Virchows Archiv (1995) 426:169–178

ORIGINAL ARTICLE

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Tumour fragment spheroids from human non-small-cell lung cancer maintained in organ culture

Received: 8 July 1994 / Accepted: 19 October 1994

Abstract Biopsy material from 17 human non-small-cell lung carcinomas (NSCLC) was maintained in agar overlay culture as tumour fragment spheroids for 40 days. A practical procedure for the formation of spheroids and organ culture is described. The mechanically dissociated tumour specimens showed a variation in their ability to generate spheroids that was not related to the ploidy or the histological differentiation of the biopsies. Light microscopic observations revealed a heterogeneous spheroid population with a mixture of tumour cells and stromal elements. Most of the histological elements normally found in human NSCLC could be seen in the spheroids. The cellular components in the spheroids varied between highly cellular to sparsely cellular, dominated by stromal elements. The squamous carcinomas were in general found to generate highly cellular spheroids more often than the adenocarcinomas. Spheroids with a different cellular content could be selected in vitro by using a morphometric technique. Diameter measurements showed a large variability in spheroid growth. Most of the spheroids decreased in size although bromodeoxyuridine labelling indicated active cell proliferation in the specimens. Frequent changes of medium did not affect spheroid growth. The culture system presented provides a model for studying the cellular heterogeneity as well as the biological characteristics of tumour tissue from individual patients in vitro.

Key words Organ culture · Spheroids · Lung neoplasms

Introduction

Carcinomas of the lung are the most common cause of death due to cancer [30]. More than 80% of primary lung malignancies consist of non-small-cell lung cancers (NSCLC), mainly squamous, adeno- and large cell carcinomas. For the NSCLC there have been no changes in the overall prognosis during the last 20 years and the only curative treatment may still be surgical resection [8]. Lung cancers show considerable cellular heterogeneity which is reflected in their biological behaviour [3, 22]. Observations in vivo and in vitro have shown that tumour cells may have an inherent genetic instability which can lead to a selective differentiation of certain cells, and this can change the overall tumour phenotype [11, 12, 13]. Permanent transformed/cloned cell lines established from tumour biopsies, therefore, show an altered phenotypic expression which may differ considerably from the original tumour in vivo [13].

In this respect monolayers, or three-dimensional culture systems derived from continuous tumour cell lines may be regarded as models not necessarily representative of the tumours in vivo. Nevertheless, three dimensional tissue culture systems mimic several of the biological characteristics of tumours in vivo [15, 27, 31]. Such culture systems have been used extensively in experimental therapy [6, 10]. Multicellular spheroids can be made from individual cells aggregating in growth medium, either in a spinner flask [32] or on an agar-medium base [36]. One advantage of the latter method is that each spheroid is easily available for manipulation and individual spheroids can be followed and studied during a specific time-period.

The main advantages of establishing three dimensional cultures from cell lines are that basic mechanisms of importance for cell growth and differentiation can be studied in a reproducible format [20]. However, much of the biological complexity of the in vivo situation is lost. Recently, interactions between tumour cells, extracellular matrix and stromal elements have been given more attention [23] and it has been shown that these components may be important in cell growth, migration and differentiation [11, 21, 23].

We recently characterized three-dimensional tumour fragment spheroids derived from glioma biopsies and

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showed that the spheroids maintained several of the phenotypic and genotypic features of the original tumours in vivo [4] and in the present work we describe a similar model for growing NSCLC as spheroids explanted directly from human tumour biopsies, using the liquid overlay technique [36]. We show that the tumour heterogeneity found in vivo is reflected in the spheroids both within and between tumour types.

Materials and methods

Fresh tumour biopsies were obtained at surgery from 17 patients with primary non-small-cell lung carcinomas, 11 with squamous cell carcinoma, 5 with adenocarcinoma and 1 with large cell carcinoma. Immediately after removal the tissue specimens were aseptically transferred to a test tube containing Dulbecco's modification of Eagle's medium (DMEM; Difco Laboratories, Detroit, Mich., USA) supplemented with 10% heat-inactivated newborn calf serum, four times the prescribed concentration of non-essential amino acids, 2% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The tumour material was brought to the tissue culture laboratory within 10 min. One part of the tumour tissue was fixed in 4% formalin and later embedded in paraffin for routine histological examination. Two other parts were prepared for cell culture and flow cytometric analysis as described below.

For spheroid cell culture one part of the tumour biopsy was cut with scalpels into pieces less than 0.5–1.0 mm in diameter and incubated in 80 cm^2 tissue culture flasks (Nunc Plastic, Roskilde, Denmark), base-coated with 6 ml 0.75% agar in DMEM (Agar Noble, Difco Laboratories) [4]. The volume of the overlay suspension (DMEM) was 15 ml and was changed during the first week and thereafter every 1–2 weeks. The cultures were maintained at 37° C in 5% carbon dioxide (CO_2) and 95% air with 100% relative humidity. They were observed regularly by a phase-contrast microscope during a 40 days culture period. The number of spheroids obtained from the tissue fragments varied between the biopsies. The incidence of spheroids formed was found by counting all specimens and tissue fragments with a diameter >250 µm after 10 days of culture. The specimens were fixed for light microscopy at intervals from day 5 to day 40.

After 10 days culture, a mixture of light and dark spheroids (see below; 200–600 µm in diameter) were selected under visual control using a dissecting microscope and a sterile Pasteur pipette, and transferred individually from the culture flasks into 16 mm multiwell dishes (Nunc Plastic). The wells were base-coated with 0.5 ml of DMEM-agar as described above. The overlay suspension was 1.0 ml DMEM and was changed every 2 weeks. Spheroid size (diameter) was measured regularly during a period of 30 days and determined as the average sum of two orthogonal diameters. The measurements were performed using an inverted microscope with a calibrated reticle in the eyepiece.

To see if frequent changes of growth medium affected spheroid growth, individual (light) spheroids from one squamous cell carcinoma (Sq-11), were put into 16 mm agar-coated multiwell dishes in four parallel series. The medium was changed every day, three times a week, once a week or once every other week.

