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ARTICLE

Identification and Characterization of Different Subpopulations in a Human Lung Adenocarcinoma Cell Line (A549)

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The morphology, cell growth, antigenic expression and tumorigenicity of cell subpopulations from the A549 lung adenocarcinoma isolated by Percoll gradient separation have been analysed. Four subpopulations were obtained (subpopulations A, B, C and D). Immunocytochemical analysis of several antigens was performed with monoclonal antibodies (MAbs): MUC1 mucin (C595, HMFG1 and HMFG2), MUC5B (PANH2); gp230 (PANH4); carbohydrate antigens including sialyl Lewis x (KM93), Tn antigen (83D4), Lewis y (C14); 5, 6, 8, 17 and 19 cytokeratins and p53. The cell population D tended to form cell aggregates that piled up on the monolayer similar to overgrowth cultures of the A549 parental cell line, whereas A, B and C cell subpopulations formed well spread monolayers. Both parental A549 and subpopulation D secreted abundant mucus. The topographic distribution and secretion production were correlated with tumorigenic assays since only subpopulation D grew in nude mice exhibiting reduced latency period; these characteristics correlated with the fast growth of the subpopulation D in vitro. Immunocytochemical analysis demonstrated that subpopulation D showed greater expression of MUC1 mucin and carbohydrate antigens such as Tn antigen, sialyl Lewis x and Lewis y and less expression of cytokeratins, p53, MUC5B and gp230; conversely, subpopulations A, B and C showed the opposite antigenic profile. Our results illustrate heterogeneity in the A549 cell line; subpopulations A, B and C retained characteristics of more differentiated adenocarcinoma while subpopulation D displayed features of a less differentiated tumor line. (Pathology Oncology Research Vol 5, No 3, 197–204, 1999)

Keywords: A549, lung adenocarcinoma, cell subpopulations

Introduction

In industrial countries, lung cancer is the most common visceral malignancy in males, and its incidence is dramatically increasing in women; moreover, about 80% of patients die with disseminated disease after surgical resection. Although many studies have been performed in order to establish genetic and phenotypic properties of lung carcinoma cells, they are still not well defined. Different embryonic origin as well as late clinical and pathological diagnosis make account for heterogeneity of tumor cell populations; divergence may increase continuously facilitating cellular adaptation to different host environmental conditions with consequences in tumor biology such as invasion and metastatic spread. In this aspect, it is probable that cells included in a parental cell line can differ in morphology, biological properties as well as in antigenic expression.[23,33]

Numerous lung tumor cell lines have been established,[19,25] among them A-549 cell line was initiated in 1972 by Giard et al[11] through explant culture of a lung adenocarcinoma. In recent years, this cell line has been largely studied and it is known to express for instance, three respiratory mucin genes (MUC5AC, MUC1 and MUC5B),[41,3] p53,[32] cytokeratins (polypeptides 7, 8, 18 and 19[4]), type I and II estrogen binding sites[7] and other antigens.[40]

In the present work, A-549 human lung adenocarcinoma cell line was employed as an *in vitro* model to: 1) obtain different subpopulations; 2) to study their morphological aspects and growth behaviour, 3) to determine their possible tumorigenicity as well as 4) to investigate their antigenic

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expression with a panel of monoclonal antibodies. Thus, in this study, the A-549 cell line has been employed as a target to increase the knowledge on lung cancer heterogeneity.

Materials and Methods

Cell culture

The parental A-549 cell line was cultured attached in plastic flasks (Corning, Glassworks, Corning, NY) in F12/HAM medium (Gibco-BRL, USA), supplemented with 10% fetal bovine serum (Gen, Argentina), 2mM glutamine (Merck), non-essential amino acids (Non essential amino acid solution No. M7145), vitamins (MEM vitamins solution No.M-6895), 50 μg/ml gentamicin and 1.5 μl/ml amphotericin B (Antibiotic-Antimycotic solution No.A-9909) in 5% CO_2/95% air atmosphere. All reagents were purchased from Sigma, Illinois, USA, unless otherwise specified.

