Characterization of Three New Variant Type Cell Lines Derived from Small Cell Carcinoma of the Lung

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ABSTRACT

Three new, well growing cell lines (GLC-1, GLC-2, and GLC-3) have been established from small cell lung carcinoma (SCLC) and characterized. A subclone (GLC-1-M13) markedly different from its parent line GLC-1 was also isolated and characterized. Cytogenetic analysis of the cell lines revealed deletions in the short arm of chromosome 3 as a most consistent chromosomal aberration. The deleted region was not identical in all metaphases, 3p(21–23) being the shortest region of overlap. Despite their SCLC origin GLC-1, GLC-2, and GLC-3 do not show pronounced SCLC differentiation features. Neurosecretory granula were very rare (GLC-1) or completely absent (GLC-2 and GLC-3), whereas the SCLC-related enzyme and hormone markers l,3,4-dihydroxyphenylalanine decarboxylase, neuron-specific enolase, creatine kinase BB, and bombesin-like immunoreactivity were variably expressed. Although the subclone GLC-1-M13 was derived from the poorly differentiated GLC-1, it behaved according to the above criteria as a differentiated “classic” SCLC cell line. When assessed with specific monoclonal antibodies the different cell lines appeared to express different subsets of intermediate filament proteins, indicative for different stages and directions of differentiation: “undifferentiated” (GLC-1 and GLC-2); “neural tissue related” (GLC-2); “simple epithelium” related (GLC-1-M13); and a combination of simple and squamous epithelium related (GLC-3).

We conclude that GLC-1, GLC-2, and GLC-3 represent dedifferentiated forms of SCLC, related to the recently described “variant” type of SCLC, whereas the clonal derive GLC-1-M13 behaves like a differentiated “classic” SCLC cell line.

INTRODUCTION

According to the latest WHO classification five major forms of lung cancer can be recognized on histological grounds, epidermoid (squamous), small cell, large cell, adenocarcinoma, and adenosquamous cell carcinoma (1). Such a classification might not be fully satisfying however, since mixing of different forms and also transitions from one form to another have been reported (2–4). In vitro similar conversions have been documented (5–7). Therefore it has been proposed that lung cancer comprises in fact a continuous spectrum of cancer types, possibly originating from one common malignantly transformed “stem cell” population, in which different histological appearances reflect preferentially expressed (normal) differentiation pathways (6). The establishment of a growing number of lung tumor-derived cell lines has been reported in the past years (6, 8, 9). The availability of continuously growing cell lines facilitates the study of the biology of lung cancer and it can be anticipated that such a study will eventually elucidate the relations between the different forms of this disease. Here we describe the isolation and characterization of three new, SCLC-derived cell lines which, although partly undifferentiated still show combinations of features characteristic for different forms of differentiated lung cancer.

MATERIALS AND METHODS

Morphological Methods. For light microscopy the tumor biopsy material was fixed in 8% formaldehyde, embedded in Epon, and stained with toluidine blue. For transmission electron microscopy the tumor biopsy or, in the case of cell lines, the isolated cell pellets were incubated in 2% glutaraldehyde in phosphate-buffered saline. Postfixation was performed in 2% osmium tetroxide in phosphate-buffered saline. The material was embedded in Epon. Further preparations were according to routine procedures.

Growth Conditions and Media. Cell lines were kept at 37°C in a humidified atmosphere in a CO2 incubator (5% CO2) and subcultured once or twice weekly by dilution in fresh growth medium. The growth medium was RPMI 1640 (Gibco, Paisley, United Kingdom) supplemented with 5 x 10^-3 m β-mercaptoethanol, 2 m glutamine, 1 m pyruvate, and gentamicin (50 μg/ml). Serum-containing medium was supplemented with 15% fetal calf serum, whereas in serum-free medium hydrocortisone (10^-8 M), estradiol (10^-8 M), sodium selenite (3 x 10^-8 M), bovine serum albumin (10^-8 M), bovine insulin (5 μg/ml), human transferrin (10 /μg/ml), 17β-estradiol (10^-6), sodium selenite (3 x 10^-6 M), bovine serum albumin (1%), arginine vasopressin (10 ng/ml), bombesin (10^-7 M), and ethanalaminephosphorylethanolamine (10^-8 M) was added according to the method of Minna et al. (10).

