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ORIGINAL PAPER

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Establishment of permanent cell lines purified from human mesothelioma: morphological aspects, new marker expression and karyotypic analysis

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Abstract This study reports the establishment of three major subtypes of human mesothelioma cells in tissue culture, i.e. the epithelioid, sarcomatoid and biphasic forms, and compares their phenotypic and biological characteristics. Primary cells isolated from biopsies or pleural exudates were subcultured for over 50 passages. We evaluated immunoreactivity using various mesothelial markers related to histological patterns of these cell lines. For epithelioid cells, calretinin and cytokeratin were found to be useful and easily interpretable markers as for control mesothelial cells. The biphasic form was only partially positive and the sarcomatoid type negative. Vimentin was expressed by all cell lines. BerEP4, a specific marker for adenocarcinoma, was negative. Interestingly, while the macrophage marker CD14 was negative, immunoreactivity for a mature macrophage marker (CD68) was expressed by all cell types, suggesting that this marker might constitute an additional tool useful in the differential diagnosis of mesothelioma. At the ultrastructural level, a cell surface rich in microvilli confirmed their mesothelial origin. PCR analysis revealed that none of the cell lines contained SV40 DNA. Karyotypic analyses showed more complex abnormalities in the epithelioid subtype than in the sarcomatoid form. These cell lines may be useful in the study of cellular, molecular and genetic aspects of the disease.

Keywords Malignant mesothelioma · Epithelioid · Sarcomatoid · Biphasic form

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Introduction

Malignant cells of mesothelial origin derived embryologically from the mesoderm exhibit different histological features and share characteristics of both epithelial and mesenchymal cells (Mutsaers 2002; Whitaker et al. 1982). Cytoarchitectural patterns have been used to describe three different subtypes of malignant mesothelioma (MM) depending on the predominant cell type present in the tumour. These subtypes range from the purely epithelioid form resembling adenomatous carcinomas, to the sarcomatoid group. Often these two cell types are mixed, thus forming the biphasic mesotheliomas which represent the most common type of MM (Attanoos and Gibbs 1997; Hoogsteden et al. 1997; Travis et al. 2003). Moreover, histiocytic infiltrates characterised by the presence of small cells in pleural effusions and biopsies, giving rise to "small cell" or "lymphohistiocytoid" mesothelioma (Attanoos and Gibbs 1997; Attanoos et al. 2001; Bielefeldt-Ohmann et al. 1994), can be confused with these cell populations in spite of the difference in size between the two cell types. The ability to perform cell and molecular biological analyses on the various MM subtypes is hindered by the scarcity of appropriate cell lines. Indeed, whereas the development of cell lines has been important

for studying the biological properties of cancer, only few mesothelioma cell lines have been established (Manning et al. 1991; Orengo et al. 1999; Pass et al. 1995; Zeng et al. 1994), as opposed to lung cancer where many established cell lines are available. Most of these cell lines could not be cultured beyond 20 passages.

Histological diagnosis of MM is rendered problematic by the structural variability between different tumours and the subtypes of mesotheliomas. There is no specific marker for mesothelioma, and antibodies that recognise molecules expressed by mesothelial cells and mesotheliomas were noted to have limited specificity or sensitivity. Differences have been noted between epithelioid and sarcomatoid subtypes of mesothelioma, as well as between the sarcomatoid form of MM and adenocarcinomas. However, it is generally agreed that no one antibody shows absolute regularity or specificity in distinguishing the epithelioid from the sarcomatoid type (Abutaily et al. 2002; Attanoos et al. 2001). Recently calretinin, a 29-kDa calcium-binding protein that is expressed in a specific set of neurons (Winsky et al. 1989), has been shown to be a potentially useful marker for differentiating MM in histological preparations (Doglioni et al. 1996; Gotzos et al. 1996; Tos and Doglioni 1998) although results have sometimes been conflicting (Leers et al. 1998; Simsir et al. 1999).

Characterisation of cell lines as being of mesothelial origin and carrying a malignant phenotype is also based on cytogenetic and karyotypic analysis (Kaplan and Hukku 1998; Pass et al. 1995; Versnel et al. 1994). Because most MM are associated with asbestos exposure, the long latency period between asbestos exposure and the onset of MM results in a multistep tumorigenesis process. Cytogenetic analysis reported from fresh patient samples, short-term and long-term cultures or established cell lines demonstrated complex karyotype abnormalities (Pass et al. 1995). However, no specific chromosomal deletions, translocations or inversions could give hints concerning changes responsible for the carcinogenic transformation.

Furthermore, many investigators have suggested that simian virus 40 (SV40) DNA sequences, possibly originating from contaminated poliovirus vaccines that were administered in the USA and some European countries between 1955 and 1963, might function as a cocarcinogen involved in the development of the disease (Carbone et al. 1999, 2003; Mossman and Gruenert 2002). The oncogenic potential of SV40 has been reported to depend on the SV40 large T-antigen (Tag) (Bocchetta et al. 2000; Rizzo et al. 2001). Moreover, the presence of Tag DNA sequences in MM cells has been shown by several groups using the polymerase chain reaction (PCR). However, the causal relationship between SV40, SV40 Tag, MM and asbestos remains uncertain (Galateau-Salle et al. 1998; Pilatte et al. 2000; Simsir et al. 2001).

We have established cell lines belonging to three different subtypes of human mesothelioma, and characterised them with regard to morphology, antigenic markers, ultrastructure, cytogenetic profile and the presence of possible cocarcinogenic factor such as SV40 associated with the onset of MM. Using some new antibodies in addition to a standard panel we suggest that the cell populations display stable, homogenous characteristics within a given line. Such cell lines are expected to help establish reliable markers useful in the characterisation of various forms of MM.

Materials and methods

Reagents and antibodies

NCTC-109 medium, L-glutamine and trypsin-EDTA were purchased from Gibco (Invitrogen Life Technologies, Basel, Switzerland) and fetal calf serum (FCS) from Inotech (Dottikon, Switzerland). Mouse monoclonal antibodies (mAb) to human cytokeratin clones AE1/AE3, anti-swine vimentin mAb clone V9, anti-human macrophage CD68 mAb clone KP1, anti-human monocyte CD14 mAb clone TUK4, irrelevant mouse IgG mAb used as isotype control and mouse mAb to the adenocarcinoma glycoprotein marker BerEP4 were all purchased from Dakopatts (Glostrup, Denmark). Monoclonal antibody to cytokeratins 8, 18 and 19 clone 5D3 was provided by BioGenex, CA, polyclonal rabbit anti-calretinin DC8 antibody by Zymed Laboratories, CA, and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG, FITC dichlorotriazinyl-aminofluorescein (DTAF)-conjugated goat anti-mouse IgG + IgM and R-phycoerythrin (RPE)-conjugated goat anti-mouse IgG by Jackson Immunoresearch, Milian Analytica (La Roche, Switzerland). Primers for SV40 assay by PCR were obtained from Invitrogen, and virus-positive control (SV40) was a gift from Dr. Peter Beard (ISREC, Epalinges, Switzerland).