Microscopy

Heterotransplanted tumors and monolayer cell cultures were prepared for light microscopy. Hematoxylin, eosin and periodic acid-Schiff staining were evaluated routinely.

Separation of A-549 cells into subpopulations

Cells were separated into subpopulations using density gradients of a polymer-coated silica colloid (Percoll; AB Pharmacia, Uppsala, Sweden). Seven stock solutions of density 1.035, 1.040, 1.050, 1.060, 1.065, 1.070 and 1.080 g/ml were prepared in 1.5 M NaCl according to the manufacturer's instructions and 2 ml of each was layered manually. Parental exponential growing A549 cells were trypsinized, washed in phosphate-buffered saline with Ca^{2+} and Mg^{2+} and layered on the top of the gradients ($3x10^6$ single cell suspensions in 1 ml of the same buffer). The gradients were centrifuged at room temperature at 800 g for 45 min in a swinging-bucket rotor. Fractions of 0.5 ml were collected from the top; the cell number in each fraction was determined in a hemocytometer and viability was assessed by trypan blue exclusion. Fractions from each identified subpopulation were pooled and washed twice with 5 volumes of phosphate-buffered saline (PBS, pH 7.2); in addition, cells from each subpopulation were plated in 35-mm plastic tissue culture dishes containing medium as above.

Morphology and growth properties in vitro

Each subpopulation was maintained in standard culture conditions and expanded to the desired cell number; they were observed daily by phase-contrast microscopy. For the study of growth properties, $1x10^4$ cells were seeded into 35-mm tissue culture dishes (NUNC, cat. 152795). Duplicate dishes were trypsinized at 48 hr and cell counted with hemocytometer; the number of viable cells was determined by trypan blue exclusion and the doubling time of replication was calculated.

Tumorigenicity studies in nude mice

Athymic nude mice (Laboratory Animal Unit, School of Veterinary, National University of La Plata) were used in all experiments.

Viable single cells suspensions ($25x10^4$) in 0.2 ml Hank's buffered saline solution were injected subcutaneously into 4–8 week old nude mice. Once tumors had obtained approximately 1–2 cm diameter, mice were sacrificed and examined for the presence of metastases; samples were excised and fixed for histopathology.

Immunocytochemical analysis

For immunocytochemical studies cells were grown on glass coverslips upon confluence and they were fixed in 10% buffered formalin. The technique was developed according to previous reports,[8] with minor differences. Cells were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes; [36] then, coverslips were incubated overnight at 4°C with mouse monoclonal antibodies. Negative controls were either incubated with PBS instead of monoclonal antibodies.

The whole area of each sample was observed by sequentially examining lower power (x10) optical fields; the

Table 1. **Panel of monoclonal antibodies employed in this studizy**

Monoclonal antibodies	*Antigens*	*Reference*
KM-93	Syalil-Lewis x	Hanai N, et al[13]
C14	Lewis y hapten	Brown A, et al[6]
C-595	Arg-Pro-Ala-Pro (MUCI protein core)	Price MR, et al[30]
HMFG-I	MUC-1 protein core	Taylor-Papadimitriou J, et al[38]
HMFG-II	MUC-1 protein core	Taylor-Papadimitriou J, et al[38]
83-D4	Tn determinat	Pancino GF, et al[29]
PANH-2	MUC-5B	Nielsen PA, et al[27]
PANH-4	Gp230	Nielsen PA, et al[28]
Anti-p53	P53	SIGMA N° P-5813
Anti-human cytokeratins	Keratins 5, 6, 8, 17, 19	DAKO N° U-7022

staining of cytoplasm, plasma and nuclear membranes were also evaluated. Cells were considered positive when at least one of these components was stained; heterogeneity was graded according to positive reactions, intensity and distribution. Staining intensity was graded as negative (○), low (◒), moderate (●) and strong (●●).[9]

With respect to p53, and considering other reports,[31] a sample was coded as positive even when only a few cells were reactive.

