ABSTRACT

Two cell lines with different in vitro growth patterns were established from the pleural fluid of a patient with malignant epithelial pleural mesothelioma. The cell line established in RPMI 1640 supplemented with human AB serum had an epithelial morphology, while the cell line established in fibroblast-like medium had a fibroblast-like morphology. Exposure of the fibroblast-like cell line to human AB serum-containing medium resulted in a nearly complete transformation of the morphology to the epithelial-like phenotype, and the epithelial-like cell line changed its phenotype to fibroblast-like upon exposure to fetal calf serum-supplemented medium. Both cell lines formed colonies in soft agarose and secreted hyaluronic acid into the culture medium. In both cell lines all the metaphases studied lacked chromosomes 5 and 9, demonstrating the same clonal origin. However, one marker and a missing chromosome 11 were found only in the fibroblast-like cell line. We conclude that human AB serum supplement can be used for the establishment of human tumor cell lines, and that the choice of serum can affect the in vitro morphology of the established mesothelioma cell lines. The mechanisms behind the different growth patterns seem to be a selective stimulation of different subpopulations of malignant cells as well as induction of changes in the morphology of individual cells.

INTRODUCTION

Malignant mesothelioma is a rare malignancy of the mesothelial cells. The basic annual incidence of this tumor is estimated to be only about one per million, but exposure to asbestos or asbestos-like minerals may lead to a thousandfold increase of the incidence (1). The expanded use of asbestos is the main reason for the gradually increasing frequency of mesothelioma all over the industrialized world (2). The tumor develops in pleural or abdominal cavities often more than 30 yr following exposure to asbestos. The biological behavior of this tumor is highly variable, with differences in the rate of progression. The mesothelial cells are of mesenchymal origin; thus, the tumor is a sarcoma. Even though sometimes the tumor cells display a epithelial appearance. During the last decade more than 100 different layers of the pleural tissue (3, 4). However, it has also been suggested that all mesothelioma types originate from the same precursor cell (5–7). The precise origin of mesothelioma within the mesothelial lining is thus subject to debate. Since mesothelioma often displays a carcinomatous growth pattern, the histological similarity to metastatic carcinoma may lead to diagnostic difficulties (8). One way by which mesothelioma reveals its mesenchymal origin is by the production of HA (9, 10). One of the most important tools so far in distinguishing between malignant mesothelioma and metastatic adenocarcinoma is therefore the detection of HA in the tumor tissue or in the resulting effusion. Another characteristic that can be given diagnostic importance is the ultrastructure of the mesothelioma cells. Here the detection of long and slender microvilli can be used to differentiate the mesothelioma from other tumors with epithelial appearance. During the last decade more than 100 permanent human lung cancer cell lines have been established. The standard procedure while establishing cell lines has been to use medium supplemented with FCS or chemicals and hormones (11, 12). To our knowledge human AB serum has, so far, not been used. The present study is a report on two mesothelioma cell lines with different morphologies, dependent on whether human AB serum or FCS was used.

MATERIALS AND METHODS

Clinical Data. The patient is a 59-yr-old male with a previous successful surgery for epidermoid lung cancer. The patient also suffers from macroglobulinemia Waldenström. The pleural effusion that developed on the side contralateral to the previous lung cancer showed an increased HA content. In subsequent effusions the HA content was still higher, and histology excluded an epidermoid carcinoma in favor of an epithelial mesothelioma. The course of the disease was relatively benign, and the patient needed only occasional drainage of pleural fluid. The patient died 33 mo after diagnosis. The autopsy revealed locally advanced malignant pleural mesothelioma. There was no evidence of recurrence of squamous carcinoma of the lung.

Culture Methods. Pleural fluid was collected for culture at one occasion 12 mo after the initial diagnosis of mesothelioma. A total of 300 ml was placed in a plastic vial containing preservative-free heparin. Within an hour the fluid was pelleted by centrifugation and separated from erythrocytes by gradient centrifugation using Lymphoprep (Nygaard & Co., Oslo, Norway). The cells were washed twice in PBS and suspended in 2 different media: RPMI 1640 supplemented with 10% FCS and 10% HS, respectively. Cells were incubated in Nunclon plastic flasks (A/S NUNC, Roskilde, Denmark) in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Media were changed twice a week, and cells were passaged at the confluence using 0.25% trypsin with 0.02% EDTA added. The initial seeding cell density was 500,000 cells/ml. After 4 wk of culture the cells were seeded at a concentration of 200,000 cells/ml.