Growth in semisolid medium was assessed after triturating the cell clumps and filtering the obtained suspension (Nylar filter, 20 μm). In this way a single cell suspension was obtained. The cells were harvested by centrifugation and subsequently suspended in serum-containing medium to which 2% (w/v) gelatin was added. (Searprep 15/45; FMC, Rockland, ME) was added.

Chromosomal Analysis. Chromosome studies were carried out on cell cultures that had been in continuous serial passage for 6–14 months after explantation. The cells received fresh medium 1 day before harvest. Vinblastine sulfate (Lilly, Indianapolis, IN) was added to a final concentration of 20 ng/ml for the last 2.5 h in order to arrest in metaphase those cells that entered mitosis. Cell were collected, treated with a hypotonic solution, and fixed as described by Yu et al. (11). Air-dried metaphases were banded by staining with 4,6-diamidino-2-phenylindole after a pretreatment with actinomycin D (12). A total of 32 metaphases were karyotyped: 10 from line GLC-1; 8 from GLC-1-M13; 5 from GLC-2; and 9 from GLC-3.

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2 The abbreviations used are: SCLC, small cell lung carcinoma; dopa, l,3,4-dihydroxyphenylalanine; RGE 53, monoclonal antibody directed against cytokeratin 18, and specific for glandular epithelium; RKSE 60, monoclonal antibody specific for keratins in keratinizing cells.
Biochemical Analysis. Culture-doubling time was assessed by measuring the increase of total culture DNA in time. DNA measurements were carried out by Gerard van Kernebeek according to the procedure of Fiszer-Szafarz et al. (13). Dopa decarboxylase determinations were carried out by Dr. D. Carney (Mater Misericordiae Hospital, Dublin, Ireland), and Dr. S. Pavlo (CKKL, University Hospital, Groningen, The Netherlands) according to described methods (14). Creatine kinase determinations and isoenzyme analysis were performed by F. Polderman (CKKL, University Hospital, Groningen) with the CK Merckotest (Mercck, Federal Republic of Germany).

Screening for polypeptide hormones (adrenocorticotropic hormone, human chorionic gonadotropin, and human placental lactogen) was carried out by A. van Zanten (Nuclear Medicine, University Hospital, Groningen) using radioimmunoassays. The presence of bombesin-like immunoreactivity and neuron-specific enolase was assessed by immunoperoxidase staining using polyclonal antibodies (Immunonuclear, Dunn, for bombesin and Pharmacia, Uppsala, Sweden, for neuron-specific enolase).

Determination of Intermediate Filament Expression. Preparation and testing of specificity of the monoclonal antibodies to cytokeratins, RGE 53 and RKE 60, have been described in detail elsewhere (15, 16). The affinity-purified polyclonal rabbit antibody to human keratin reacts with most epithelial tissues, but not with nonepithelial tissues (16). Rabbit antisera to calf lens vimentin, chicken gizzard desmin, and human spinal cord glial fibrillary acidic protein have been reported before (16) and were shown to react specifically with the respective antigens.

The monoclonal antibody BF 10 directed against a M, 155,000 protein present in neurofilaments was a kind gift from Dr. B. Anderton, London, United Kingdom, and has been described before (17). MNF (Eurodiagnostics, Apeldoorn, The Netherlands) is another monoclonal antibody directed against neurofilaments. Procedures for immunoperoxidase and indirect immunofluorescence staining have been described earlier (18, 19).

RESULTS

Patient History and Establishment of Cell Lines. Cell line GLC-1 (previously designated OC-Rol; see Refs. 18 and 20) was established from a pleural effusion of a 59-year-old man who presented with a tumor in the hilum of the left lung with subpleural metastases in the left lung and bone marrow. A short description of this cell line has been given before (21, 22). Fig. 1a shows the histology of the tumor focus. The patient died 2 months after s.c. injection in nude mice. Electron microscopy of GLC-1 tumor cells grown in nude mice occasionally shows dense core vesicles (Fig. 2b).