Cultures and isolation of clones for cell proliferation

Tumour specimens (surgical samples and fluids of pleural effusions collected via a sterile evacuative thoracentesis system) were obtained from patients with untreated MM after informed consent through collaboration with clinicians and in full compliance with ethical guidelines. Two samples were from the epithelioid form of MM, two from the sarcomatoid and two from the mixed type. All cultures were performed in NCTC-109 medium supplemented with 10% heat-inactivated FCS and 0.02 mg/ml L-glutamine and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

For pleural effusions, samples were centrifuged (400 *g* for 5 min) and the pellets washed and distributed in 75-cm^2 flasks (Falcon, Becton Dickinson; 2×10^6 cells in 20 ml medium). Importantly, the medium was changed every 3 days in order to remove contaminating inflammatory cells, which were floating or spread out on the tumour cells. After confluence, the cells were further kept in the same medium for 2 weeks or more, to allow for death of the remaining inflammatory cells. Cells were then treated by trypsin-EDTA for 5 min at 37°C. Pure populations of MM cells were obtained after three to four passages. Control pleural effusions from patients with benign inflammation were similarly processed in order to obtain cultures of normal mesothelial cells. Monolayer cultures of these normal cells could be maintained for three to six passages.

Biopsy samples were cut into small pieces (approximately 1–3 mm) and plated in large Petri dishes (100×20 mm; Falcon) containing 12 ml medium. They were cultivated for 2–4 weeks without changing the medium, during which time colony formation was observed. To prepare cell lines, colonies were isolated by scraping all other cells in the dish with a rubber policeman, washed and then detached with trypsin-EDTA; after washing, the cells were plated and allowed to grow to confluence. Cultures from biopsy samples were found to be more efficient in developing into homogeneous populations than cultures from pleural fluids. For all cell lines, the NCTC medium was changed every 8 days and cells were replated according to their growth rate. Antibiotics were used

only in the first preparation of biopsies and the following cultures were performed without antibiotics.

Immunoreactive assay and cell identification

To confirm their mesothelial origin, the cells were detached with trypsin-EDTA, washed twice and centrifuged on microscope slides in a Cystospin 2 centrifuge (Shandon, UK), fixed for 10 min in absolute ethanol, air-dried and stored at 4°C. For staining for cytokeratins, calretinin and CD68, a second fixation was performed with buffered formol solution (PBS/formol 4%) at room temperature (RT) for 15 min and the preparations were then rinsed with running distilled water for 5 min at RT. Prior to staining with antibodies, the smears were boiled for 10 min in 10 mM citrate buffer solution pH 6.0, allowed to cool down in the same solution at RT for 20 min and rinsed once in distilled water, in PBS and then in PBS/BSA 0.5% for 15 min. They were then processed using a Dako autostainer Universal staining system (Dako). The slides were incubated with diluted primary antibodies, i.e. a pool of monoclonal anti-cytokeratin AE1/AE3 (diluted 1:25 dilution in PBS/BSA 0.5%), and anti-cytokeratins 8, 18 and 19 antibodies (diluted 1:100), anti-calretinin (1:100) and anti-CD68 (1:200) antibodies for 1 h at RT. Without formol postfixation or boiling, preparations were treated with anti-vimentin (1:20), anti-CD14 (1:20) or anti-BerEP4 antibodies (1:100) in PBS/BSA for 1 h at RT and then washed twice in PBS/BSA. All samples were incubated with biotin-labelled anti-mouse or anti-rabbit Ig secondary antibody (1:250 in PBS/BSA) for 30 min at RT followed by washing in TRIS-buffered saline (TBS). Final immunostaining was obtained using streptavidin-alkaline phosphatase and the appropriate chromogen for 30 min at RT. All slides were counterstained for 10 min with fast red, rinsed in water, covered with a coverslip using Aquatex (Merck, Glattbrugg, Switzerland) and visualised with a Zeiss microscope (Axiophot, Oberkochen, West Germany). For controls, isotype-matched antibodies (Dakopatts) were used or the primary antibodies were omitted. The presence of BerEP4 and CD68 was also tested for on frozen samples of adenocarcinomas as controls in order to confirm that CD68 mAb reacts as a selective marker for cells of mesothelial origin, but not for adenocarcinoma cells. Furthermore, representative biopsy samples from tumour corresponding to each cell line were routinely processed as formalin-fixed paraffin-embedded material. Sections were cut for morphological examination using the avidin-biotin-peroxidase complex (ABC) method and examined using a Zeiss Axiophot microscope.

Flow cytometry analysis

Phenotypic analysis was confirmed by exploring cell expression of calretinin, keratin, vimentin, CD68, CD14 and BerEP4 by fluorescence-activated cell sorting (FACS). Cells detached with trypsin-EDTA were centrifuged in NCTC medium + 10% FCS, washed twice in PBS and fixed in a solution of 2% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.4) at 4°C for 45 min. After three washes in PBS, the last one in PBS/BSA 0.1%, they were incubated with antibodies. Each staining step lasted 30 min at 4°C and was followed by two washes in PBS/BSA. A double staining method to detect the CD68 antigen in combination with keratin or calretinin was also used. The preparations were then treated with a secondary DTAF-conjugated goat or rabbit anti-mouse IgG, or RPE-conjugated goat anti-mouse IgG, depending on the experiment. FACS data acquisition and analysis were performed using CELL-Quest software (Becton Dickinson).

Transmission electron microscopy (TEM)

Detached cells were washed and centrifuged at 400 *g*, and the pellets were prefixed for 10 min at 4°C in 0.2% glutaraldehyde in cacodylate buffer and then recentrifuged, refixed for 1 h in 2% glutaraldehyde in cacodylate buffer at 4°C, washed in the same buffer and postfixed in 1% osmium tetroxide at 4°C for 1 h. Samples were dehydrated in a graded alcohol series, then propylene oxide and embedded in Epon. Ultrathin sections were examined in a Philips 400 electron microscope.

Chromosomal analysis

Cytogenetics and karyotyping were performed using cells in tissue culture following in situ treatment with colcemid for 3 h at a final concentration of 0.1 μg/ml. The cells were then treated with a hypotonic solution and fixed in a methanol-acetic acid solution. Trypsin-Giemsa staining banding was effected for assignment of all chromosomes in the karyotypes (Seabright 1971). Chromosome counts were done on well-spread metaphases.