At regular intervals between day 5 and day 40, 5–10 spheroids from each tumour were collected for light microscopy. The specimens were fixed for 24 h in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.2 M sucrose (pH 7.2, 300 mosmol). Postfixation was performed for 1 h in 1% osmium tetroxide using the same buffer and dehydrated in increasing concentrations of ethanol up to 100%. After three 5 min periods in propylenoxide and 12 h in a 1:1 mixture of propylenoxide and Epon 812 (Fluka, Buchs, Switzerland), the spheroids were embedded in pure Epon 812. The final polymerization was carried out at 37° C for h and at 60° C for 48 h. Semithin (1.5 µm) sections were cut on a microtome (LKAB, Bromma, Sweden) and stained with toluidine blue.

After 2 weeks of culture spheroids between 180 and 630 µm with different light microscopic density, were transferred individually into 16 mm agar-coated multiwell dishes. The overlay suspension consisted of 1.0 ml of DMEM. By using an inverted microscope and an image analysis system (Kontron, Eching, Germany) the total area of a single spheroid could be divided into subareas with different greytones. A light penetration index for each spheroid was then determined by using the formula:

$$\text{Light penetration index} = \frac{\text{area with greytone } 0-20}{\text{area with greytone } 0-200} = \frac{\text{area of dark tones}}{\text{total area of spheroid}}$$

By keeping the light source constant a light penetration index <0.20 was found to be consistent with the visual impression of a translucent (=light) spheroid as seen in the inverted microscope, whereas an index >0.40 indicated a dark spheroid. Values between 0.20 and 0.40 were visually interpreted as intermediate spheroids. After the morphometric measurements, spheroids were individually prepared for light microscopic examinations as described above. This was done in order to compare the histological architecture of the spheroids with the morphometric light penetration indices.

Based on the results observed by light microscopy and morphometric analysis dark and light spheroids from different squamous tumours were studied by scanning electron microscopy (SEM). Five dark and five light spheroids were prepared for SEM after 5, 20 and 40 days of culture. The specimens were fixed and dehydrated using the same procedure as described for light microscopy. The specimens were then critical-point dried with CO_2 according to the technique described by Anderson [1]. They were then carefully mounted on stubs using tape and silver conducting paint. The specimens were coated with a conductive film of gold-palladium in a vacuum evaporator (Sputtercoater, Bio-Rad, Polaron Division, England) and examined by a Philips SEM 500 microscope (Philips, Eindhoven, Holland).

Material for flow cytometric deoxyribonucleic acid (DNA) analysis was either taken directly from the tumour biopsy or from paraffin embedded tumour tissue. The tumour biopsy was minced with scalpels, fixed in ice-cold 100% ethanol, and stored at 4° C until flow cytometric measurements. The specimens were prepared for DNA analysis according to the procedure by Barlogie [2]. Briefly, the samples were incubated for 15 min in 0.5% crude pepsin (pH 1.5 at 37° C). The isolated nuclei were then washed once in 0.9% sodium chloride (NaCl) and incubated for 5 min with four drops of ribonuclease (1 mg/ml in 0.9% NaCl). Staining of DNA was obtained with propidiumiodide 50 µg/ml in phosphate-buffered saline (PBS). The cellular DNA content was measured with a FACScan flow cytometer (Becton Dickinson, San Jose, Calif., USA) with a 488 nm argon laser. By analysis of the area against the peak signal and gated analysis, doublets were discriminated; thus only the DNA content of the single cell population was measured. Normal peripheral blood lymphocytes were used as a standard diploid reference. For eight of the tumours, the material for flow cytometry came from tumour-tissue embedded in paraffin. These specimens were prepared for flow cytometric analysis according to a procedure described by Mørkve [26]. The first peak on these DNA histograms was taken as the diploid peak. For all tumours the DNA index (DI) was calculated as described elsewhere [14]. The cut off points to discriminate aneuploid tumours from diploid tumours were set at a DI of 0.9–1.1.

To test viability, after 40 days of culture, a mixture of light and dark spheroids from one squamous tumour was transferred individually into 16 mm multiwells with 0.5 ml of propidiumiodide 50 µg/ml in PBS with glucose 3 mg/ml and incubated at 37° C for 10 min. The spheroids were then transferred to 16 mm wells with PBS and glucose and studied either under an inverted fluorescence microscope using rhodamine filter optics or by a scanning confocal microscope (Biorad MRC-1000, Hemel Hempstead, England) using a krypton-argon mixed gas laser. Cells with red fluorescence were determined as non-viable whereas living cells were unstained. The spheroids were then fixed individually for light microscopy, as described above, to compare their histological picture with their fluorescence microscopy image. A qualitative impression of live and dead cells was then obtained.

Table 1 Human non-small-cell lung carcinomas grown as spheroids in vitro (*M* male, *F* female, *ND* not determined)