Electron Microscopy of the Cell Lines. The electron microscopica of the cell lines GLC-1, GLC-1-M13, GLC-2, and GLC-3 is shown in Fig. 2, c–f. GLC-1 (Fig. 2c) is characterized by the presence of finely dispersed chromatin, prominent nucleoli, a few desmosomes, and the absence of dense core vesicles. Compared with the tumor cells in the original pleural fluid, the occurrence of prominent nucleoli is remarkable. GLC-1-M13 (Fig. 2d) has the same characteristics as does GLC-1, except that this cell line contains a large amount of dense core vesicles. GLC-2 (Fig. 2e) is characterized by finely dispersed chromatin, prominent nucleoli, the presence of only a few desmosomes, a sparse occurrence of microvilli, and the absence of dense core vesicles. GLC-3 (Fig. 2f) is also characterized by the presence of finely dispersed chromatin and prominent nucleoli. Desmosomes are present and dense core vesicles are absent. As an additional feature GLC-3 contains a considerable amount of small villous structures.

Chromosome Analysis. The chromosome number in the metaphases studied varied predominantly between 51 and 69. Numerically both GLC-1 and GLC-2 had a marked overrepresentation (three to six copies per metaphase) of chromosomes 13.
Table 1

<table>
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<th>GLC-1</th>
<th>GLC-1-M13</th>
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<tr>
<td>Growth in serum-free medium</td>
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<td>+</td>
<td></td>
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<tr>
<td>Growth in nude mice after s.c. injection of 10^7 cells</td>
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<td>++ (4/4)</td>
<td>- (0/6)</td>
<td>- (0/6)</td>
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<td>Predominant culture morphology in serum-containing medium</td>
<td>Loosely packed floating aggregates</td>
<td>Tightly packed floating aggregates</td>
<td>Loosely attached monolayer</td>
<td>Tightly packed floating aggregates</td>
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<td>42</td>
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Table 2

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<td>MM</td>
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<td>+</td>
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<td>Bombesin</td>
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Enzyme and Hormone Markers. The occurrence of SCLC markers, i.e., neuron-specific enolase, bombesin-like immunoreactivity, creatine kinase BB, and dopa decarboxylase, in the different cell lines is summarized in Table 2. Creatine kinase BB and neuron-specific enolase proved to be present in all cell lines, whereas the expression of dopa decarboxylase and bombesin-like immunoreactivity varied. Dopa decarboxylase and bombesin-like immunoreactivity were present in only moderate amounts in GLC-1-M13. At a very early passage number GLC-1 also contained a detectable amount of dopa decarboxylase (passage 10; 8.3 units dopa decarboxylase per mg protein). Apparently the expression of this enzyme was lost during prolonged in vitro culture. GLC-2 and GLC-3 never had bombesin-like immunoreactivity or dopa decarboxylase in detectable amounts. The present of adrenocorticotropic hormone could be demonstrated in all cell lines, whereas human choriongonadotropin hormone was absent.

Expression of Intermediate Filament Proteins. Table 3 summarizes the occurrence of the different types of intermediate sized filaments in the different cell lines as assessed by immunoperoxidase and immunofluorescence staining on cytospins using both monoclonal and polyclonal antibodies to intermediate filament proteins. Some examples of reaction patterns are shown in Fig. 6. No reactions were seen in any of the cell lines with antibodies to glial fibrillary acidic protein, while all cell lines but one (GLC-1-M13) expressed vimentin filaments. It is noteworthy that cell line GLC-3 showed in only 50% of the cells a filamentous staining pattern for vimentin. A strongly reverse correlation is seen between the compactness of the cell clusters and the expression of vimentin (compare results from Tables 1 and 3). The rabbit antiserum to human skin keratins and the monoclonal antibodies to cytokeratin 18 (RGE 53) and to the keratins specific for keratinizing cells (RKSE 60) gave only positive staining reactions in lines GLC-1-M13 and GLC-3. All cells of subclone GLC-1-M13 showed a filamentous staining both with the polyclonal antiserum and with RGE 53. These results indicate a simple

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epithelial," nonsquamous differentiation pathway for this cell line. On the contrary the presence of RKSE 60-positive cells in cell line GLC-3 indicates keratinization in some cells and thus a squamous differentiation. However, a considerable part of the cells were also positive for RGE 53 which is supportive for an adenocarcinomatous component in the cell line. It is striking, however, that only 25% of the cells are positive for the antikeratin rabbit serum in this latter cell line. Staining for neurofilament proteins was found only in a minority of the cells in cell line GLC-2, when using monoclonal antibodies, while desmin staining was seen in a few cells in cell line GLC-1.