Monoclonal antibodies (Mabs)

The following monoclonal antibodies (Mabs) were employed: C14 Mab, an IgM anti-Lewis y hapten against the difucosylated Type-2 blood group chains,[6] Mab KM93 (IgM), an anti syalil-Lewis x.[13] Three anti MUC1 Mabs were employed: Mab C-595 (IgG3) defines the tetrameric epitope Arg-Pro-Ala-Pro in the MUC1 protein core,[30] HMFG1 and HMFG2[38] define the epitopes PDTR and DTR respectively.[30] Mab 83D4 (IgM) against Tn determinant,[29] an anti MUC5B (PANH2 Mab) (IgG1) raised against partially deglycosylated MG1,[27] and PANH4 Mab (IgM) an anti gp230 derived from buccal mucosa.[28] Also, anti-p53 Mab (mouse IgG2a isotype) (SIGMA No. P-5813) and finally, an anti-human cytokeratin (CK)/HRP against keratins 5,6,8,17 and 19 (Dako EPOS, Code No. U 7022).

Results

Subpopulation distribution in Percoll gradient

Several Percoll cell separation tests employing the A549 cell line were performed. The parental A549 cell line produced abundant mucus gel that made its separation in Percoll gradients difficult; it was necessary to determine a minimal inoculum to obtain the fractionation without mucus disturbance. Decreasing cellular concentrations were employed; $3x10^6$ was found to be the adequate inoculum to obtain good separation.

Figure 1 shows the distribution of cells among subpopulations obtained by Percoll density gradient centrifugation of the cell line at exponential growth. In a typical and reproducible experiment, four different subpopulations were isolated: subpopulation A at the top of the gradient, subpopulation B, fractions 5–8; subpopulation C, fractions 9–12; subpopulation D, fractions 13–16. The distribution was heterogeneous; subpopulation D was the most abundant, fraction C intermediate whereas fractions A and B contained the lowest number of cells.

Morphology and growth properties

Phase-contrast microscopy revealed that the parental cell line A-549 grew as a well-spread monolayer with prominent cellular processes; a regular epithelial morphology was observed with two types of cells: first, small polygonal cells and second, large cells with abundant vacuoles. Cells assumed typical pavement-like structures as cultures approached confluence; after this growth stage, they started to pile up with anchorage independency. The pavement pattern was also observed in subpopulations A, B and C. However, differences within these subpopulations were detected: subpopulation A comprised a homogeneous cellular type of small polygonal cells with frequent mitotic figures. Subpopulations B and C presented a heterogeneous pattern of larger cells with abundant vacuoles with prominent nuclei, mostly with dispersed chromatin and numerous nucleoli. The presence of more than one nucleus was a frequent observation as were apoptotic elements; nuclear budding was occasionally seen while only a few mitotic figures were observed.

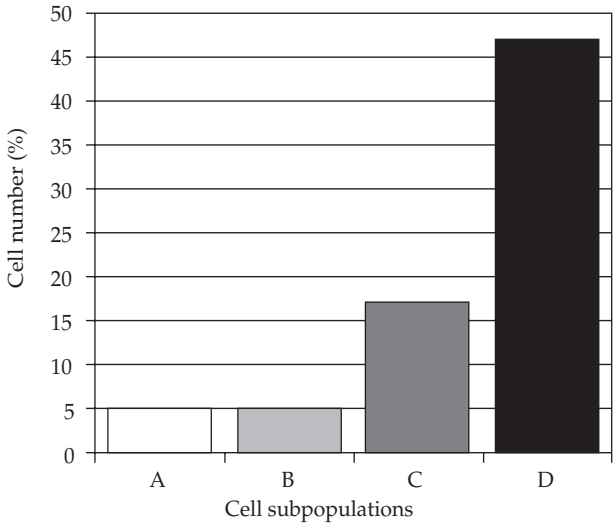

Figure 1. *Distribution of exponentially growing of A, B, C and D cell subpopulations in Percoll density gradients. Exponentially growing cells ($3x10^6$) were submitted to a density gradient centrifugation and counted as described in Materials and Methods.*

In contrast, subpopulation D grew as a monolayer, but some cells piled up on each other in the center of colonies showing anchorage independency. Mainly, these cell aggregates were found at the top of the monolayer together with suspended cells and mucoid threads. Cells exhibited characteristics such as refractivity and disorganisation while cell processes were rarely seen in this variant. Even in sparse culture with low density, subpopulation D formed cell aggregates rich in mucoid threads.