Tumour type	Patient's sex, age (years)	DNA-index	Fraction of spheroid formation	Main histological findings[1]	
				Biopsy (in vivo)	Three-week-old spheroids (in vitro)[2]
Squamous carcinoma					
Sq-1	M, 62	1.0	5%	Well differentiated, abundant keratinization and parakeratosis	Solid tumour spheroid, several mitoses, sparse stroma
Sq-2	M, 68	2.1	8%	Moderately differentiated, scattered giant cells	Polarized spheroid. Partly compact squamous tumour cells, moderately differentiated, with several mitoses and scattered giant cells. Partly stromal tissue
Sq-3	M, 62	2.7	7%	Poorly differentiated tumour with necrosis and abundant stroma	Dense stromal tissue with scattered tumour cells. No mitoses
Sq-4	M, 61	1.0	53%	Moderately differentiated tumour with lymphocyte infiltration	Compact spheroid with large squamous cells, moderately differentiated, scattered mitoses
Sq-5	M, 59	1.5	37%	Poorly differentiated, large cell pattern with atypical nuclei, numerous mitoses and leukocytes	Dense connective tissue with numerous macrophages. Few if any tumour cells
Sq-6	M, 66	1.0	16%	Moderately-to-poorly differentiated tumour	Polarized spheroid. Partly compact squamous tumour cells, moderately differentiated, with several mitoses. Partly connective tissue
Sq-7	M, 74	3.6	22%	Poorly differentiated with vacuolated cells and massive necrosis. Negative mucus staining	Solid tumour spheroids with frequent mitotic figures. Carcinoma with a few glandular like structures
Sq-8	M, 76	1.9	5%	Well-to moderately differentiated with horny pearls and abundant stroma	Heterogenous tumour spheroids. Partly large cell with stroma, vacuoles and several mitoses. Squamous pattern, moderately differentiated. Partly stroma with plasma cells
Sq-9	M, 70	1.8	43%	Well differentiated tumour with several mitotic figures and abundant stroma	Solid heterogenous tumour spheroids. Poorly differentiated carcinoma. Partly large and small cells. Partly stroma with plasma cells
Sq-10	F, 79	1.6	55%	Poorly differentiated, some stroma and mitoses	Compact highly cellular tumour spheroid. Poorly differentiated carcinoma
Sq-11	M, 66	1.5	ND	Well differentiated with horny pearls	Solid tumour spheroid with nests of large and small cells in a whorl like pattern. Moderately differentiated squamous type. Scattered mitoses
Adenocarcinoma					
Ad-1	M, 66	1.4	13%	Well differentiated with necrosis and scattered mucus producing cells	Spheroids containing large tumour cells with mitotic figures
Ad-2	M, 68	3.2	5%	Large cell, solid growth, numerous mitoses, highly invasive. Moderately differentiated. Abundant stroma.	Stroma with patches of calcium and a small rim of tumour cells with intercellular bridges at the periphery
Ad-3	M, 67	1.0	15%	Well differentiated, infiltrating	Dense connective tissue with scattered macrophages
Ad-4	M, 69	3.1	41%	Well differentiated, some stroma	Dense connective tissue, mostly collagen, with scattered macrophages
Ad-5	M, 69	1.5	ND	Well differentiated	Mostly collagen, scattered cells. A mixture of fibroblasts, macrophages and tumour cells
Large cell carcinoma					
L-1	F, 50	1.0	17%	Tumour with numerous mitoses, abundant stroma, inflammatory reaction and some necrotic areas	Compact and loose tumour spheroids. Carcinoma. Scattered mitoses, plasma cells and macrophages

[1] Biopsy and spheroid histology were studied independent of each other
[2] Identifiable cellular spheroids

Bromodeoxyuridine (BrdU) labelling was used to evaluate any cell proliferation in the spheroids. Ten spheroids from a squamous carcinoma were selected after 10 and 20 days of culture and pulse-labelled for 1 h with 10 μM BrdU in complete growth medium containing 10 μM deoxycytidine. The spheroids were then fixed in 70% ethanol for 16 h and embedded in paraffin. The specimens were cut into 4 μm sections that were placed on adhesive microscope slides (Starfrost, Knittel Gläser, Germany). After deparaffinization in xylene and rehydration, the sections were exposed to 0.3% hydrogen peroxide in methanol for 20 min and denaturated for 30 min in 2 M hydrochloric acid. The slides were then incubated with horse serum followed by an incubation (in a humidity chamber) for 45 min at room temperature with a BrdU monoclonal antibody (1:20 dilution in PBS; DAKO, Glostrup, Denmark). The primary antibody was visualized using the Vectstain indirect avidin-biotin peroxidase immunostaining kit (Vector, Burlingame, Calif., USA). The sections were counterstained with haematoxylin and the labelling index was determined as the percentage of labelled nuclei. All cells on two separate sections from each spheroid were counted. Only sections containing more than 100 cells were used. Depending on the size of the spheroid a total of 300–1000 cells was counted to determine the labelling index for each spheroid.

Changes in spheroid size (between 10 to 40 days of culture) were evaluated by the Student's paired *t*-test. Only two tailed tests were applied on the 5% level. To see if frequent changes in medium had any significant effect on spheroid growth, analysis of variance for repeated measurements was performed.

Results

Spheroid cell culture

Spheroid formation was observed in all the tumours studied (Table 1). This process was characterised by the formation of light (translucent) spheroids within 1–3 days of culture. Within a week dark looking, non-translucent spheroids were formed. The fraction of spheroids from the tissue fragments varied considerably between the tumour specimens (5–55%; Table 1) and this was reflected in the number of spheroids available for further studies. After 10 days culture the mean size of the spheroids from the different tumours varied between 250 µm and 450 µm. While all tumours formed dark spheroids, the formation of light spheroids was observed in 10 of the 11 squamous carcinomas, one of the five adenocarinomas and in the one large cell carcinoma (Table 1 Fig. 1). The light spheroids became gradually more yellow/yellow-brown coloured during the second week of culture. A small fraction of these (~10%) disintegrated during the fourth week of culture. The dark looking spheroids on the other hand were seldom found to disintegrate. Cell shedding from the surface of the spheroids was observed, the number of cells and the fraction of spheroids showing this phenomenon differed between the tumour specimens. Besides the spheroids, single cells, small cell clusters, stromal debris and larger tumour fragments (>750 µm) were observed. The larger fragments, while also developing smooth edges, showed a tendency to stick together into even larger fragments. These fragments were not studied further.