DISCUSSION

In the past years the establishment of a considerable number of SCLC-derived cell lines has been documented (6-9, 23). Most of these cell lines are characterized by the expression of high levels of dopa decarboxylase, neuron-specific enolase, bombesin-like immunoreactivity, and the creatine kinase BB isoenzyme. In addition the presence of neuroendocrine tissue-related dense core vesicles is considered as a characteristic feature of these cell lines. According to these criteria most of the described cell lines resemble SCLC in vivo and therefore are called "classic" SCLC cell lines. More recently and until now small numbers of SCLC-derived cell lines have been described which express only a subpopulation of the above mentioned markers (5, 7). Whereas high levels of creatine kinase BB and neuron-specific enolase are still present in these cell lines, bombesin, dopa decarboxylase, as well as dense core vesicles seem to be absent. These cell lines are called "large cell-like" (5), "converter" (23), or "variant" (8) SCLC cell lines. Since such variant cell lines were obtained both directly from patients with a very poor prognosis (23) and after conversion of originally classic SCLC cell lines in vitro, the suggestion was put forward that variant SCLC cells are derived from the classic SCLC cell type (23). As an additional argument for their SCLC origin, these variant lines were found to contain the SCLC-related chromosomal aberration (del)3p (24, 25). Since variant SCLC cell lines resemble end stage, treatment-resistant SCLC in vivo morphologically and with respect to radiation sensitivity (26), these cell lines could offer an in vitro model for this clinically most important aspect of SCLC. By fulfilling the criteria for variant SCLC cell lines in a number of aspects, the three cell lines described and characterized in this report extend the available number of such lines.

It has been reported that only a minority of established SCLC derived cell lines are variant (27). Our experience is in accordance with such a finding; over the past years we have established 14 cell lines from SCLC patients. From these according to morphological criteria (27), only five lines were variant, whereas the others were classic.

The SCLC origin of the three cell lines described in this report is deduced from the characteristics of the clinical material from which they are derived. All three biopsies could be classified as SCLC of the intermediate type. In addition in the tumor biopsy of the patient from whom GLC-3 was established an admixture of larger cells with prominent nucleoli could be identified.

The growth behavior of the cell lines in vitro (growth in floating aggregates, growth in serum-free media, short population-doubling times, high cloning efficiencies in semisolid media) largely corresponds with the characteristics of variant SCLC-derived cell lines as observed by others (26). GLC-3, however, had a relatively low cloning efficiency and did not grow in serum-free medium, indicating that this cell line needs additional, serum-derived growth factors.

