molecular biological examinations did not reveal any mutations of the \( p53 \) gene or overexpression of the \( c-myc \) gene, which are frequently detected in human lung cancers, in the HPL1 lines, whereas expression of Rb protein also appeared to be normal (data not shown). These findings suggested that the established cell lines were derived from a normal
NOVEL HUMAN PERIPHERAL LUNG EPITHELIAL CELL LINES

CC10 mRNA expression in the presence of higher than 10% FCS was almost 100-fold more abundant than that with 1% FCS (data not shown). Expression of other surfactant proteins including SP-A, SP-B, and SP-C mRNAs was not detected in these five cell lines.

Effects of EGF and HGF. We also examined effects of EGF and HGF on the growth and morphology of HPL1 lines, because these polypeptide growth factors are known to have roles in proliferation of normal and cancerous cells of the adult lung as well as in fetal lung development (20-23). The addition of 20 ng/ml of EGF was found to greatly change cell shape and arrangement in monolayer culture, i.e., cells became elongated and piled up on each other, forming meshworks (results for HPL1D with and without EGF are shown in Fig. 1, E and D, respectively). Responsiveness to EGF addition was more pronounced with HPL1A, HPL1C, and HPL1D than in the HPL1B and HPL1E cases. Northern blot analysis showed that all of the lines expressed EGF receptor transcripts, and the expression levels in HPL1A and HPL1D cells were higher than in the others (data not shown). No TGF-α or EGF-like activity could be detected in the

Fig. 3. Growth curves of HPL1 lines. •, HPL1A; O, HPL1B; A, HPL1C; △, HPL1D; ■, HPL1E. The data are representative of three independent experiments.

peripheral lung epithelial cell, although the possibility that the cell lines might have been derived from a preneoplastic cell that was more susceptible to immortalization than a truly normal cell cannot be entirely ruled out.

Growth Characteristics. None of the five lines piled up under the standard culture conditions, even after reaching confluence. As shown in Fig. 3, their growth rates and saturation densities varied considerably, with doubling times ranging from 37.9 to 72.2 h, and saturation densities ranged from $3.6 \times 10^4$ cells/cm$^2$ to $23.5 \times 10^4$ cells/cm$^2$. With the exception of HPL1A, none of the lines could form colonies in an efficient manner in the standard medium supplemented with 1% FCS and 0.33% agar overlayed onto 0.5% agar with 1% FCS (Fig. 6B).

Transplantability to Nude Mice. Athymic nude mice were s.c. injected with $5 \times 10^6$ cells of each cell line and sacrificed 100 days after injection. None of the five cell lines formed palpable tumors. Microscopic examination of several injection sites confirmed this observation.

Integration of the SV40 Large T Antigen Gene. Southern blot analysis was performed to examine integration of the SV40 large T antigen gene (Fig. 4). Genomic DNA from the cell lines were cleaved with either EcoRI or NcoI. There were no EcoRI recognition sites and only a single NcoI site within the expression construct. Hybridization signals of the same length (7.0 and 7.4 kb in EcoRI and NcoI digests, respectively) were detected, suggesting that these five cell lines had been derived from a single transfected cell and that a single copy of the transduced gene had been integrated. Additional faint bands detected in HPL1B (9.1 and 3.8 kb in EcoRI and NcoI digests, respectively) probably reflect the presence of a minor population of cells carrying a rearrangement after the integration.

Expression of Peripheral Lung Cell-specific Genes. RT-PCR analysis revealed the presence of CC10 mRNA, which is known to be expressed specifically in Clara cells of the bronchioles (19), in HPL1A cultured in the standard medium supplemented with 1% FCS. In addition, we also noted that CC10 mRNA expression in HPL1A could be enhanced by raising the FCS concentration in the medium (Fig. 5A), whereas CC10 mRNA were easily detectable in HPL1D and HPL1E as well as HPL1A in the presence of 20% FCS (Fig. 5B). RT-PCR analysis using serially diluted template cDNAs showed that

Fig. 4. Southern blot analysis of integration of the SV40 large T antigen gene into genomic DNA of the HPL1 lines. Derivation of the HPL1 cell lines from a single transfected cell is suggested by the presence of major hybridization signals of identical sizes (arrowsheads) with both EcoRI and NcoI digests (7.0- and 7.4-kb bands, respectively), because the expression construct used has no EcoRI site and a single NcoI site. Faint bands (arrows) detected in HPL1B (9.1- and 3.8-kb bands with EcoRI and NcoI, respectively) probably reflect the presence of a minor population of cells with an additional rearrangement.