PCR analysis

The possible presence of SV40 infection of the cells growing in culture from biopsies or pleural effusions was determined by PCR using as primers SV3.for 5′-TGA GGC TAC TGC TGA CTC TCA ACA-3′ and the reverse, SV.rev 5′-GCA TGA CTC AAA AAA CTT AGC AAT TCT G-3′, which amplify a 105-bp fragment of Tag. The reaction mixture had a final concentration of 100 ng genomic DNA, 50 μM of each dNTP (Sigma), 0.2 μM of each primer, 0.5 U of Taq DNA polymerase (Sigma) and 1.5 mM $MgCl_2$ contained in the PCR buffer (Sigma) to a final volume of 25 μl. The mixture was assembled for 2 min at 95°C followed by 35 cycles of 10 s at 94°C, 30 s at 62°C, 40 s at 72°C and a final extension of 5 min at 72°C. The amplification products were resolved in an ethidium bromide-stained 5% acrylamide gel. We analysed the three tumour cell lines and included positive and negative controls.

Western blot analysis

Cells were harvested with 0.2% EDTA and solubilised in a lysis buffer supplemented with a protease inhibitor cocktail and PMSF (Complete; Roche, Basel, Switzerland). Lysates were clarified by centrifugation at 4°C and an equivalent amount of proteins from each sample was used to analyse the profile of keratins and CD68 expression. Lysate samples were mixed with an equal volume of SDS buffer and boiled at 95°C for 5 min. Samples were then subjected to SDS-polyacrylamide gel electrophoresis using a denaturing 6–20% gradient. Running and transfer conditions were as recommended by the manufacturer (Amersham Biosciences Basel, Switzerland). Proteins were transferred to nitrocellulose, blocked for 1 h in TBS containing 0.1% v/v Tween 20 and 5% non-fat dry milk. The membranes were incubated for 18 h at 4°C with primary antibodies, goat anti-actin (C-11) SC-1615 (Santa Cruz Biotechnology, CA) 1:250, mouse anti-CD68 mAb (1:200) and a pool of anti-cytokeratin AE1/AE3 (4 μl) and anti-cytokeratins 8, 18, and 19 mAbs (2 μl) diluted 1:400. Antibody-reactive bands on the blots were visualised by incubation with anti-mouse and anti-goat horseradish peroxidase-conjugated mAbs diluted 2,500-fold for 2 h at RT. The chemiluminescent signal was captured using ECL detection reagents according to the manufacturer's instructions (Western Blotting kit; Amersham) and exposure on Kodak BioMax Light film (Kodak, Switzerland). Purified macrophages, which served as positive controls, were obtained from leukapheresis samples after Ficoll Hypaque density gradient and 5 days adhesion culture on collagen-coated plastic Petri dishes at 37°C in 5% CO_2 atmosphere in the presence of GM-CSF 200 U/ml in DMEM and 10% of FCS. Negative control, human normal fibroblast cell line is a generous gift of Dr. J.M. Dayer, Cantonal Hospital of Geneva, Switzerland.

Results

Characterisation of primary tumour

To determine whether the contaminant cells can be totally eradicated in tissue culture, biopsies were cultured and monitored for cell proliferation. A typical mixed-type MM culture developing from a tissue explant (Fig. 1A) shows a heterogeneous population with multiple histiocytic cells. A more homogeneous cell population develops from a sarcomatoid-type MM biopsy (Fig. 1B). This population was separated from the biopsy after a 3-week culture and shows colony formation 4 weeks after replating (Fig. 1C). Cells were then detached from the dish as described in Materials and methods and replated in a flask for proliferation, giving rise to a representative sarcomatoid variant (Fig. 1D).

Three cell lines identified as JL1, DM3 and PJ2, of the epithelioid, sarcomatoid and biphasic forms, respectively, were successfully established from three out of six biopsy specimens (JL1 and DM3) or pleural effusions (PJ2) from patients. The three cell lines exhibited marked differences in morphology. We considered as "epithelial" polygonal cells those whose phenotype was nearest to that of mesothelial control cells, with centrally placed large nuclei; the sarcomatoid-type cells consisted of spindle-shaped cells which appeared triangular with long thick processes, and indistinct oval nuclei; and the biphasic pattern was composed of both elements. Three others cell lines progressed in tissue culture. At the time of writing this paper, these cells have been maintained for over 40 (PJ2) and 52 (JL1, DM3) passages and could be regrown 2 years after cryopreservation at different culture stages in vitro with maintenance of their original morphology. Growth as multilayers is a feature of all three cells lines. Even for the PJ2 cell line, the mixed phenotype was still apparent after 30 passages. All cell lines grew as adherent layers and no floating cells were observed at confluence in the culture media. Sometimes dead cells in aged, multilayered cultures of confluent epithelioid or biphasic forms could be observed. The time for development of a line endowed with stable characteristics was approximately 3–8 months.

The morphological differences between epithelioid and sarcomatoid mesothelioma forms is illustrated by the JL1 cell line (Fig. 2A) and DM3 cell line (Fig. 2B) which present the cells 41 and 22 passages after explantation, respectively. After 4 weeks of culture, normal control mesothelial cells become confluent (Fig. 2C). A typical biphasic form originating from a pleural effusion (4 weeks of culture) is contaminated with a noticeable number of histiocytes (Fig. 2D), which is clearly different from the pure epithelioid or sarcomatoid forms of MM. This culture provided the PJ2 line; no contaminating histiocytes could be detected beyond the sixth passage (not shown). Interestingly, a strong correlation was observed between the presence of histiocytic cells and poor proliferation of tumour cells in the same culture. As a consequence, it was found to be very important to carefully remove these