After trypsination of the parental line as well as subpopulations A, B and C, homogeneous single cell suspensions were obtained, while subpopulation D was difficult to disaggregate and frequently its cells remained clustered.

When isolated subpopulations were grown separately, subpopulation D grew faster than subpopulations B and C, with subpopulation A attaining an intermediate rate.

Growth rate analysis was performed one day after Percoll separation; cells were plated for 48 hr, which was sufficient time to achieve their maximal morphological homogeneity coincident with the highest mitotic activity. Doubling time during exponential growth was as follows: the parental line, 18.3 hr; D subpopulation, 19.7 hr; B subpopulation, 25.7 hr; C subpopulation, 26.2 hr and A subpopulation, 22.4 hr. Growth characteristics of cell lines and derived subpopulations are summarized in *Table 2*.

Tumorigenicity studies

Subcutaneous injection of $25x10^4$ cells belonging to A549 parental cell line as well as A549 Percoll derived subpopulations were xenotransplanted into the supercapsular region of nude mice. The parental line and subpopulation D grew subcutaneously producing tumors with different latency periods. With respect to the A549 parental line a subcutaneous tumor appeared at about 90 days postinoculation. On the other hand, subpopulation D developed tumors at the site of inoculation with a latency period from 56 days; during this period, tumors were neither visible nor palpable.

Histologically, tumors developed in nude mice showed a solid growth pattern forming glandular acini; two cellular types were found: first, large cells with abundant vacuoles, clear cytoplasm and delicate nucleus where the presence of numerous nucleoli was usually observed; a second type of small and polygonal cells with dark chromatin was also described showing both cell types marked nuclear atypia and several apoptotic bodies. Necrotic areas were not an infrequent event and an invasive pattern of growth reaching the surrounding tissues were observed.

In contrast to the parental A549 line and subpopulation D, the remaining A549 derived subpopulations (A, B and C) were not tumorigenic in nude mice.

Antigenic expression by immunocytochemistry

The A549 parental cell line as well as Percoll derived subpopulations were analyzed for antigenic expression with a panel of monoclonal antibodies *(Table 2);* all experiments were performed with cells at confluence.

In general, samples assayed showed a differential reactivity with most Mabs although parental A 549 cell line developed a positive reaction with all antibodies. In this cell line, MUC1 mucin yielded its highest expression compared with other samples, although moderate levels of expression were observed. Antibody reactivity with MUC5B was also moderate but lower than for MUC1 staining while gp230 (PANH4Mab) showed weak staining reactivities. Carbohydrate antigens presented a positive reaction, mainly in Tn

Table 2. **Morphology, growth behaviour and expression of associated tumor antigens as determined by immunocytochemistry of A549 original cell line and its derived subpopulations**

	Cell samples				
Parameters	*A549 parental cell line*	*Subpopulation A*	*Subpopulation B*	*Subpopulation C*	*Subpopulation D*
Epithelial morphology	yes	yes	yes	yes	yes
Tumorigenicity	yes	no	no	no	yes
Anchorage independency	at confluence	no	no	no	yes
Doubling time	18.3 h	22.4 h	25.7 h	26.2 h	19.7 h
MUC5B	●	●	●	●	◒
MUCI	●	◒	◒	◒	●
Gp230	◒	●●	●	●	
Le y	●●	○	○	○	●●
SLe x	●	○	○	○	●
Tn antigen	●●	●	●	●	●●
5, 6, 8, 17 and 19 CK	●●	●●	●●	●●	◒
p53	◒	●	●●	●●	◒