Fig. 5. Expression of Clara cell-specific CC10 mRNA in HPL1 lines detected by RT-PCR. A, marked induction of CC10 expression by FCS in a dose-dependent fashion. B, expression of CC10 in HPL1 lines in the presence of 20% FCS. RT-PCR of human β-actin was conducted as a control for the quality of the cDNAs. FCS conc., fetal calf serum concentration.
Fig. 6. EGF-induced stimulation of anchorage-dependent and -independent growth. A. growth stimulation of HPL1 lines by EGF (20 ng/ml) in monolayer cultures. The cells were cultured in a standard medium in the presence (▲) or absence (□) of EGF (20 ng/ml) for 4 days and counted on day 5. The data show means for three cultures; bars, SD. Similar results were obtained from three independent experiments. B. colony formation by HPL1 lines in soft agar under the standard medium in the presence (▲) or absence (□) of EGF (20 ng/ml). Data are means for three cultures; bars, SD. Similar results were obtained from three independent experiments.

conditioned media of HPL1A and HPL1D with the colony formation assay using NRK cells (Ref. 14; data not shown).

EGF also stimulated anchorage-dependent growth in all five HPL1 lines, with higher response in HPL1A, HPL1C, and HPL1D than in HPL1B and HPL1E (Fig. 6A). In addition, colony formation in soft agar was also markedly induced by the addition of 20 ng/ml EGF in the HPL1 lines except for HPL1B and HPL1E (Fig. 6B), showing that EGF can confer anchorage independence, a characteristic feature of malignant cells in vitro.

We also examined whether HGF could stimulate anchorage-dependent and -independent growth using HPL1D in comparison with EGF (Fig. 7). EGF stimulated both anchorage-dependent and -independent growth in a dose-dependent fashion, with plateaus after 1.0 and 3.0 ng/ml, respectively. HGF also stimulated anchorage-dependent cell growth, although the effective concentration was approximately 10 times higher than that with EGF. Notably, in contrast to EGF, HGF failed to elicit anchorage-independent growth, even at the concentration of 100 ng/ml.

Effect of TGF-β. We also examined whether responsiveness to TGF-β had been maintained during the immortalization process, because this pluripotent polypeptide is known to induce inhibition of proliferation, stimulation of extracellular matrix formation, and modulate cytoskeletal organization in various normal epithelial cells, including primary cultures of bronchial epithelial cells and type II pneumocytes (24, 25), with the response often being lost in malignant cells. TGF-β even at a relatively low concentration (0.2 ng/ml) caused HPL1A cells to adopt an enlarged, flattened and angular shape and stimulated formation of thick and straight actin stress fibers (Fig. 8A). Proliferation was also found to be significantly inhibited by the addition of TGF-β (Fig. 8B). Furthermore, Western blot analysis revealed that the expression of cellular fibronectin was drastically induced, and the amount of high molecular weight tropomyosin isoforms was increased by the addition of TGF-β in a dose-dependent manner. These results provided a clear demonstration that HPL1A cells retain a responsiveness to TGF-β.

DISCUSSION

In the present study, we established immortalized peripheral lung epithelial cell lines, termed HPL1A to HPL1E, that retain several features of NHPLE cells. Considering the uniform integration pattern observed for the SV40 large T antigen gene, these clonal lines were concluded to be probably derived from a single transfected cell, produced from seven independent attempts. A rare occurrence of immortalization was also reported for establishment of proximal airway cell lines (2), suggesting that the underlying processes are difficult to be enacted in both proximal and peripheral lung epithelial cells. This is, to our knowledge, the first report of successful establishment of immortalized human peripheral airway cells.