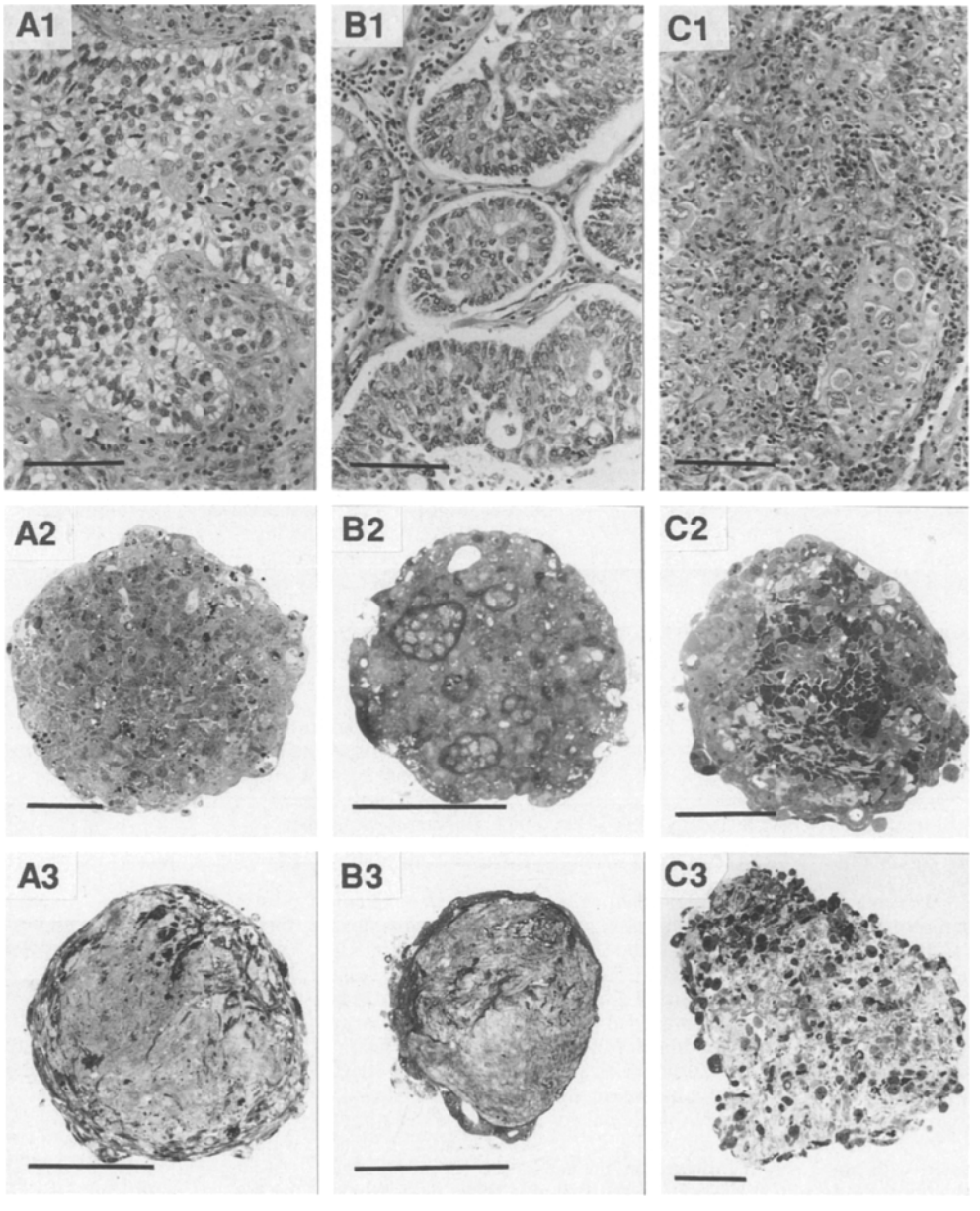


Fig. 1 Photomicrographs of biopsies (**A1, B1, C1**) and semithin sections of highly cellular (**A2, B2, C2**) and stromal spheroids (**A3, B3, C3**) from a squamous carcinoma (Sq-7; **A1, A2, A3**), and adenocarcinoma (Ad-1; **B1, B2, B3**) and a large cell lung carcinoma (L-1; **C1, C2, C3**). *Bar*=100 µm

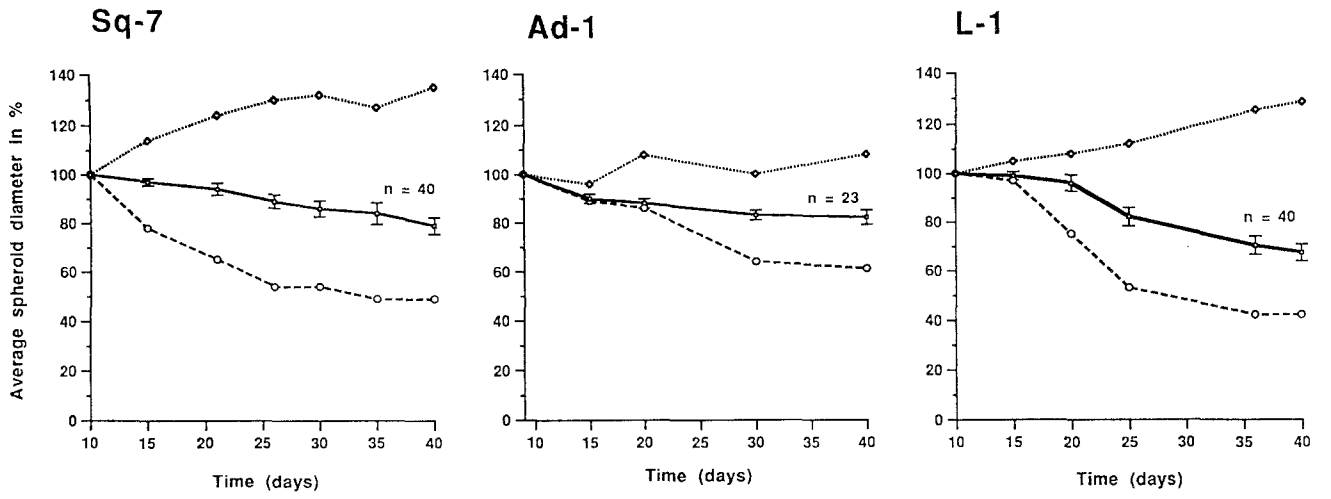


Fig. 2 Spheroid growth curves from Sq-7, Ad-1 and L-1, measured between 10 and 40 days in culture. The mean diameter of all spheroids is shown, as well as that of the two spheroids with the highest and lowest growth, respectively. Spheroid diameter is given in % of initial size. *Bars*=±standard error of the mean

Spheroid growth

The mean size of the spheroids was found to decrease between 10 and 40 days of culture in 11 of the 13 tumour specimens studied. Representative growth curves are shown for Sq-7, Ad-1 and L-1 (Fig. 2). Only spheroids from one tumour (Sq-4) showed a significant increase in size during 30 days culture (data not shown) whereas spheroids from still another tumour specimen (Sq-11) showed a tendency to increase in size (p=0.054). Changing the medium at different time intervals had no significant effect on spheroid growth in the one tumour tested (Sq-11). However, a heterogeneous growth pattern was observed between individual spheroids obtained from the same tumour. For instance, if the mean size of spheroids declined there were occasionally spheroids that increased in size (Fig. 2). The spheroids that grew were all found to be of the light type.