Sera. Different batches of FCS were obtained from Biochrom KG (Berlin, West Germany) and Northumbria Biologicals, Ltd. (Cramlington, United Kingdom). Several batches of HS were obtained from healthy donors who were serologically negative for human immunodeficiency virus and hepatitis B surface antigen. Every batch was pooled from 8 to 9 different donors.

Incubation of FCS-cultured Cells in HS-supplemented Medium and Vice Versa. FCS-cultured cells were trypsinized, washed 3 times in PBS, and resuspended in medium containing 10% HS. They were then grown in HS medium for 6 wk. Passages were made at the confluence, and medium was changed twice a week. The equivalent procedure was applied to the HS-cultured cells, substituting the culture medium with FCS for 6 wk.

Transmission and Scanning Electron Microscopy. Cell suspensions were fixed overnight in cold (4°C) 2% glutaraldehyde, also containing 0.1 M cacodylate buffer at pH 7.3 and 0.1 M sucrose. After rinsing in 0.1 M cacodylate buffer at pH 7.3 and 0.1 M sucrose. After rinsing in...
cold buffer, cells were postfixed in 2% OsO4 in a 5-collidine buffer, pH 7.4, for 2 h. The cells were then centrifuged at 300 × g for 5 min, whereupon the obtained pellets were dehydrated in ethanol, taken through propylene oxide, and embedded in epoxy resin (LX-112; Ladd, Burlington, VT). Ultrathin sections were made using an ultramicrotome (LKB Produkter, Stockholm, Sweden). To further visualize the growth patterns and cell surface structures, cells were also cultured on glass slides inserted into the Nunc 6-well culture dishes. After 1 wk the slides were taken for critical point drying and subsequent coating for scanning electron microscopy.

Immunocytochemistry. In order to visualize intermediate filaments, cells were allowed to grow on the glass slides, fixed in cold (~20°C) methanol, and stained with monoclonal antibodies against vimentin and keratin (Bio Genex Laboratories, Dublin, CA) using the biotin-avidin-peroxidase system (Vectastain PK 4002 ABC kit; Vector Laboratories, Burlingame, CA) to visualize antibody reactivity. Enzyme label was demonstrated using diaminobenzidine (Sigma, St. Louis, MO), and monoclonal anti-Leu 4 antibody (Becton Dickinson, Immunocytochemistry Systems, Mountain View, CA) was used as a negative control.

Demonstration of Hyaluronic Acid Production. Hyaluronic acid was analyzed in the cell-free media collected from both cell lines on Days 1 to 4. On Day 4 the cell density was 400,000 cells/ml. We used as control PCS- and HS-supplemented media as well as media from a small cell lung cancer cell line grown in both PCS- and HS-supplemented media at the same cell density. The glycosaminoglycans were precipitated from 20 μl of the fluid by the addition of 80 μl of ethanol. Following centrifugation (10,000 × g for 5 min) the supernatants were removed, and the pellets were digested and analyzed using high-performance liquid chromatography as previously described (13).

Colony Formation in Agarose and Determinations of Doubling Time. For each cell line a single cell suspension of 100,000 cells/ml was prepared in medium supplemented with FCS or HS and 0.3% agarose. Each suspension was then poured onto the prehardened layer of 0.5% agarose also containing serum supplement (14). Colonies of 20 or more cells were counted 14 days later. For growth rate determinations 100,000 cells/ml from each cell line were seeded into plastic flasks and incubated in HS- or FCS-supplemented medium. The number of viable cells/flask was determined every 24 h for 6 successive days. A growth curve was made to permit calculation of cell doubling time.

Effect of Different Growth Factors on the in Vitro Morphology of Mesothelioma Cells. In order to investigate if certain growth factors...
SERUM-DEPENDENT GROWTH PATTERN OF MESOTHELIOMA CELLS

Fig. 3. Transmission electron microscopy showing a mesothelioma cell cultured in HS (a) demonstrating an epithelial appearance with tight junctions (arrow, inset) and numerous long and slender microvilli. Cells cultured in FCS-supplemented medium (b) displayed only a few microvilli. Scanning electron microscopy of mesothelioma cells cultured in HS (c) shows epithelial morphology with large amounts of long and slender microvilli while FCS-cultured cells (d) show a fibroblast-like appearance with few and short microvilli. X 7000 for both transmission and scanning electron microscopy. Bar, 10 μm.