The HPL1 lines were found to exhibit morphological and biochemical characteristics of both types of epithelial cells in the peripheral
airways, i.e., Clara cells and type II pneumocytes. The observed features include formation of cell sheets of polygonal shape, presence of multivesicular bodies, and incomplete multilamellar body-like structures, as well as expression of cytokeratin and the Clara cellspecific protein, CC10. Furthermore, we also observed growth stimulation in response to the addition of HGF, which is produced by mesenchymal cells and stimulates proliferation of epithelial cells (26). It is, therefore, suggested that HPL1 lines may be derived from some immature NHPLE cell, capable of differentiating into both Clara cells and type II pneumocytes, in line with the suggested existence of common precursor cells (27).

Mice lacking the EGF gene have been shown to demonstrate defects in lung morphogenesis, including collapsed alveoli and dilated terminal bronchi (20). HGF is believed to play a role in tissue repair after acute lung injury (28). It is thus notable that monolayer cultures of the HPL1 lines respond to both of these peptide growth factors. Furthermore, anchorage-independent growth, one of the characteristics of transformed cells, can be conferred by EGF on HPL1A, HPL1C, and HPL1D. These findings are intriguing, because overexpression of TGF-α, a family member of EGF, has been reported to be frequent in pulmonary adenocarcinomas (29, 30). Interestingly, in contrast to EGF, HGF did not stimulate anchorage-independent growth, even at very high concentrations, although EGF and HGF receptors with tyrosine kinase activities share several common signal transduction pathways including ras, phospholipase C-γ, and phosphoinositol-3-kinase (31). Future studies on the signal transduction pathways as well as of transcriptional and cell cycle controls using the HPL1 cell lines should shed light on the mechanisms underlying acquisition of a malignant phenotype by NHPLE cells.

Recent studies on cell cycle control elements in relation to anchorage dependence have revealed that suppression of cyclin E-cyclin-dependent kinase 2 kinase activity by cyclin-dependent kinase inhibitors and/or reduced cyclin A expression may play a pivotal role in blocking fibroblastic cells from undergoing anchorage-independent growth (32, 33). However, differences between fibroblasts and epithelial cells may exist in this context, because it was reported previously that unlike fibroblasts, normal epithelial and endothelial cells undergo apoptosis instead of G₁ arrest under anchorage-independent conditions (34–36). Our HPL1 cell lines may thus be valuable to clarify the roles of cyclin E and cyclin A as well as other as yet unidentified molecules involved in the anchorage independence, specifically of peripheral lung epithelial cells.

TGF-β has been suggested to be important for the development of the peripheral airways (37) as well as their physiological functions in adults. Unresponsiveness to TGF-β has been reported for lung cancer cell lines (38, 39), and we showed recently that a fraction of lung cancers carry mutations in the DPC4/Smad4 and Smad2 (Jv18–1) genes at 18q21, both of which encode Smad family proteins active in the TGF-β signaling pathway (40, 41). The fact that demonstrated marked responses to TGF-β in the present study means that it might find application in an in vitro system to investigate functional consequences of alterations in the TGF-β signaling pathway in the carcinogenic process of peripheral lung epithelial cells.

There is increasing evidence that transformation of human normal cells results from a complex series of genetic and epigenetic events. Recent molecular biological studies have identified a number of tumor suppressor genes and dominant oncogenes involved in the pathogenesis of human lung cancers (1). The present first successful establishment of immortalized peripheral lung epithelial cell lines will allow us to examine physiological functions, in relation to carcinogenicity, in precise detail. In addition, HPL1 lines may also be useful for development of in vitro systems to study other human lung disorders such as cystic fibrosis, as well as to elucidate how differentiation toward distinct peripheral lung epithelial cell types is controlled.
ACKNOWLEDGMENTS

We thank Drs. T. Kiyono (Aichi Cancer Center Research Institute) and K. Kimata (Aichi Medical University) for their gifts of an expression vector for the SV40 large T antigen gene and anti-chicken cellular fibronectin polyclonal antibody, respectively.

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