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Establishment and characterization of spontaneous mesothelioma cell lines derived from F344 rats

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Abstract Three mesothelioma cell lines (MeET-4, MeET-5, and MeET-6) established from ascitic fluid of F344 rats with spontaneous abdominal mesothelioma have been maintained through at least 60 passages on the DMEM with 10% FBS. Two of original tumours consisted of epithelioid cells growing in a papillary patern, while one (original tumour of MeET-5) had sarcomatous areas composed of spindle-shaped tumour cells. The cell line originating from MeET-5 showed a constantly beiphasic growth pattern during the repetitive subcloning, while the other two lines retained a monophasic growth pattern. Although the growth pattern was different, the tumour cells in all three lines were positive for vimentin and keratin and ultrastructurally showed an abundant distribution of glycogen granules in the cytoplasm and numerous long microvilli on all surface. The modal chromosome number of cell lines varied from 41 to 71, and abnormal chromosomes were frequently seen. All cell lines established formed colonies on semi-solid medium and could be successfully transplanted, growing tumour masses in syngeneic rats and thus indicating their malignant nature. Cell lines grew even on a medium with a low concentration of FBS. The evidence suggests that they may produce growth factors that enable them to survive unfavourable medium conditions.

Key words Mesothelioma · Cell line · F344 rat

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Introduction

Malignant mesothelioma is a neoplasm arising from the serosal membrane covering the visceral organs in the pleura and peritoneum. In humans, asbestos fibres are suspected, on the basis of epidemiological studies of being the primary cause of malignant mesothelioma. There is a long latent period following exposure [6]. The incidence of the disease has been rising in the world, in parallel with the worldwide development and increasing use of asbestos. In the laboratory, the carcinogenic potential of asbestos has been identified in mice and rats exposed to the chemicals through inhalation or by inoculation directly into the lung [7–9, 18, 41]. Many attempts have been made to characterize the nature of malignant mesothelioma in animal models. The implantation of human malignant mesotheliomas into mice was reported by Bielefeldt-Ohmann et al. [3] and by others [27, 30] using nude mice that had pieces of the tumour mass inoculated directly into the subcutis or abdominal cavity. Several in vitro cell lines of human malignant mesothelioma have been also established [2, 24, 29, 32, 39, 40]. In the course of the experiments using these in vivo and in vitro systems, it has been shown that there is a close relationship between growth of mesothelioma cells and several classes of cytokines [10, 13, 20, 25, 37, 38].

In an investigation on the nature of a tumour, an animal model derived from naturally occurring tumours will sometimes provide an excellent tool, better than those yielded by experimentally induced lesions; the naturally occurring lesions can be free of chemical stress, which can bring about additional effects on the tumour cells, resulting in monstrous changes in their physiological and morphological properties.

In Fischer 344 (F344/DuCrj) rats, a mesothelioma is a rather rare spontaneous tumour, with a male predominance (incidende 2–4% in males, 0–0.3% in females) [16, 22, 23] at an average age of 98.7 weeks [33]. They are usually found in the abdominal cavity, especially on the serosal surface of the tunica vaginalis. Morphologically, most spontaneous mesotheliomas in F344 rats are

similar to the epithelioid type of their human counterpart, and their growth pattern is more nearly uniform than that of asbestos-induced malignant mesotheliomas [14, 15, 36]. Recently several cell lines of spontaneous mesotheliomas in F344 rats have been reported [11, 31, 34], but few data are available on their biological features. The present report describes the estabslihment and chracterization of three cell lines derived from spontaneous abdominal mesotheliomas in F344 male rats.