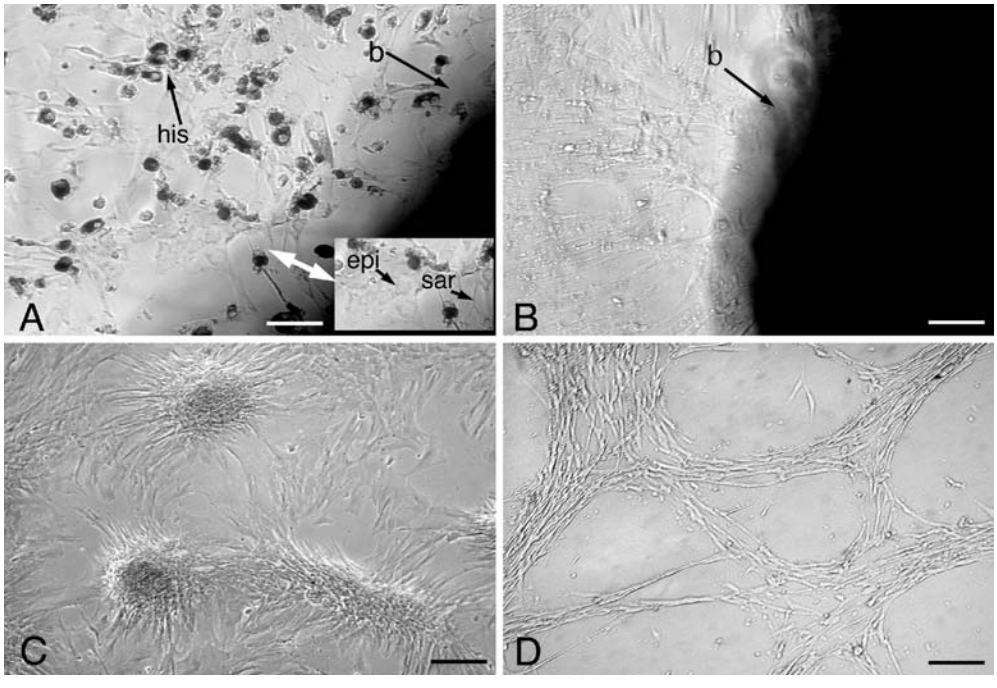

Fig. 1 **A** Phase-contrast micrographs of culture originating from a mixed-type malignant mesothelioma (MM) biopsy (*b*; dark, lower part of the picture), which shows both epithelioid (*epi*) and sarcomatoid (*sar*) cell types migrating out of the explant. Histiocytic cells (*his*) can also be observed. *Inset:* scale identical to main figure. **B** Biopsy (*b*) of a sarcomatoid mesothelioma (dark spot at the right half of the picture) with cells migrating out of the explant. This biopsy was cultured for 3 weeks and then treated with trypsin-EDTA; the detached cells were plated and maintained for 4 weeks (**C**). **D** is a representative sarcomatoid type MM culture. *Scale bar* 10 μm

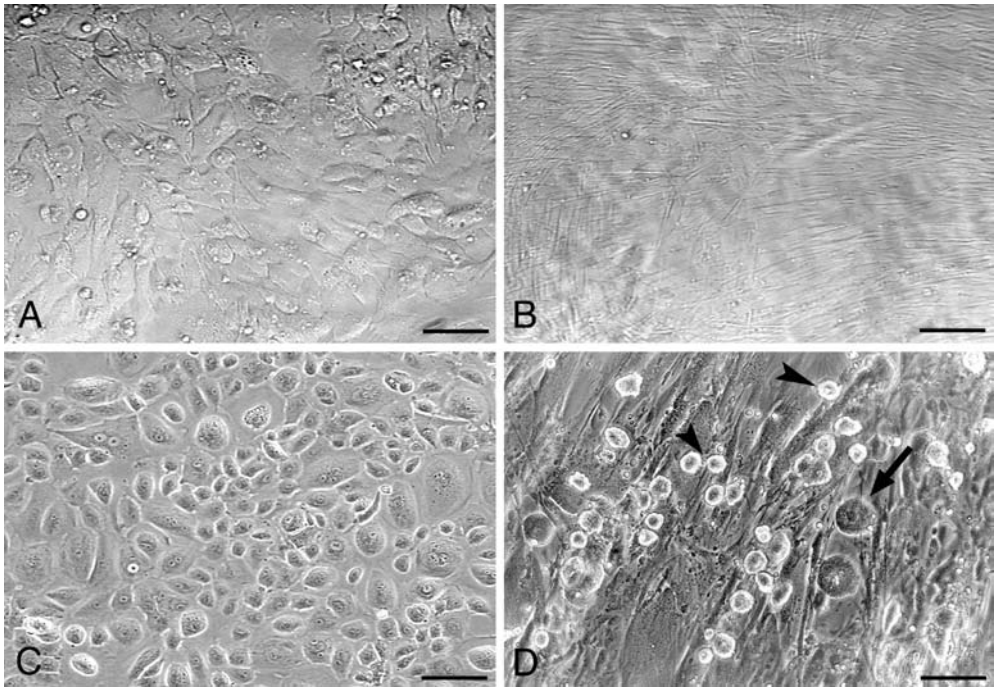

Fig. 2A–D Phase-contrast micrographs of MM cells in tissue culture. **A** JL1 epithelioid-type mesothelioma cells (passage 41); the cells grow in multilayers. **B** Sarcomatoid type MM cells (DM3 line, passage 22). Note the typical elongated cells growing as multilayers. **C** Control, normal mesothelial cells. **D** Mixed-form MM culture (PJ2, 4-week cultures) containing both round, epithelioid cells (*long arrow*) and elongated, sarcomatoid cell types. Many histiocytes (*arrowheads*) are also present. *Scale bar* 20 μm

histiocytic cells to obtain a permanent culture of the tumour cells.

Ultrastructural analysis

By TEM, the majority of cells from epithelioid-type MM cultures were round, and exhibited typical long, thin, surface microvilli or lamellar projections (Fig. 3) similar to their parental normal mesothelial cells (not shown). Cytoplasmic vesicles and vacuoles were abundant, and well-developed structures such as rough endoplasmic reticulum, mitochondria, lysosomes, intermediate filaments and glycogen granules were evident in the three cell types, which also contained large nuclei with nucleoli.

Immunophenotypic analysis of MM cell lines

Immunocytochemical studies were performed on the subtypes of MM to evaluate the cell type-specific expression of various mesothelioma markers (Fig. 4). By immunocytochemistry, calretinin and keratin were exclusively expressed by the entire epithelioid cell population (Fig. 4A, B). They are found to be useful and easily interpretable markers with >98% positive cells as also observed for normal mesothelial cells (not shown). These markers are only partially positive by the mixed form (30–50% cells) which also contained some epithelioid-positive cells (Fig. 4C, D). No immunoreactivity was observed for the pure sarcomatoid form (not shown). Furthermore, the epithelioid- and sarcomatoid-type MM cells, as well as the mixed type and control normal mesothelial cells, were all positive for both vimentin (>99%)