○ = negative staining, ◒ = low staining, ● = moderate staining, ●● = strong staining

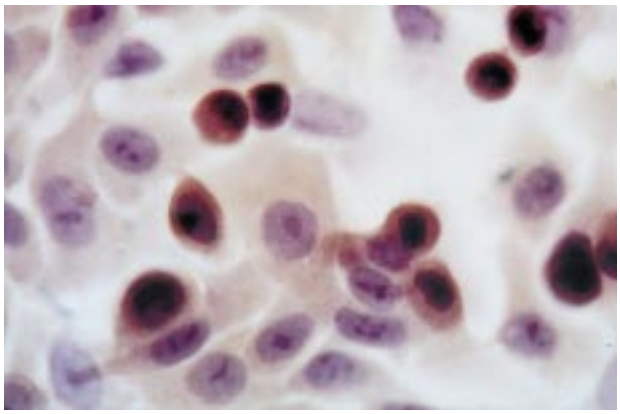

Figure 2. *Staining of D cell subpopulation with anti-sialyl Lewis x (KM93) Mab. A colony of cells are stained with a homogeneous pattern and variable intensity. Anisocytosis and anysocaryosis are observed (x80).*

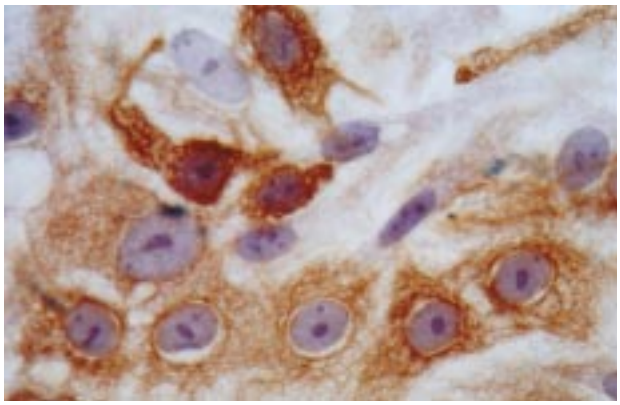

Figure 3. *Staining of A 549 cell line with anti-cytokeratin Mab. Some cells show an intense perinuclear staining; the cytoplasm shows a heterogeneous pattern and clear vacuolae; cytoplasm processes are seen betwen cells; some of them are negative. Several nucleoli are identified in most cells (x80).*

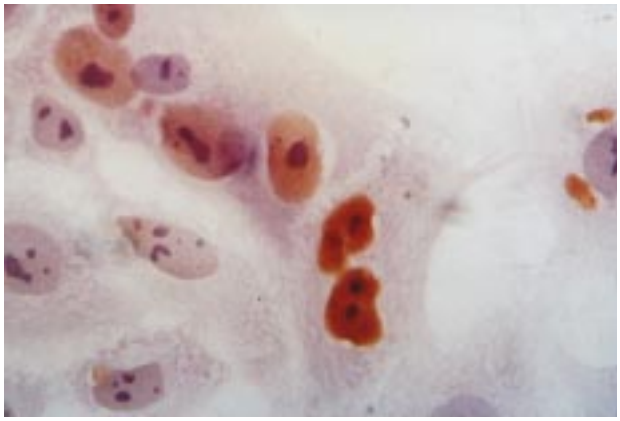

Figure 4. *Staining of C cell subpopulation with anti-P53 MAb. Several nuclei were positive showing a variable nucleoli number (x40).*

expression and Lewis y hapten, while sialyl Lewis x showed a moderate staining.

Subpopulation D showed a significant expression of carbohydrate antigens and MUC1 mucin, with Tn and Lewis y antigens being the most intense and widely detected; anti sialyl Lewis x stained mostly cells that *(Figure 2)* were grouped in piled up areas. Anti MUC5B Mab reacted in a low number of cells of the subpopulation D while gp230 displayed the lowest level of expression; these mucins were principally expressed by subpopulations A, B and C. On the contrary, in these three subpopulations (A, B and C), MUC1 as well as Tn antigens showed a low level of expression while sialyl Lewis x and Lewis y were not expressed at all.