Materials and methods

The mesothelioma samples were obtained from three male F344 rats (Charles River, Japan, Tokyo, Japan) that belonged to the treatment groups at different dose elevels in a 104-week chronic toxicity and oncogenicity study. The test substance, a novel acaricide, was administered by incorporating it into the basic diet for each dose level at five dietary levels, including 0 ppm for male and female animals for a period of 104 weeks. At the initiation of treatment, each dose group consisted of 50 males and 50 females. Animals found dead or killed in extremis during the treatment period were subjected to necropsy, as were survivors after 104 weeks of treatment. The incidence of malignant mesothelioma in males ranged from 8% (middle low dose group) to 2%. This range was similar in all groups, and the mean incidence was 4%. The first male seen to be bearing a malignant mesothelioma was 90 weeks of age (middle low dose group). Two of the three rats killed in extremis at 97 and 106 weeks of age, respectively, showed signs of anaemia, debility and abdominal distension. The other one survived until the terminal kill (111 weeks of age), though the same clinical signs were observed. Under ether anaesthesia, abdominal fluid (2–3 ml) was collected into a syringe in sterile conductions. After centrifugation (1000 rpm, 5 min), the cells in the pellet were seeded onto a 90-mm Premiere dish containing 6 ml of DMEM (Nissui Seiyaku, Tokyo, Japan) with 10% fetal bovine serum (FBS, Gibco, N.Y.). 2 mM L-glutamine, antibiotics (kanamycin, 5 µg/ml, ICN Biomedicals, UK), and amphotericin B (2.5 µg/ml; ICN Biomedicals). After 6 h incubation at 37 °C/5% CO_2, the medium containing nonadherent cells was removed and replaced by fresh. Confluent cells were detached with 0.25% trypsin (ICN Biomedicals), with a 0.02% EDTA added. At the 5th passage, subcloning was carried out by the limit dilution method using 96-well culture plates and then over 60 passages were repeated.

The original tumour specimens and cultured cells scraped from the culture dish were fixed in 2.5% phosphate-buffered glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy resin for electron microscopy. Aultrathin sections were stained with uranyl acetate and lead citrate and examined using an electron microscope 1200EXII (JEOL, Tokyo, Japan) operated at 80 kV.

To visualize intermediate filament, the cells cultured on Lab-Tek chamber slides (Nunc, Ill.) were fixed in methanol for 5 min and subsequently an avidin–biotin complex (ABC; Dako, Glostrup, Denmark) method was applied. Anti-bovine keratin rabbit antiserum (Dako, Calif.) and mouse anti-swine vimentin antibody (Dako) were used as primary antibodies. Likewise, methacarn-fixed, paraffin-embedded tumour specimens were used for the immunohistochemical staining of these two antigens.

When the culture cells attained approximately 50% confluency, they were harvested for chromosome number analysis. After exposure to 0.25 µg/ml colchicine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 2 h, the cells were suspended in a hypotonic solution containing 0.4% KCl for 20 min. Then the cells were fixed in 33% acetic acid in methanol, air-dried, and stained with Giemsa. To assess the distribution of chromosome number, at least 50 cells in metaphase were counted in each cell line.

To examine the anchorage-independent growth of the mesothelioma cell lines, 10^3 cells suspended in 1.5 ml of DMEM containing 10% FBS and 0.5% bacto-agar were seeded onto a 60-mm dish with a solid layer of 7 ml of 0.5% bacto-agar in the same medium. Dishes were incubaed for 2 weeks at 37 °C. The number of colonies containing more than 50 cells was counted under the microscope.

The animals used in the present studies were reared, treated, and killed in accordance with the provisions for animal welfare in The Institute of Environmental Toxicology, which are based on the Guidelines for Animal Experimentation issued by Japanese Association for Laboratory Animal Science [17].

To clarify the in vivo growth capacity of the three cell lines in syngeneic rats, each cell line was transplanted intraperitoneally (i.p.) to four males and four females and subcutaneously (s.c.) to three females (all adult F344 rats). Single-cell suspension of 10^6 cells in 2 ml of DMEM was inoculated into each animal. Animals were observed daily for tumour growth, and were sacrificed when the tumours reached about 4 cm in diameter following s.c. inoculation and when increased ascites and/or marked debility were recognized following i.p. inoculation.

To assess single-cell suspension of mesothelioma cell lines (10^4 cells/well) was seeded in triplicate onto 24-well plastic plates, incubated for 6 h in DMEM containing 10% FBS and then incubated overnight in serum-free DMEM. To examine the factors influencing the growth rate of the cell lines, serum-free medium was subsequently replaced by the following four test media: 1% FBS medium with or without epidermal growth factor (EGF, Boehringer Manheim, Ind.) or hydrocortisone (HC, Sigma, Mich.), 10% FBS medium. The cells in wells were harvested and counted at 24-h intervals for 7 days. The doubling time was calculated to evaluate the effects of FBS, EGF and HC on cell growth.