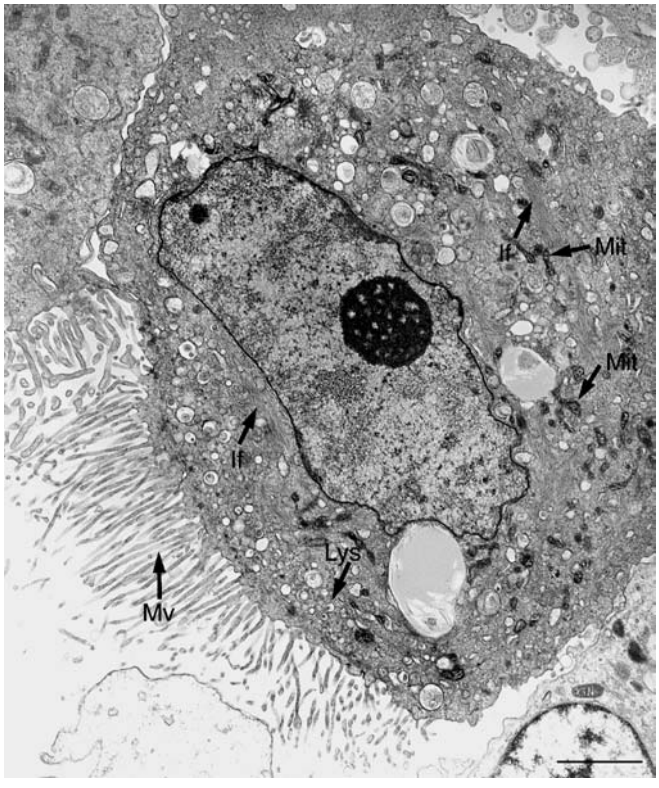

Fig. 3 Electron micrograph of a typical epithelioid MM cell exhibiting slender, long surface microvilli (*Mv*), with abundant mitochondria (*Mit*), lysosomal structures (*Lys*) and intermediate filaments (*IF*). *Scale bar* 2 μm

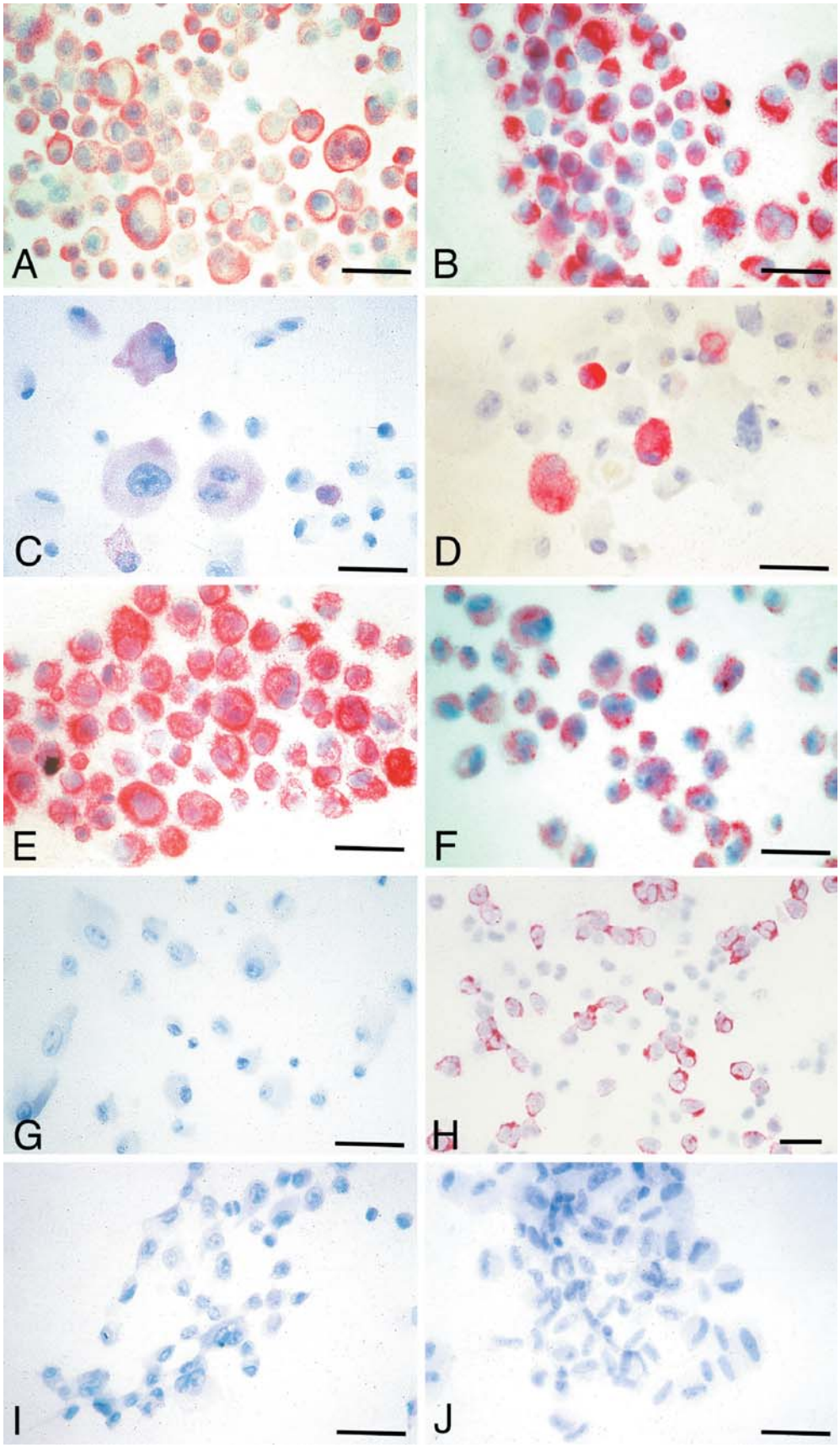

Fig. 4A–J Immunocytochemical staining of MM cells in culture. **A** Epithelioid (JL1) cells are positive for calretinin with diffuse cytoplasmic staining pattern. **B** JL1 cells showing intense staining for keratin. **C**, **D** Cells of the mixed form PJ2 line: only the large, epithelioid cells are positive for calretinin (**C**) and keratin (**D**). **E** Epithelioid-type (JL1) and **F** sarcomatoid-type (DM3) cells show staining for vimentin and CD68, respectively. **G**, **H** Cells of the mixed type are negative for CD14 (**G**), whereas control peripheral blood leukocytes are positive for this marker (**H**). **I** Sarcomatoid cells are negative for BerEP4. **J** IgG control antibodies. *Scale bar* 10 μm for **A–G**, **I–J**; 5 μm for **H**

and CD68 (a marker for macrophages) (>97%); epithelioid cells were stained for vimentin (Fig. 4E) and the sarcomatoid cells for CD68 (Fig. 4F).

Anti-CD68 antibody appeared to stain intracytoplasmic granules. Whereas this marker was also positive on frozen material from MM biopsies (not shown), it was negative for adenocarcinoma cells (not shown). CD14, another macrophage marker, was negative for all cell lines (Fig. 4G), and was significantly expressed by monocytes from the peripheral blood (Fig. 4H). The absence of detectable staining for MM cell lines using BerEP4, a marker for adenocarcinoma (Fig. 4I), supports the difference between the two cell types. Normal mesothelial cells from the pleural cavity of patients with benign inflammation were found to be positive for the same markers as they were positive for MM (not shown) thus confirming the mesothelial origin of the tumour cells. Comparative studies by immunohistochemical staining of representative tissue samples from MM biopsies revealed the same results, indicating that the cultured cells maintained the immunohistochemical characteristics of the parental tumour cells (not shown).