All three cell lines as well as the clonal derivate show as the most common chromosomal abnormality a deletion in the short arm of chromosome 3 (del3p). This result extends the findings of Whang-Peng et al. (24, 25), who described the occurrence of 3p deletions in at least one chromosome 3 in all metaphases studied from a total of 16 SCLC lines (both classic and variant), whereas the investigated non-SCLC cell lines did not show this aberration. The deleted region was highly variable in Whang-Peng's study with p(14-23) described as the shortest region of overlap. We identified 3p deletions in 28 of 32 metaphases studied from the three cell lines and the clonal derivate GLC-1-M13. Also in our material the deleted region was not identical for all metaphases, p(21-23) being in our opinion the common deletion (see Fig. 5). Although the short arm of chromosome 3 is known to be generally susceptible to rearrangements as documented for spontaneous breaks in noncancerous cells (28, 29) and also for renal cell carcinoma (30, 31), the common deletion of 3p(21-23) in a relatively large number of SCLC-derived cell lines might be causally related to the etiology of SCLC. In contrast to such a notion the studies of Wurster-Hill and Maurer (32) and Bergh et al. (33) did not reveal 3p deletions in their SCLC-derived cell lines. The reason for this discrepancy is not clear. An additional finding in the metaphase plates of cell lines described in this report was the presence of a small number of double minutes in cell line GLC-1 and GLC-2, but not in GLC-3. These double minutes persist after prolonged in vitro culture and, at least in the case of GLC-2, could not be related to in vivo-acquired drug resistance, as has been described for a methotrexate-resistant SCLC cell line (34), since GLC-2 was isolated before the patient received chemotherapy. The clonal derivate M13 showed in addition an extremely high number of these double minutes.
The electron microscopic picture of the cell lines is indicative for a dedifferentiated large cell or variant nature, except for clone GLC-1-M13. All cell lines show round or oval nuclei with finely dispersed chromatin and prominent nucleoli. In cell clusters molding of nuclei was sometimes observed. In GLC-1 a few clear dense core vesicles could be observed in the nude mouse-grown tumor, whereas GLC-2 and GLC-3 were devoid of these granules. The clonal derivate GLC-1-M13, isolated from GLC-1, was characterized by a relatively high number of dense core vesicles. This is a somewhat surprising finding, which might be explained either by the fortuitous selection of one classic clone from a majority of variant cells or by a differentiation process induced by the harsh growth conditions during the limiting dilution in serum-free medium. In favor of this last explanation is the fact that other clones obtained during this experiment also contained large amounts of dense core vesicles (not shown). GLC-2 did not show any differentiation features, whereas GLC-1 showed, to a low level, some desmosomes, which could be taken as an indication for an epithelial differentiation. GLC-3 had some adenodifferentiation judged from the presence of desmosomes, microvilli, as well as some secretory material containing vacuoles in the cytoplasm.

In agreement with previous studies (23) we found the presence of the classic SCLC markers dopa decarboxylase and bombesin-like immunoreactivity to parallel the presence of dense core vesicles, since these markers were clearly detectable only in GLC-1-M13. Neuron-specific enolase and high levels of creatine kinase proved to be present in all cell lines. In addition to the expression of high amounts of the BB isoenzyme of creatine kinase, the presence of comparable amounts of the MM isoenzyme could also be demonstrated in all cell lines, which is in contrast to previous studies with classic SCLC (23, 35). The reason for this is not clear for the moment. One possible explanation could be the rather undifferentiated nature of GLC-1, -2, and -3, which may be accompanied by a changed isoenzyme composition. If so redifferentiation, as proposed to have occurred during the isolation of GLC-1-M13, does not necessarily imply a matching shift back to a sole expression of the BB isoenzyme form of creatine kinase, however.

The presence of specific subsets of intermediate filaments in the different cell lines can be taken as an indication for different differentiation potentials in the otherwise relatively undifferentiated cells. As a result of tissue culturing three of four cell lines express vimentin intermediate filaments. It is striking, however, that in cultures which grow as tightly packed floating aggregates, vimentin expression is reduced (as in the case of GLC-3 where 50% of the cells are positive for vimentin) or completely absent (as in the case of GLC-1-M13). These findings support the hypothesis that vimentin expression in epithelial cells may be related to a reduced cell-to-cell contact (36, 37).

GLC-1 cells do not express cytokeratins. Therefore it is very surprising to see that a classic subclone from this variant line, i.e., GLC-1-M13, does express cytokeratins. The reaction of RGE 53 with this cell line shows the presence of cytokeratin 18, normally found in adenocarcinomas and small cell carcinomas, but not in squamous cell carcinomas.