Light microscopy

The histological observations of the resected tumour biopsies and the spheroids are described in Table 1 and in Figures 1, 3 and 4. Among the squamous carcinoma specimens, all degrees of differentiation were represented with three well, one moderately and four poorly differentiated carcinomas. Two tumours (Sq-6 and Sq-8) showed a variable type of differentiation in different areas of the biopsies. Four of the five adenocarcinomas (Ad-1, 3, 4 and 5) were well differentiated whereas the fifth (Ad-2) showed a medium grade of differentiation.

In general, the spheroids showed variability in histological morphology. This varied from highly cellular spheroids consisting mainly of tumour cells to nearly acellular ones consisting of connective tissue components. Among the specimens there were spheroids with more or less fibrous tissue (Figs. 1, 3, 4). A few spheroids were polarized with tumour cells located at one end of the spheroid and stromal elements at the other end (Table 1). This variance in morphology was seen with spheroids from all the three carcinoma types studied and was found throughout the culture period of 40 days.

In squamous carcinomas the highly cellular spheroids obtained from the individual biopsies generally showed carcinoma cells in a compact pattern with visible intercellular bridges and mitotic figures scattered throughout. This architecture was preserved during most of the culture period. In addition, spheroids with a looser appearance with vacuolated and scattered degenerative cells were observed during the last 20 days of culture. Larger cellular spheroids (>500 μm) with a necrotic centre were observed after 2 weeks of culture. No differences in the histological picture were found between highly cellular spheroids increasing in size and those which decreased or showed a stable size. The same was true for the spheroids from Sq-11 that had their medium changed at different intervals. However, a variance in morphology was observed between cellular spheroids from different tumour biopsies (Table 1; Figs. 1, 3).

Highly cellular spheroids were observed from tumours of all degrees of differentiation. The differentiation in the tumour biopsies and in the cellular spheroids was found to be the same in 5 of the 11 squamous tumours studied (Sq-2, 4, 6, 8 and 10; Table 1). In four other carcinomas the cellular spheroids showed either a lower degree of differentiation (Sq-9 and 11) or showed no differentiation at all (Sq-1 and 7). In one biopsy (Sq-7), which was described as a poorly differentiated squamous carcinoma, a glandular like pattern was observed in the cellular spheroids that was not seen in the tumour biopsy (Table 1; Figs. 3, 4). The histological picture of the latter spheroids was rather uniform and stable. Of the last two tumour biopsies, which both showed poor differentiation, Sq-5 was found to form only a few highly cellular tumour spheroids (Fig. 5) whereas none were formed from the Sq-3 tumour.