In addition, analysis of individual cells by flow cytometry using a double-staining immunofluorescence procedure was also performed (Fig. 5). A large percentage (see Fig. 5A) of the JL1 tumour cells expressed both calretinin (FITC) and CD68 (RPE) (Fig. 5A *a*, *b*). A double-colour immunofluorescence staining showed coexpression of CD68 and calretinin (Fig. 5A *c*). Staining for CD14 was negative confirming a cell population different from macrophages (Fig. 5A *d*). The same experiment (see Fig. 5B) was performed using the PJ2 cell line prior to elimination of histiocytes. The line was positive for CD68 and partially so for calretinin (Fig. 5B *f*, *g*). This was confirmed with partial coexpression of CD68 and calretinin (Fig. 5B *h*). CD14 expression was detected in a population, most likely representing the contaminating histiocytes (Fig. 5B *i*), that was clearly distinct from the rest of the cells, which were essentially negative for this marker. The same histiocytic population was negative for calretinin, but also positive for CD68 (Fig. 5B *h*).

Using these antibodies, we also evaluated the expression of keratins and CD68 in cell lines by western blot analysis. The lysate obtained from Triton X-100-treated cells (Fig. 6A) gave results that support the immunocytochemistry analysis for keratins with a strong positive staining for normal mesothelial cells and epithelioid form (JL1 cell line) (*lanes 1* and *2*, respectively), against a partial staining of the mixed form PJ2 cell line (lane 3). Cells from the sarcomatoid form (lane 4), macrophages (lane 5) and fibroblasts (lane 6) are negative for this protein.

In Fig. 6B, anti-CD68 antibodies stained a band with a molecular weight of 110 kDa in NP40 lysates of the PJ2 cell line and macrophages (*lanes 3* and *5*, respectively). Fibroblastic cells were not stained by anti-CD68 (lane 6). Except for macrophages (*lane 7*), no staining of material at this position was seen with Triton X-100 lysis treated

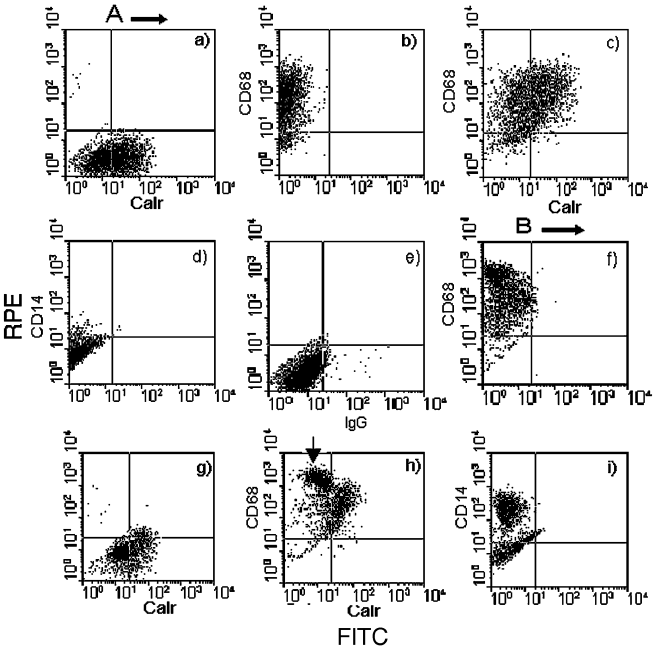

Fig. 5 A Flow cytometric analysis of antigen expression by cells of the JL1 line. *a* Calretinin (*Calr*), *b* CD68, *c* double staining showing a coexpression of CD68 and calretinin, *d* CD14, *e* IgG control. **B** Experiment with a cell line contaminated with histiocytes. *f* 84% of 90% gated cells stain for CD68. Calretinin (*g*) is positive for 43%; *h* Double staining where 47% of the cells stain for both calretinin and CD68, whereas a discrete population (presumably histiocytic and sarcomatoid type) is positive for CD68 but not for calretinin (*arrow*). *i* 38% of cells are positive for CD14

cells (Fig. 6B *lanes 1, 2, 4*). Actin was positive in all conditions (Fig. 6A, B).

Karyotypic examination and PCR results

Cytogenetic analysis revealed abnormal karyotypes with both numeric and structural chromosomal abnormalities. The most frequent structural changes were fragment deletions and/or translocations, trisomies and monosomies, particularly in the epithelioid form which presents more restructurings and chromosomal alterations with many amplifications or deletions (Fig. 7A). Many karyotypes from the sarcomatoid form are normal or close to those of normal cells showing few chromosomal alterations (Fig. 7B). Unassignable chromosomes, particularly in the epithelioid form, will be those that are neither normal nor consistently present from one karyotype to another.

By PCR analysis, no SV40-positive amplification was detected in any of the three cell lines of MM studied. In contrast the SV40-positive control was always detected (not shown).