Fig. 4. Production of hyaluronic acid by mesothelioma cells cultured in HS- and FCS-supplemented media. □, FCS; □, HS.

could affect the in vitro morphology of mesothelial cells, we incubated single cell suspensions of 200,000 cells/ml of both cell lines in serum-free RPMI 1640 with or without different concentrations of EGF (0.1 to 10 ng/ml), PDGF (0.1 to 20 ng/ml), or IGF I (1 to 400 ng/ml).

We also incubated both cell lines in the respective serum-supplemented medium and supplemented the cultures with different concentrations of growth factors (see above). The cells were passaged at the confluence and maintained in culture for 6 wk. The morphology was assayed 2 times a wk using an Olympus IMT 2 phase-contrast microscope.

Tumorigenicity in Nude Mice. On two different occasions 7 to 30 million cells were injected under the dorsal skin of 2-wk-old female BALB/c-nu/nu athymic nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN). The animals were kept according to standard procedures, observed for 180 days, and were then sacrificed and examined for the presence of tumor cells.

Chromosome Analysis. The cell lines were treated with Colcemid at a final concentration of 0.05 μg/ml. Hypotonic treatment was performed with 0.56% potassium chloride for 10 min, and the cells were then fixed in methanol:acetic acid (3:1) for 60 min. Following centrifugation and resuspension in fixative, one drop was allowed to dry on a glass slide, whereupon the material was stained by the Q-banding technique (15). Metaphases were photographed using a Zeiss UV microscope.

RESULTS

Growth Properties. Tumor cells obtained from the patients' pleural fluid were present as morula-like spheroids and as single cells. Between 1 and 4 wk, both cultures gradually developed two distinctly different growth patterns. HS-cultured cells grew attached to the bottom of the dish in an epithelial-like monolayer (Fig. 1a) with very few spindle-shaped cells. Occasionally, during the first 2 mo, morula-like spheroids were released into the medium. The cells cultured in FCS medium were also growing attached to the substrate. However, all these cells displayed a fibroblast-like morphology (Fig. 1b). The in vitro morphology of both cell lines remained unchanged during 24 mo in culture despite the fact that different batches of FCS and HS, respectively, were used. Both cell lines were Mycoplasma free as established by electron microscopy and DNA-4-6-diamine-2-phenyl-indole dihydrochloride fluorescence staining (Boehringer Mannheim, Mannheim, West Germany).
Effect of the Serum Change on the Mesothelioma Cell Lines. FCS-cultured cells were rapidly affected by a change to HS-supplemented medium. Already after 1 day of incubation in the HS medium = 10% of cells adopted the epithelial growth pattern. The proportion of cells with such morphology steadily increased and, at the end of the 6 wk culture period, they were a majority (70%) (Fig. 2a). When the HS-cultured cell line was given FCS supplement, a change of morphology occurred but at the lower rate. Thus, after 6 wk in FCS medium, 50% of the cells had changed their morphology to a fibroblast-like phenotype (Fig. 2b).

Electron Microscopy. Most cells cultured in HS-containing medium were rounded or polygonal with numerous long and slender microvilli. Their cytoplasm contained large amounts of microfilaments and, within the cell spheroids, there was a considerable number of desmosomes and tight junctions (Fig. 3a). The cells obtained from FCS-containing medium had numerous myelin configurations in their cytoplasm. Tight junctions and desmosomes were fewer than in HS-cultured cells. The intact cell surface exhibited only sparse and coarse microvilli (Fig. 3b). The difference in surface structures as regards cell shape and microvilli density was also demonstrated by scanning electron microscopy (Fig. 3, c and d).

Immunocytochemistry. Regardless of the differences in cell morphology a coexpression of keratin and vimentin was demonstrated in both cell lines. No binding of anti-Leu 4 antibodies could be detected using equivalent dilutions.

Hyaluronic Acid Production. HA was secreted from both cell lines to give chemically detectable amounts in the media already after 1 day. Higher levels of HA were observed in the HS-cultured cells (Fig. 4). The control cell line (small cell lung cancer) did not produce detectable amounts of this glycosaminoglycan when cultured in both HS- and FCS-supplemented media at the same cell concentration.