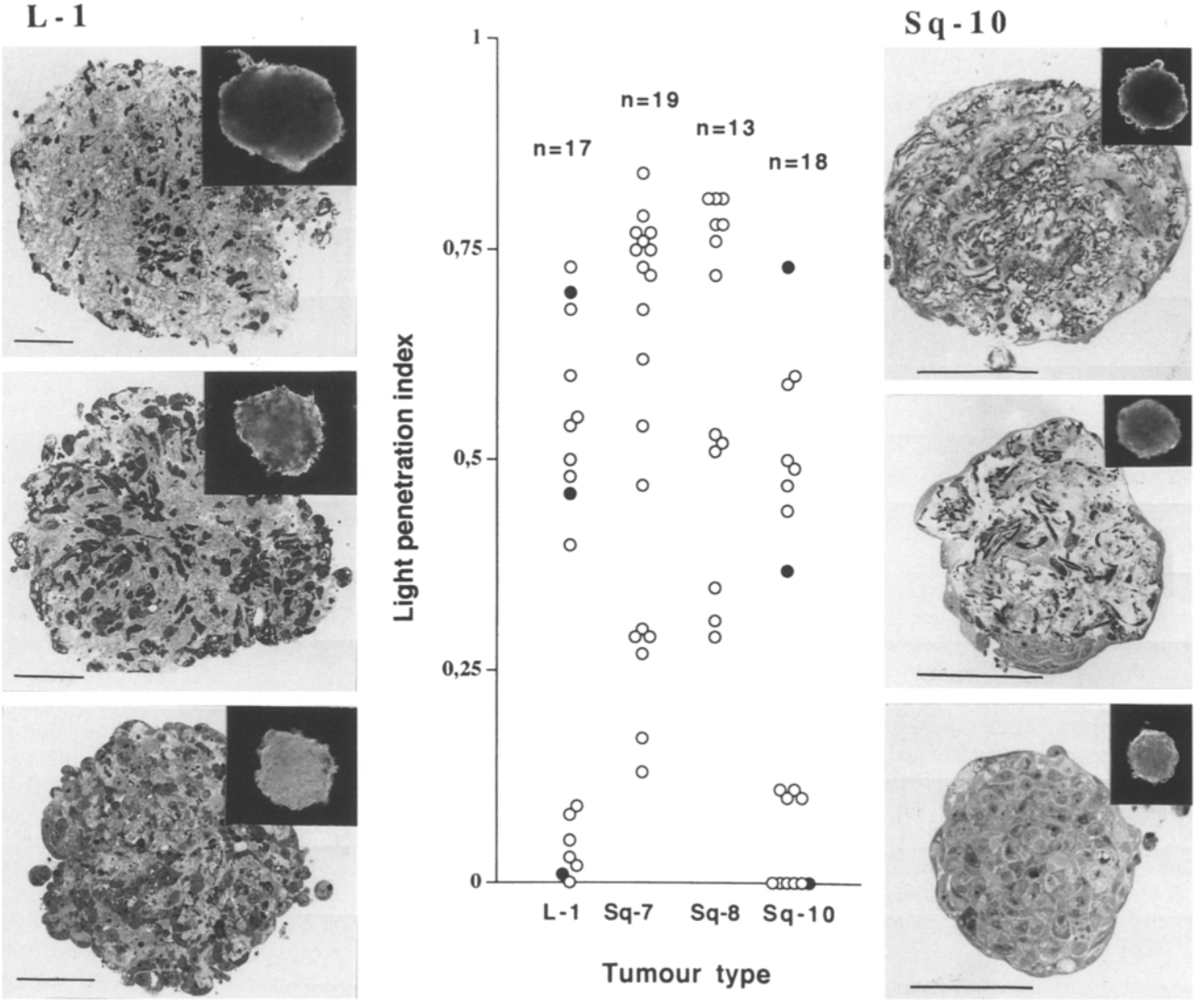


Fig. 3 Light penetration indices obtained from spheroids from four different tumour biopsies (L-1, Sq-7, 8 and 10). The *closed circles* in the diagram represent the spheroids shown in the semithin light microscopic sections. *Insets* show the same spheroids in the tissue culture wells (light, intermediate and dark spheroids). *Bar*=100 μm

In adenocarcinomas the spheroids contained few cellular components and expressed mostly fibrous tissue. In these cultures macrophages and fibroblasts were observed together with atypical tumour cells (Table 1). Occasionally, patches of calcium and elastic fibres were found. In addition, spheroids from one tumour (Ad-2) showed stroma with a 1–3 layer thick rim of cells with intercellular bridges indicating squamous cells. Only spheroids from one of the five adenocarcinomas studied (Ad-1) were found to be highly cellular showing large tumour cells with a grandular organization and scattered mitotic figures (Fig. 1).

The large cell carcinoma spheroids expressed a mixture of highly cellular as well as stromal elements (Figs. 1, 3). The histological picture was in general quite uniform among the cellular spheroids with the tumour cells located in a compact arrangement. Frequent mitotic figures and macrophages with anthracotic pigment were seen throughout the culture period. Solitary as well as groups of plasma cells were seen during the first 3 weeks of culture (Fig. 1). After 40 days, some amorphous, hyaline material was seen intermingling between the tumour cells. At this time scattered vacuolated cells were found in a few of these highly cellular spheroids. Central necrosis was not seen even in the larger spheroids. Also spheroids with mainly stromal elements were seen throughout the culture period.

Selection of spheroids

Figure 3 shows the light penetration index for individual spheroids from four different tumours. For the three squamous carcinomas (Sq-7, 8 and 10) and the large cell carcinoma (L-1) a relationship between the light penetration index and the cellularity of the spheroids

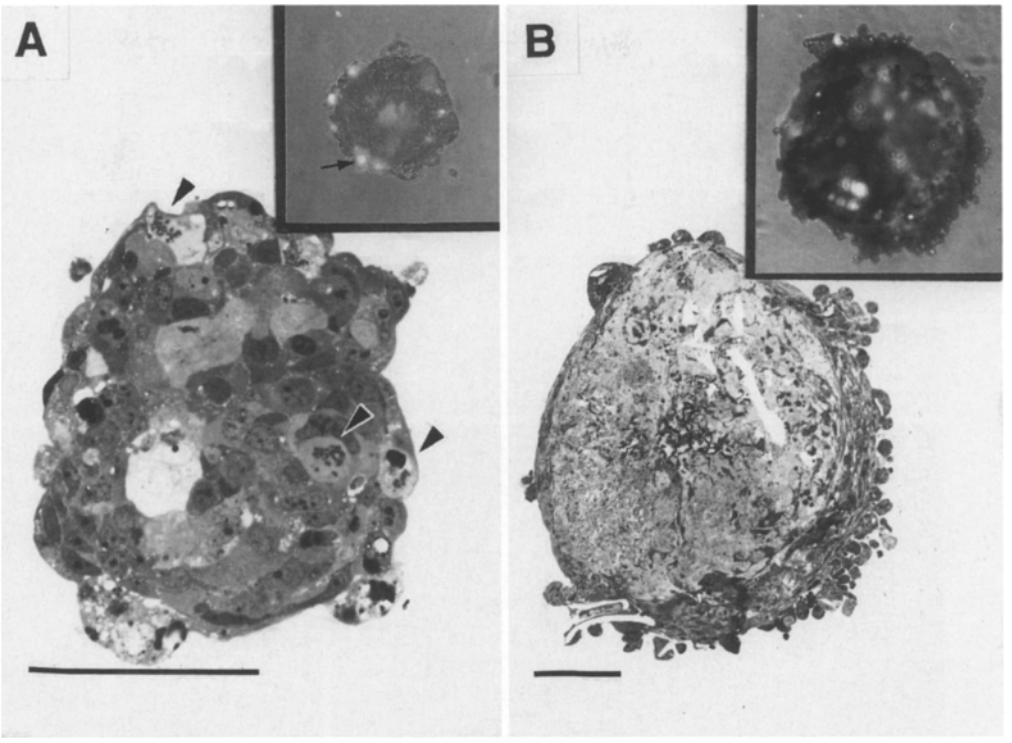


Fig. 4A, B Semithin sections obtained from two Sq-7 spheroids after 40 days culture. The specimens were exposed to propidiumiodide and *insets* show the number of dead cells as observed by inverted fluorescence microscopy. **A** shows a highly cellular spheroid with mitotic figures (*arrowheads*). In addition, fluorescent dead cells are also observed in the same spheroid (*arrow*). **B** shows a spheroid with low cellularity. *Bar*=100 μm

was observed. Regardless of size and tumour type, an index below 0.25–0.30 indicated a highly cellular spheroid whereas an index above 0.5 indicated a mostly fibrous spheroid. Spheroids which had a light penetration index between 0.3 and 0.5 expressed a mixture of cells and connective tissue components. This varied among the spheroids from the same tumour as well as between different tumours. However, if the highly cellular spheroids had a size >500 μm, a light penetration index between 0.3 and 0.5 was observed, particularly if the spheroids had necrotic centres. A light penetration index was not determined for the adenocarcinomas since the tumour tissue formed mainly fibrous spheroids.