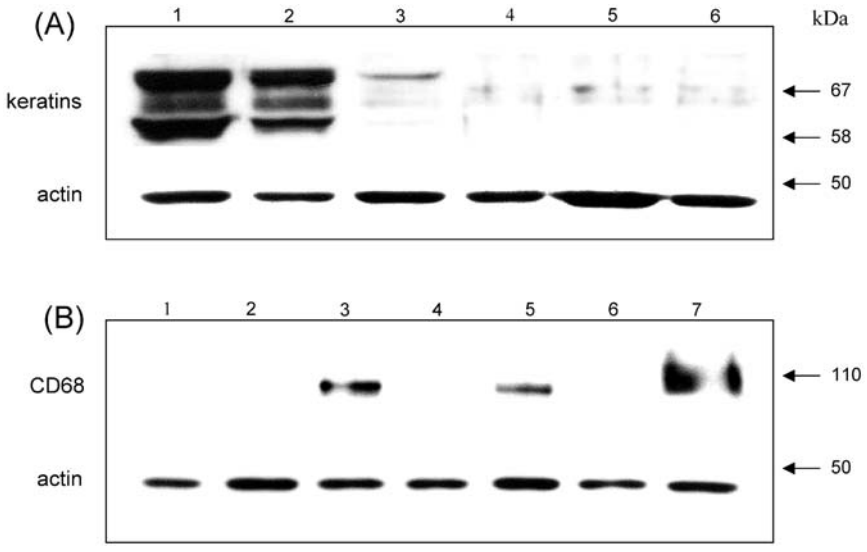

Fig. 6 A Cells were lysed in a medium containing 1% Triton X-100 and a mixture of protease inhibitors. The proteins were then treated as indicated in Materials and methods. Western blot analyses were performed with the antibodies/antisera against the indicated keratins in human mesothelial cells, cell lines and controls. *Lanes 1–6* represent marker proteins under reducing conditions in extracts of normal mesothelial cells (*lane 1*), epithelioid cells (JL1, *lane 2*) and mixed cells (PJ2, *lane 3*). Keratins were not expressed by the sarcomatoid type (DM3, *lane 4*), macrophages (5 day culture, *lane 5*) and normal fibroblasts (*lane 6*) as negative control. All samples were loaded with 25 μg of proteins. **B** Western blot analysis of CD68 expression. Samples in *lanes 1*, *2*, *4* and *7* are from extracts obtained using Triton X-100; cells from *lanes 3*, *5* and *6* were homogenised in 50 mM HEPES, pH 7.5, containing 1% Nonidet P40, 0.25% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthonovanadate and protease inhibitors. *Lane 1* Normal mesothelial cells, *lane 2* Jl1 cells, *lane 3* PJ2 cells, *lane 4* DM3 cells, *lanes 5* and *7* normal macrophages, *lane 6* fibroblasts. All samples were performed under reducing conditions. *Lanes 3*, *5* and *7* show 110-kDa bands detected by the anti-CD68 KP1 antibody in extracts of PJ2 cell line and purified macrophages after 5 days of culture. In each condition 20 μg of proteins were used

Discussion

Establishing stable and permanent primary cell lines represents an essential tool to study MM at the cellular and molecular level and to test therapeutic approaches. Up to now, no human mesothelioma cell lines have been successfully established. Using cells from tumour biopsies and fluids from pleural effusions of patients with the disease, homogenous cultures of different MM subtypes were obtained. These lines have been used to analyse differences in marker expression between the epithelioid and sarcomatoid forms, which may be of use in the further histological characterisation of primary tumour.

Markers capable of identifying MM unambiguously are scarce. MM are considered negative for antibodies to carcino-embryonic antigen (CEA), CD15, the epithelial glycoprotein Bg8 and the tumour glycoproteins BerEP4 and MOC-31, while these markers are usually positive for adenocarcinomas (Oates and Edwards 2000; Riera et al. 1997; Wirth et al. 1991). The antibodies most commonly used for identification of MM cells are anti-calretinin, which reacts with epithelioid-type mesotheliomas (Attanoos et al. 2001; Doglioni et al. 1996; Gotzos et al. 1996; Tos and Doglioni 1998), and anti-cytokeratins (Clover et al. 1997; Whitaker 2000); both antibodies have been found useful in separating epithelioid mesothelioma (positive staining) (Clover et al. 1997; Doglioni et al. 1996; Gotzos et al. 1996; Whitaker 2000) from adenocarcinoma (negative staining) (Ordonez 1998a, b). Other positive markers for mesotheliomas have been described recently, including HBME-1 (Ordonez 1997), mesothelin (Chang and Pastan 1996), N-cadherin (Han et al. 1997), thrombomodulin (Collins et al. 1992; Ordonez 1997) and Wilms' tumour gene product (Amin et al. 1995). Although most attention has been given in recent years to the immunostaining separation of epithelioid MM from metastatic adenocarcinoma, antibodies used for that purpose are generally not helpful in the differential diagnosis of non-epithelioid MM variants (Wick and Mills 2000). Distinction in calretinin and cytokeratin expression have been recognised as potential means of differentiating between the epithelioid form, positive, and the sarcomatoid type, negative for these antibodies (Clover et al. 1997; Gotzos et al. 1996). In the present study, positivity of the JL1 cell line for calretinin and cytokeratins excludes the presence of a subpleural carcinoma growing in a pseudomesotheliomatous pattern.

The morphology of the JL1 and DM3 cell populations was homogeneous for the epithelioid and the sarcomatoid form, respectively. The use of well-differentiated cell types produced impressive discriminatory expression of the markers recognised by anti-calretinin and anti-cytokeratin antibodies. Interestingly, western blotting results which are consistent with immunocytochemistry findings indicated that only epithelioid cells express these proteins. Some reports have cast doubt on the value of calretinin as a diagnostic marker probably because of the use of less well differentiated tumour or mixed cell populations