An interesting observation was the positive reaction obtained with anti-CK Mab and the A549 parental cell line *(Figure 3);* subpopulations A, B and C showed an intense expression while subpopulation D displayed a low staining.

On the other hand, anti p53 Mab showed its most significant reactivity in subpopulations B and C *(Figure 4),* the subpopulation A presented a moderate staining like the A549 parental line while with subpopulation D anti p53 showed a reduced staining.

Discussion

The A549 cell line has been established from a human lung adenocarcinoma[11] having properties of type II alveolar epithelial cells.[24] As is well described, carcinogenesis constitutes a stepwise process and 10 to 20 mutations are present in lung cancer; these mutations induce tumor heterogeneity that may account for diverse cell subpopulations present in a cancer cell line; cell variants may differ for instance, in activation of oncogenes, morphological aspect, growth behaviour, antigenic expression and metastatic potential.[10,17,23,33,35]

In the present research, we have analysed a non-small cell lungcancer (NSCLC) cell line in order to identify different cell subpopulations; we have also characterized these cell subpopulations with respect to features of malignancy such as morphology, growth behaviour and antigenic expression. To study cell growth, we have included anchorage independency and doubling time which are considered expressions of cellular motility and behaviour.[21]

Different procedures were employed for the *in vitro* determination of cell heterogeneity such as cell cloning[21,39] or density gradient centrifugation.[33] Using the latter, we have demonstrated that several subpopulations can be isolated and subsequently characterized. For this purpose, we performed a detailed immunocytological investigation with a panel of Mabs, which detect antigens possibly involved in diverse tumor biology aspects such as development, invasion and metastasis.

We have shown some evidence that emphasises a differential expression of mucins usually related to the airway tract. MUC1, MUC4, MUC5AC and MUC5B were found to be relevant airway mucins; furthermore, MUC1 has been described as a novel marker for type II pneumocyte lineage during lung carcinogenesis.[18] By Northern blot analysis, Berger et al[3] found that A549 express MUC1, MUC5/5AC and MUC5B. In our study, MUC 1 showed a positive expression restricted mostly to the original A 549 cell line and subpopulation D. MUC5B was also detected in all samples assayed.

In lung epithelia, these two mucins have been related to important functions influencing cell-cell interactions; they have been described in normal tissue differentiation[12] as well as in metastasis facilitation.[26]

The alteration of mucin expression in A549 cancer cell line and its derived variants may be accompanied by changes in carbohydrate determinants since during oncogenic transformation, glycoproteins and/or glycolipids expressed in cell membrane are often altered.[14] The availability of a Mab panel detecting these antigens has been employed to facilitate the identification of possible A549 cell subpopulations; in this context, anti-Tn, anti sialyl Lewis x and anti Lewis y antigens may be of interest.

The demonstration of a strong and extense expression of these determinants in A549 parental cell line as well as in subpopulation D constitutes an interesting observation; for instance, Lewis y has been often described in lung carcinoma cells[2] and other tumors.[1]

On the other hand, it is known the association of sialyl Lewis x and cancer cell spreading through the binding of this carbohydrate to ELAM-I (E-selectin, endothelial cell leucocyte adhesion molecule-I) expressed in endothelial cells.[37] Furthermore, Kawai et al[20] found a high expression of sialyl-Lewis x in lung adenocarcinoma, mainly in poorly differentiated tumors.

Tn antigen has been recognized as a tumor associated antigen,[34] and subsequently. Hirohashi et al[15] supported this concept in studies with anti-Tn monoclonal antibodies which reacted against a lung carcinoma cell line. Tn antigen has also been associated with tumor growth and spreading in different tumor localisations.[15] We have found high expression of Tn antigen in the A549 cell line as well as in subpopulation D and less in subpopulations A, B and C. Recently, carbohydrate expression has been employed to select and characterize subpopulations belonging to heterogeneous established tumor cell lines.[17]

We have performed assays which show that the alteration of mature glycoproteins in A549 cell line also comprise mucin molecules determined by PANH4 (anti-gp 230 Mab) since A549 express gp230. A different pattern of reaction was described, being more reactive in A, moderate in subpopulations B and C while a low expression was observed in subpopulation D as in the parental A549 cell line.