Scanning Electron Microscopy

The surface architecture of the light and dark spheroids from the squamous tumour biopsies differed considerably on SEM (Fig. 5). Light spheroids showed a compact outer layer of living cells with high surface activity. This surface architecture was quite uniform between spheroids from one tumour as well as between spheroids from different squamous tumours. The dark spheroids, however, showed either of two different surface architectures. One was dominated by a compact network of fibroblast-like cells with scattered round cells clustered at the surface (Fig. 5C–D). The other had flat epithelioid-like cells at the surface (Fig. 5G–H). These three main architectural types were stable during the whole culture period.

Flow Cytometry

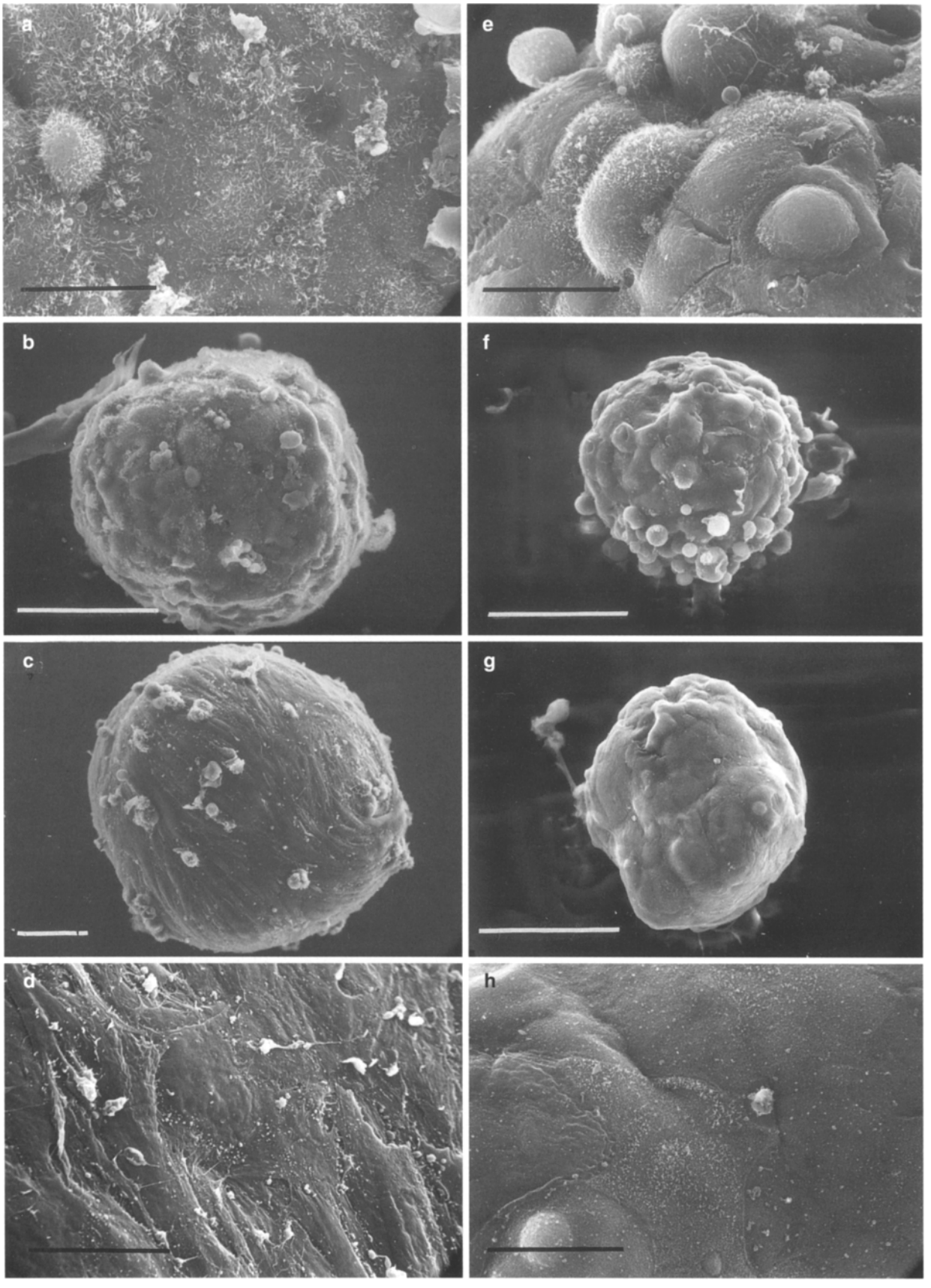
By flow cytometry 5 of the 17 tumours studied had a diploid DNA content, the other 12 were aneuploid with a DI larger than 1.4 (Table 1). No relationship was found between the ploidy of the lung tumours and their ability to form spheroids.

Viability

Light and scanning confocal microscopy of the 40-day-old spheroids that were incubated with propidiumiodide showed both fluorescent and non-fluorescent cells at the surface and at the centre indicating that the dye had penetrated to the centre of the specimens. As shown in Figure 4 (*insets*) a mixture of dead and viable cells were seen, with the majority of cells being unstained. This was also reflected in the histological sections of the same spheroids. In the cellular spheroids several mitotic figures were seen.