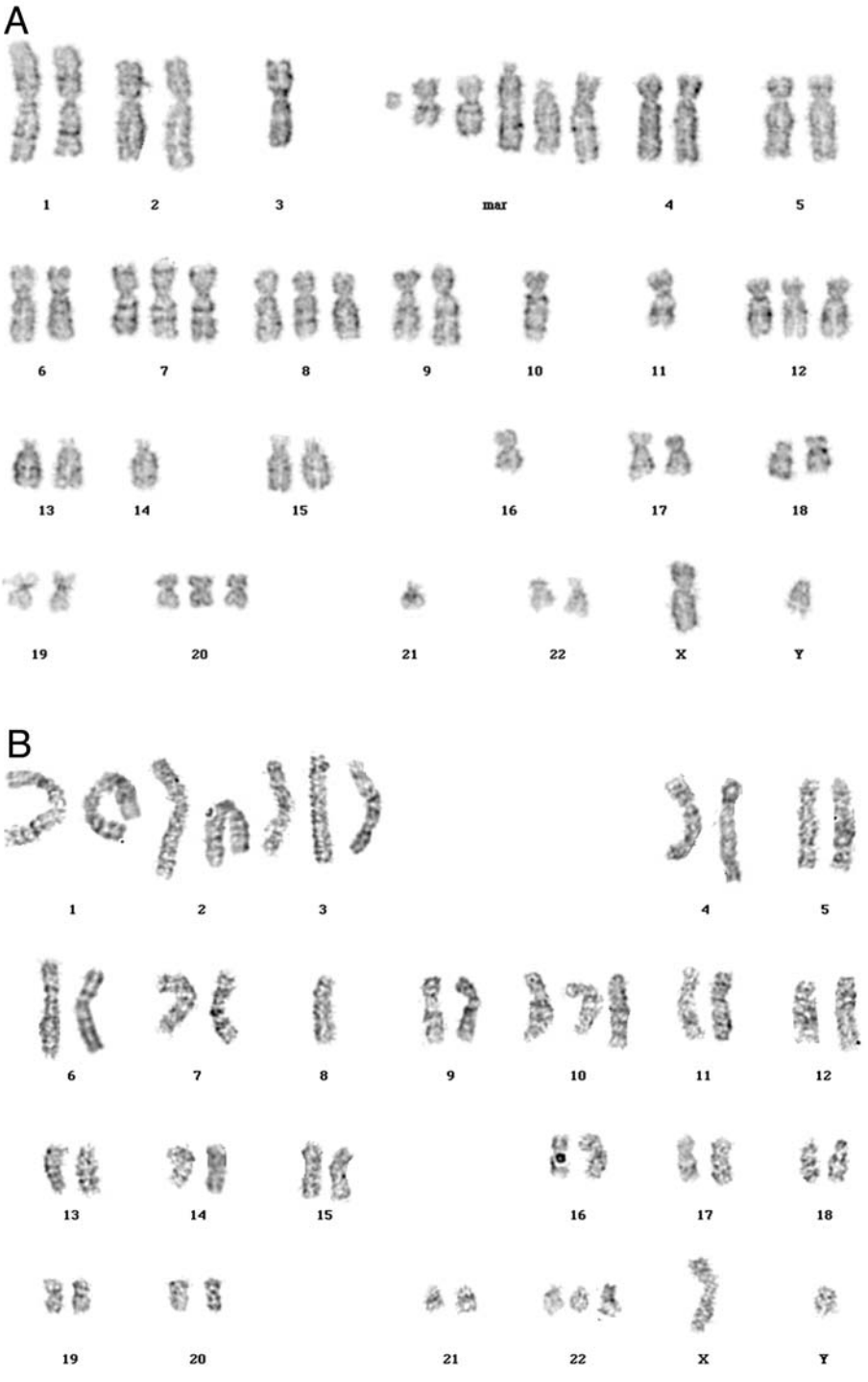

Fig. 7 A Representative karyotypic analysis of the epithelioid JL1 cell line showing many structural anomalies, monosomies and trisomies. Unassignable chromosomes (*mar*), grouped between chromosome numbers 3 and 4, represent non-identifiable chromosomes with structural anomalies not consistently present from one karyotype to another. **B** Representative karyotype from the DM3 sarcomatoid line with far fewer chromosomal abnormalities

(King and Hasleton 2001). The detection of calretinin- and keratin-positive epithelioid cells does not serve to identify the sarcomatoid type of MM (Clover et al. 1997; Doglioni et al. 1996; Gotzos et al. 1996). However, these markers may nevertheless be useful because the biphasic form represents the most current group of mesothelioma-containing areas with both sarcomatous and epithelial features.

While the cell lines were negative for CD14 immunoreactivity, staining for CD68 was strongly positive for all cell lines as well as for control normal mesothelial cells by immunocytochemistry and flow cytometry. Constitutive expression of the CD68 antigen was also demonstrated by western blot analysis of cells extracted by NP40 (but not with Triton X-100). Conversely, fibroblastic cells were negative. This finding confirmed that KP1 is a valuable addition to a panel of mAbs for

phenotyping mesothelioma cells. Few authors have described immunoreactivity for CD68 in mesothelioma cells. Positivity for this marker was attributed to the proliferation of histiocytic cells, based on morphological features and immunohistochemical stains (Chan et al. 1997; Wu et al. 2000). However, the immunoreactivity of CD14 to help determine the true histiocytic identity of these cells was not established. CD68 is a 110-kDa intracellular glycoprotein primarily associated with cytoplasmic granules and to a lesser extent with the membrane of macrophages, monocytes and some other granular leucocytes (Holness and Simmons 1993). Designated as macrophage marker, anti-CD68 antibodies also demonstrated a variety of staining patterns in lymphocytes and some non-haematopoietic cells, which are related to the presence of lysosomal glycoproteins (Hameed et al. 1994; Pulford et al. 1990). The exact function of CD68 remains undefined. However, based on the structural homology with mononuclear cells, the expression of this molecule in MM could be associated with some properties of phagocytic cells, such as pinocytosis and phagocytosis. As previously described by others for epithelial cells (Doussis et al. 1993), our observation confirms that anti-CD68 antibodies, especially the KP1 clone used in our experiment, are specific mainly for organelles and not for a cell type, thus, the staining of mesothelial cells containing multiple granules and lysosomes is possible. Our data are consistent with other reports showing that anti-CD68 also reacts with granular cell neoplasms (Doussis et al. 1993; Tsang and Chan 1992) and malignant melanomas (Facchetti et al. 1991). Consequently, the constitutive expression of CD68 by MM cells extends their immunophenotypic similarities with mesenchymally derived mononuclear phagocytes and provides an additional antigenic marker for the diagnosis of mesothelioma.

Ultrastructural observations provided convincing evidence that the epithelioid form of tumour cells represented the fully differentiated mesothelial cells, except that sarcomatoid cells tend to lose the specialised features of epithelial cells when undergoing spindle cell transformation (Hammar and Bolen 1985). Moreover, the JL1 cell line showed the typical characteristics of mesothelial cells, presenting microvilli with a length to diameter ratio >15 and intracytoplasmic vesicles with basal lamina as reported elsewhere (Ordonez and Mackay 1998; Oury et al. 1998).

Recent studies have suggested that SV40 may be a factor in the development of MM, although the pathogenic mechanism remains unclear (Carbone et al. 1999, 2003; Testa et al. 1998). However, other laboratories have reported different results and concluded that SV40 is not a contributing factor in the pathobiology of MM, or argued that the SV40 DNA detection is probably a PCR contamination artefact (Galateau-Salle et al. 1998; Pilatte et al. 2000; Simsir et al. 2001). The effect of infection of human mesothelial cells by SV40 is unknown, because few mesothelial cell lines have been studied. Hence the role of SV40 in the pathogenesis of MM, as suggested by the susceptibility of human mesothelial cells to SV40 virus infection, remains an open question (Galateau-Salle et al. 1998; Pilatte et al. 2000; Rizzo et al. 2001; Simsir et al. 2001). In any case, the presence of SV40 DNA sequences was not detected in any of the three cell lines studied.

Cytogenetic analysis, performed on epithelioid and sarcomatoid cell lines between the 8th and 30th passages, revealed complex patterns of chromosomal abnormalities. Interestingly, karyotypes of the epithelioid cell lines revealed more chromosomal alterations than the sarcomatoid form. Translocations, deletions and inversions have been reported in the literature (Balsara et al. 1999; Hagemeijer et al. 1990; Hansteen et al. 1993). Further studies will be required to determine whether malignant transformation in this type of cancer is associated with identifiable chromosomal abnormalities.

To summarise, this study reports methodological conditions allowing purification and maintenance of MM cell lines in tissue cultures. These cell lines display the specific morphological characteristics of the epithelioid, sarcomatoid and biphasic forms, and show distinct expression patterns for specific markers as well as exhibiting various karyotypic abnormalities. These findings provide a structural basis for a better understanding of the cellular, molecular and genetic aspects of the disease.

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