Nielsen et al[28] reported that normal mucosal stratified epithelia usually express gp 230 mainly in non-keratinized type epithelia; it was also postulated that in oral mucosa gp230 may be the principal carrier of Tn antigen. Since A549 parental and variants also express other mucins, it is possible that concomitant expression of Tn antigen could be explained as a result of the alteration of other glycoproteins different from gp230. This is in agreement with the observation that samples which show more reactivity with anti carbohydrate Mabs show their highest reaction with anti MUC 1 and the lowest with anti gp230 and MUC5B.

In a tumor population, different clones may be identified by tumor antigens. However, clonal expansion of cells carrying alterations of oncogenes and/or suppressor genes defines neoplasia at a fundamental genetic level. The frequency of p53 abnormalities in lung cancer has been largely evidenced by different methods.[16,31] A was previously described,[32] we found p53 expression in the A549 parental cell line. This expression was restricted to the nucleus but with differential staining since subpopulations B, C and A have been more reactive than the A549 parental cell line as well as subpopulation D. Ledinko and Constantino[22] have pointed out that in the A549 cell line, retinoid-induced suppression of invasive ability was accompanied by increased amounts of p53 mRNA and protein and a CK18 decrease; moreover they found a correlation between p53 levels and the degree of suppression of invasion. In our assays, an interesting observation was the quantitative difference in CK expression among samples since 100% of cells from the parental A549 line were reactive, while subpopulations A, B and C showed a strong reaction with anti-CKs, with subpopulation D showing the lowest expression of staining. Broers et al[5] have observed that during progression from classical to variant lung cancer cell lines, different CK expression paralleled the transition from one cell type to another. The anti-cytokeratin Mab employed in this work reacts with CK 5, 6, 8, 17 and 19. It is well known that lung adenocarcinoma usually expresses CK 7, 8, 18, 19 and that it is unlikely to express CK10 and 13.

The panel of Mabs enabled us to establish that subpopulation D is marked different to the other gradient variants since it showed high percentage of cell expression of MUC1 as well as carbohydrate antigens such as sialyl Lewis x, Lewis y and Tn antigen. These observations correlated with a decreased expression of p53, cytokeratins, gp230 as well as MUC5B in subpopulation D.

When these observations were compared with morphology and growth behaviour, it was possible to emphasise that the variant D tended to form cell aggregates that piled up on the monolayer. The same pattern was observed in overgrowth cultures from A549 parental cell line whereas subpopulations A, B and C formed well spread monolayers. We have also detected that both A549 and subpopula-

tion D formed abundant mucus, although we could not identify its constituents.

The topographic distribution and secretion production were correlated with tumorigenic assays; subpopulation D was the only A549 derived variant that grew in nude mice giving rise to tumor after subcutaneous transplantation with a reduced latency period. Furthermore, these characteristics could be associated with fast *in vitro* growth in subpopulation D since its doubling time during exponential growth was the lowest compared with the other subpopulations.

The morphological and growth properties presented here combined with the antigenic expression of protein core mucins and oligosaccharides suggest that A549 is a heterogeneous cell line; subpopulations A, B and C retained characteristics of more differentiated adenocarcinoma while subpopulation D is a more undifferentiated line. The differential expression of cytokeratins as well as p53 constitute vital features in addressing this hypothesis. These observations are not surprising because lung epithelium is complex with diverse cell types, contributing to secretion and carcinogenesis which themselves extend this tissue heterogeneity.

The significance of this study on cell line variants is that it identifies the development and/or expression of certain characteristics specific for the acquisition of an undifferentiated cell phenotype; these may account for a phenotypic diversification including the acquisition of invasive and metastatic behaviour.

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