Statistical significance was evaluated by applying Mann-Whitney's *U*-test [12] to the data obtained to growth rate analysis.

Results

The three mesotheliomas occurred as multiple nodules of various sizes on the genital serosa and peritoneum, accompanied by bloody ascites. No metastatic foci were observed. Histopathological alterations other than mesothelioma in the abdominal cavity in these rats were those usually observed in aged male rats, including Leydig cell tumours in the testis, bile ductal proliferation and altered cell foci in the liver.

Histologically, the original tumours of cell lines MeET-4 and MeET-6 represented typical papillary mesotheliomas in which single or several layers of epithelioid cells covered the vaso-fibrous connective tissue (Fig. 1a). Tumour cells were cuboidal, and had abundant eosinophilic cytoplasm and round to oval nuclei. The original tumour of MeET-5 showed predominantly typical papillary growth of epithelioid cells, and in limited areas there were sarcomatous regions consisting of spindle-shaped cells under the epithelioid cell layers. Both types of cells were positive for vimentin and keratin. The reaction of spindle-shaped cells was partial and weaker than that of epithelioid cells (Fig. 2).

Ultrastructurally, intracytoplasmic glycogen granules, intermediate filament and desmosome-like structures between the cells were frequently observed. In the tumour cells on the surface of papillary nodules microvilli were observed on the cell surface, and basement membrane separated the tumor cells from the vaso-fibrous connective tissue (Fig. 3a). In the cells in the sarcomatous regions of the original tumour of cell line MeET-5, there were only scarce microvilli on the cell surface and no clear basement membrane was identified between the neighbouring parenchymal tissues.