Colony Formation in Agarose and Growth Rates. Both cell lines formed colonies in agarose with a cloning efficiency of 2% in FCS- and 0.2% in HS-cultured cells. Both cell lines had a doubling time of approximately 96 h.

Culture of Mesothelioma Cells with Different Growth Factors. The attempt to culture the cells in serum-free medium with or without EGF, PDGF, and IGF I led to a continuous decrease in cell viability. Due to the poor growth these cultures were discontinued. The supplement of different concentrations of EGF, PDGF, or IGF I in the medium supplemented with FCS or HS did not influence the in vitro morphology of mesothelioma cells during an observation time of 6 wk.

Tumorigenicity in Athymic Nude Mice. In repeated experiments we failed to obtain detectable tumor formation during 180 days. Microscopic examination of the injection site could not demonstrate any remains of the tumor cell suspension.

Chromosome Analysis. The chromosome analysis showed very complex abnormalities as presented in Table 1. Six and five metaphases, respectively, were analyzed. Deletion of chromosomes 5 and 9 and a translocation between chromosome 13 and an unidentified chromosome were detected in all metaphases, indicating the common origin of the cell lines. The other abnormalities were not constantly present in the two cell lines. A marker (M 1) and a deletion of chromosome 11 were only seen in FCS-cultured cells while 5 of 6 metaphases in FCS-cultured cells, but only in 1 of 5 metaphases in AB-cultured cells.

DISCUSSION

Our results demonstrate that pleural mesothelioma specimens can be maintained in culture for several months in both HS- and FCS-supplemented media, producing lines of continuously replicating cells. Microscopically as well as chemically and immunohistiochemically, the described cell lines showed properties which are characteristic for malignant mesothelioma in vivo.

The difficulty in obtaining tumors in athymic nude mice is in good accordance with reports where human malignant cell lines (16) including mesothelioma (17) failed to produce tumors in these animals.

The reason why malignant mesothelioma shows such variations in morphology has as yet not been clarified. It has been proposed that the epithelial form originates from the mesothelial cavity-lining cells, while the sarcomatous forms would arise from the underlying stromal tissue. However, it may well be that both phenotypes could arise from the same precursor cell, the differences in phenotypic expression being caused by other factors. In the present mesothelioma cell lines, the type of serum supplement used (HS of FCS) was important for the altered phenotypic expression. FCS supplement resulted in a culture of fibroblast-like cells, while the presence of human serum induced the growth of an epithelial-like papillary mesothelioma. The influence of the medium used was further demonstrated by the change of morphology following the change of serum.

There seem to be two different mechanisms behind the alterations in morphology of mesothelioma cells. One of these, influencing the majority of cells, was slowly acting and changed the growth pattern after weeks of culture. This supports the concept of HS and FCS inducing the proliferative activity of

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different subpopulations of mesothelioma cells. This is further supported by karyotypic characterization. Thus, a marker chromosome (M 1) and a deletion of chromosome 11 were present only in the FCS-cultured cells (5 of 6 metaphases). Similarly, a deletion of chromosome 13 was seen only in HS-cultured cells (4 of 5 metaphases).

However, a selective growth advantage of different subpopulations cannot solely explain our data, since approximately 10% of the cells changed their morphology rapidly within 24 h. This indicates that the two sera used differ in their contents of factors which influence the morphology of individual cells. This theory is supported by in vitro experiments with benign mesothelial cells which in the presence of FCS, hydrocortisone, and EGF adopted a fibroblast-like morphology, while in the absence of EGF, the cells grew in an epithelial monolayer (18). Interestingly, in our study, addition of different growth factors including EGF to either FCS- or HS-cultured cells did not alter the morphology of mesothelioma cells. In conclusion, malignant mesothelial cells can be maintained in culture over a long period of time in either FCS- or HS-supplemented medium. Different sera contain factors that have the capacity to both alter the morphology of individual cells and also stimulate a selective growth of subpopulations of mesothelioma cells.

Our data indicate that the epithelial and sarcomatous forms of mesothelioma have the same cellular origin and that the differences in morphology are due to secondary processes such as clonal evolution and environmental influence of the phenotypic expression.

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Serum-dependent Growth Patterns of Two, Newly Established Human Mesothelioma Cell Lines


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