Cell proliferation

The labelling index representing cells in DNA synthesis ranged from 2.0%–5.0% (mean 3.4%) in 10-day-old spheroids and 0.6%–5.0% (mean 3.5%) in 20-day-old spheroids. This is in the same range as observed for NSCLC in vivo and by in vitro studies of fresh tumour biopsies [33, 34]. BrdU positive cells were found both in highly cellular spheroids and in spheroids with a mixture of cells and stromal elements.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Discussion

We have shown that it is possible to initiate and maintain spheroids from human NSCLC biopsy specimens in organ culture. In general, the spheroids showed most of the different histological elements found in the parent tumour including tumour cells, host cells, fibrous stroma, calcium deposits and anthracotic pigment. There was, however, considerable variation in the histological architecture between individual spheroids obtained from the same tumour which may reflect the cellular heterogeneity present in the parent tumour in vivo.

The mechanically dissociated biopsy fragments consist of both tumour cells and stromal elements. These fragments are the origin of the spheroids described in this article and this system may therefore be regarded as an organ culture model. To distinguish them from multicellular spheroids generated from cell lines in suspensions we call them tumour fragment spheroids. However light tumour fragment spheroids are indistinguishable from multicellular spheroids as seen through the inverted microscope. Furthermore we cannot exclude that some of the smaller, highly cellular spheroids are induced by cells reaggregating in the culture medium as is the case when generating multicellular spheroids [4, 16].

Morphological studies have shown that lung tumours possess an extensive histological heterogeneity and can express features of both squamous and glandular differentiation [22, 25]. In addition, changes in cellular differentiation may occur during tumour progression in vivo. In the present study no mixed differentiation was seen by light microscopic examination of the tumour biopsies. The spheroids showed in general the same morphological features as their parent tumours except in two cases (Sq-7 and Ad-2) where different histological differentiation was observed. This may be due to differentiation induced by the culture conditions or it may be caused by the inherent biological heterogeneity of lung tumours.

The different tumours showed a variability in spheroid formation. This variation was in particular evident in their ability to form highly cellular spheroids and may in this respect reflect the variability observed when generating spheroids from tumour cell lines [7, 9, 16, 29]. In the present study the tumour biopsies were taken immediately after the lung was removed from the thoracic cavity. The transit time to the laboratory was only a few minutes and the minced biopsies were incubated within an hour after resection of the lung. We found no certain relationship between the time used to prepare the fragments and spheroid formation. Neither the ploidy nor the histological picture of the biopsies was found to be a good predictor of spheroid formation. However, the adenocarcinomas were in general found to be less able to generate highly cellular spheroids when compared with the squamous and large cell carcinomas. The reason for this is unclear.

The spheroids obtained from the NSCLC specimens showed little volume growth. This has also been observed in other studies generating spheroids from primary human tumour material [4, 16, 35] and may partly be explained by a balance between cell proliferation and cell loss as illustrated by the findings of both mitotic figures, BrdU incorporation and cell shedding [5]. The special micromilieu and the lack of blood vessels in vitro may also contribute to the limited volume growth. Our observations of a different growth potential between spheroids from the same tumour may reflect the extensive cellular heterogeneity present in the biopsy material. This can be explained by the presence of tumour cell subpopulations with a higher proliferation potential in some spheroids as reflected in the variation in BrdU labelling index between spheroids. Such subpopulations have been postulated in human tumours in vivo and the phenomenon has been named "clonal dominance" [18, 24]. Primary NSCLC in vivo have been found to have a doubling time between 20–380 days with a high cell loss (>70%) [19]. This may also be reflected in the spheroid model and can explain the relative low growth potential (Fig. 2). Earlier studies in other tumour models have shown changes in spheroid growth by manipulating the culture conditions [7, 17, 28, 29, 35]. However, we found no effect on spheroid growth by changing the growth medium at different intervals.

The spheroids obtained in the present study showed a considerable variation in their cellular content from highly cellular to nearly acellular consisting mainly of connective tissue elements. By using a morphometric technique (Fig. 3) we were able to make a quick estimate of spheroid cellularity without interfering with their microenvironment. Highly cellular as well as spheroids with low cellularity can therefore be selected for further studies. There are however several factors that can interfere with the optical density measurements making the interpretation of the values obtained more difficult. Necrotic material in the spheroids will give them a lower translucency, that is a higher light penetration index, as we observed in larger highly cellular spheroids. Completely necrotic intact spheroids were not observed after 2 weeks of culture and other necrotic tissue pieces could be distinguished both by form (not being spherical) and by a grey or black appearance. Spheroids with a high stromal content on the other hand, were found to have a brown colour as observed in an inverted microscope. In time the highly cellular spheroids also acquired a more yellow brown colour. We therefore recommend that the morphometric technique described is used only on spheroids with a size <500 μm for up to 2 weeks of culture.

It is concluded that the use of tumour fragment spheroids from human lung cancer biopsies may supplement

Fig. 5A–H Scanning electron microscopy of 20-day-old light and dark spheroids from two squamous carcinomas. Sq-5, left column (**5A–D**), and Sq-10, right column (**5E–H**). The light, highly cellular spheroids (**5A, B, E, F**) show cells with a high number of surface processes indicating living tumour cells. The dark, stroma rich spheroids show fibroblast-like (**5C, D**) and epithelial-like (**5G, H**) living cells with few microvilli-like processes. *White bar*=100 μm; *black bar*=25 μm

the existing uses of spheroids from cell lines. The heterogeneity found in tumour fragment spheroids implies that this model can be used for studying the influence of stromal elements on tumour cells. The model may also be used to study cellular heterogeneity as well as biological characteristics of tumour tissue from individual patients. At present we are using these microtumours in confrontation studies with normal human bronchial mucosa with the aim of making a three-dimensional in vitro invasion system. Future applications should attempt to extend the model to include biopsies taken bronchoscopically, when material from non-resectable advanced lung tumours, including the small-cell lung cancers, may also be studied.

Acknowledgements The authors wish to thank Dr. Lodve Stangeland for supplying the biopsies used in this study and Geir Egil Eide for statistical advice. This work was supported by the Norwegian Cancer Society, grant 92105.

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