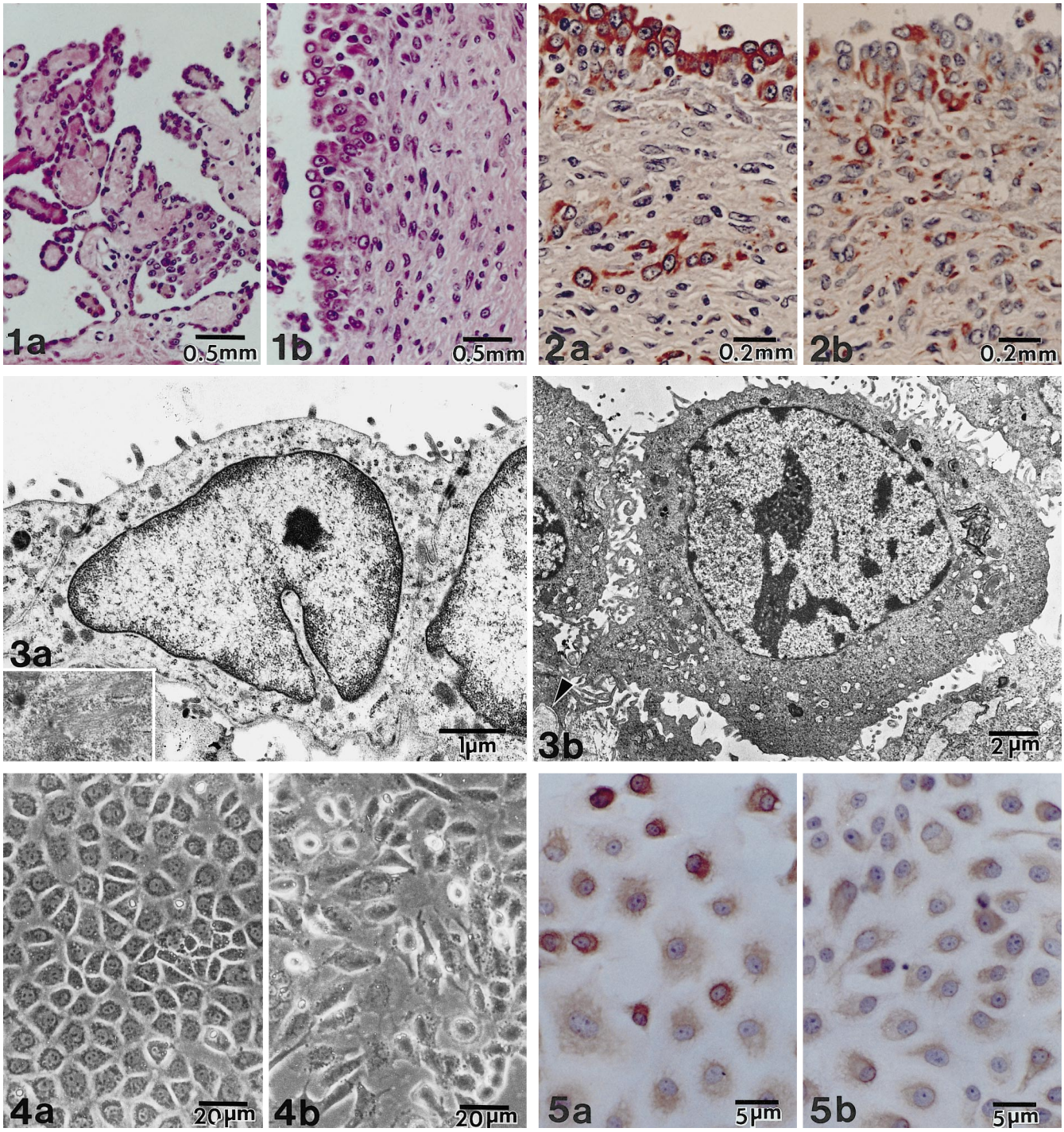

Fig. 1 a Typical papillary growth of the original tumour of MeET-4 and **b** solid area consisting of epithelioid and spindle-shaped cells of the original tumour of MeET-5. Haematoxylin and eosin stain, ×170

Fig. 2 a Anti-keratin and **b** anti-vimentin staining of the original tumour of MeET-5. Epithelioid cells are strongly positive for both antibodies, while the reaction of spindle-shaped cells is weakly positive. ×340

Fig. 3 a Transmission electron microscopy of papillary region of original tumour and **b** cultured cell of MeET-6 showing numerous microvilli, intracytoplasmic glycogen granules and basement membrane (*arrowhead*). Desmosome-like structures are seen between the tumour cells (**a**). Intermediate filament (**a** *inset*) and lysosomes (**b**) were also prominent. **a** ×10000, *inset* ×10000; **b** ×4500

Fig. 4a, b Mesothelioma cells in culture. **a** MeET-4 showing epithelioid phenotype and **b** MeET-5 consisting of epithelioid and spindle-shaped cells. Phase-contrast microscopy, ×400

Fig. 5 a Anti-keratin and **b** anti-vimentin staining for MeET-4. All cells express positive reactions to both antibodies. ×1400

Table 1 Characteristics of three mesothelioma cell lines (*ip* intraperitoneal inoculation, *sc* subcutaneous inoculation)

Cell lines	Morphology	Immunocytochemistry for intermediate filament		Modal chromosome number	Frequency of large submeta-centric chromosome	Tumorigenicity in syngeneic rat (weeks)	Colonies /dish[a]
		Keratin	Vimentin				
MeET-4	Epithelioid	+	+	41 (38–43)	50/50	10–12 (sc)[b] 3–5 (ip)[c]	162
MeET-5	Epithelioid and spindle-shaped	+	+	71 (65–74)	43/52	15–17 (ip)	44
MeET-6	Epithelioid	+	+	63 (59–69)	33/51	19–22 (ip)	104

[a] The number of colonies containing more than 50 cells was determined at 2 weeks after seeding of triplicate 10^3 cells in soft agar onto solid bottom of a 60-mm dish

[b] Subcutaneous mass reaches over 40 mm in diameter

[c] Animal shows marked debility

Mesothelioma cell lines MeET-4 and MeET-6 were epithelioid in appearance, forming monolayer sheets reminiscent of cobblestones at the confluent stage (Fig. 4a), while the cell line MeET-5 showed a biphasic character with both epithelioid and spindle-shaped cells (Fig. 4b). Ultrastructurally, the cultured cells had numerous microvilli on the cell surface, intraplasmic glycogen granules, basement membrane and intermediate filaments, all visible with the electron microscope, with good retention of many of the morphological properties of the original tumours (Fig. 3b). These were also seen in the spindle-shaped cells of MeET-5. Immunocytochemically all mesothelioma cell lines showed positive reactions, mainly around the nucleus, for both keratin and vimentin (Fig. 5).