Apparently the presence of differentiation features in this clone such as dense core vesicles, bombesin-like immunoreactivity, and dopa decarboxylase is coexpressed with the occurrence of cytokeratins. Although the majority of the cells of GLC-2 is unreactive with anti-keratin or anti-neurofilament antibodies, in agreement with the undifferentiated variant nature of this cell line, a small percentage of the cells shows a pronounced and filamentous reaction with the monoclonal antibody to neurofilaments, suggesting a neural or possible neuroendocrine differentiation of some of the cells in this cell line. This finding confirms that SCLC can express not only cytokeratins as shown by Sappino et al. (38), Makin et al. (39), and Van Muyten et al. (40) but also neurofilaments as has been found by Lehto (41) and Bergh et al. (42). Cells of the cell line GLC-3 showed, next to a partial staining with the antibody to vimentin, a heterogeneous staining pattern with the monoclonal and polyclonal antibodies to cytokeratins, which indicate an epithelial differentiation of this cell line. Since more than 60% of the cells show a positive reaction for RGE 53, the line predominantly exhibits a SCLC or adenocarcinoma like differentiation status. However, the finding of 9% cells positive for RKSE 60 shows that in this cell line also profound keratinization could occur. Therefore this SCLC-derived cell line seems to have adapted an adenocarcinomatous as well as to a lesser extent a squamous pathway of in vitro differentiation. This finding partly matches with the electron microscopically detected microvilli present in part of the cells, which can also be taken as an indication of an adenocarcinoma feature of part of this cell line. Electron microscopy did not clearly indicate a squamous cell differentiation status in (a subset of) GLC-3 cells as a further support of the intermediate filament staining of 9% of the cells with RKSE 60. Probably the number of such cells or the degree of keratinization herein is too small to be detected by this technique.

In conclusion the cell lines described in this report are all SCLC derived but lack a variable number of classic SCLC markers. Therefore they can be classified as variant SCLC cell lines. The combination of several differentiation features in otherwise undifferentiated cell lines as well as the remarkable finding that a classic SCLC clone can be isolated from one of these cell lines can be explained in terms of the stem cell hypothesis of lung cancer histogenesis (23). In this view therapy-resistant SCLC, either initially or at relapse, consists of dedifferentiated SCLC cells that are closely related to the original undifferentiated normal "stem cell" from which all different normal and malignant lung cells originate (23). In vivo but possibly also in vitro, these cells are able to switch in differentiation, i.e., from the SCLC line to the "adenoc" line. Further studies including differentiation induction with specific agents are needed to support this hypothesis.

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REFERENCES


Fig. 1. Histology of bronchial biopsies taken from patients from whom the cell lines were isolated (a, b, and c for GLC-1, -2, and -3, respectively). In a, the nuclei show a finely dispersed chromatin pattern with inconspicuous nucleoli. Crushing and molding of nuclei can also be noted (i.e., in the area indicated with an asterisk). In b the tumor cells are monomorphic with finely dispersed chromatin. Sporadically a nucleolus was seen in the tumor cells (examples are indicated with arrows). In c the tumor cells are polymorphic. Both large cells with nuclei regularly containing prominent nucleoli (see arrows) and small cells with nuclei containing inconspicuous nucleoli are present. Toluidine blue staining, × 350.
Fig. 2. Electron microscopic appearance of a tumor cell in the pleural fluid from which GLC-1 was established (a) and tumor cells in a nude mouse grown tumor after the injection of GLC-1 cells (b). c, d, e, and f, tissue culture grown cells of GLC-1, GLC-1-M13, GLC-2, and GLC-3, respectively. Arrows, location of dense core vesicles in a, b, and d, the appearance of desmosomes in b and c, and the presence of small villous structures in e and f. a, x 10,752; b, x 11,261; c, x 21,043; d, x 56,024; e, x 7,142; f, x 7,142.
Fig. 3. Culture morphology of GLC-1 (a), GLC-2 (b), and GLC-3 (c). Cells are grown in serum-containing medium and viewed with phase-contrast microscopy. × 100.
Fig. 6. Indirect immunofluorescence assay of several antibodies to intermediate filament proteins on the different types of small cell lung cancer-derived cell lines. The staining reaction is given of GLC-1 cells incubated with polyclonal anti-vimentin (a) and with polyclonal anti-desmin (b); of GLC-1-M13 cells incubated with polyclonal anti-keratin (c) and with RGE 53 (d); of GLC-2 cells incubated with polyclonal anti-vimentin (e) and with BF-10 (f); of GLC-3 cells incubated with polyclonal anti-vimentin (g); with polyclonal anti-keratin (h); with RGE 53 (i); and with RKSE 60 (j), respectively. a, ×250; b, ×250; c, ×370; d, ×370; e, ×250; f, ×400; g, ×350; h, ×490; i, ×300; j, ×360.
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