The chromosome numbers in these cell lines varied considerably. MeET-5, especially, showed marked variation in the number of chromosomes ranging from 65 to 71. The modal chromosome numbers of MeET-5, MeET-5, and MeET-6 were 41, 71, and 63, respectively (Table 1). The major chromosomal aberration detectable in solid stained specimens was a large submetacentric chromosome suggesting translocation (Fig. 6). The abnormality was observed in 50/50, 43/52 and 33/51 cells in metaphase of MeET-4, MeET-5 and MeET-6, respectively.

The average number of colonies per dish formed in semi-solid medium were 162 and 104 for MeET-4 and MeET-6, respectively, while MeET-5 formed only 44 colonies during 2 weeks after seeding of 10^3 cells (Table 2).

Although all the transplanted mesothelioma cell lines produced tumours in F344 rats, the kinetics of tumour development varied substantially (Table 1). When inoculated into the peritoneal cavity, all the transplanted cell lines developed multiple nodules or a large single mass on the visceral surface, associated with retention of bloody ascites. After subcutaneous (s.c.) inoculation of the MeET-4 cell line, 3 recipients developed masses about 4 cm in diameter at the inoculation site after 10–12 weeks. One of them developed metastatic foci in the pleural organs and thorax. Neither MeET-5 nor MeET-6 was successfully transplanted by s.c. inoculation.

In the FBS-free medium, all cell lines remained alive for as long as 7 days. There were no significant differ-

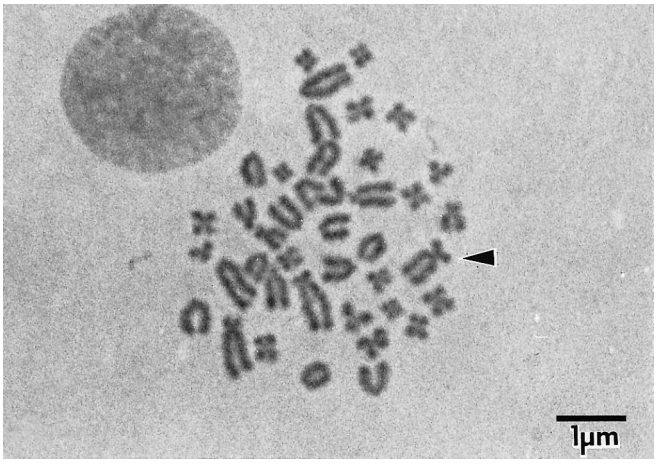

Fig. 6 Metaphase smear of a MeET-4 cell showing a large submetacentric chromosome (*arrowhead*). ×10000

Table 2 Growth rate of three mesothelioma cell lines (The influence of serum concentration and the effect of supplement of EGF or HC to growth of the cell lines were evaluated on treatment 1 versus 4, and treatment 1 versus 2 or 3, respectively [( ) number of trials, *FBS* fetal bovine serum, *EGF* epidermal growth factor (10 ng/ml), *HC* hydrocortisone (0.4 μg/ml)])

Group	Doubling time (h, mean±SD)		
	MeET-4	MeET-5	MeET-6
1 1% FBS	18.1±3.5 (8)	18.4±2.7 (7)	25.2±6.8 (9)
2 1% FBS+EGF	18.4±1.0 (4)	18.1±4.6 (4)	20.5±3.5 (4)
3 1% FBS+HC	20.0±7.5 (4)	18.3±4.1 (4)	25.9±5.8 (6)
4 10% FBS	15.3±2.2 (9)	15.1±2.7 (8)	23.0±4.8 (9)

ences in any of the cell lines between the two FBS concentrations. The average doubling times of MeET-4, MeET-5, and MeET-6 cultured in DMEM with 10% FBS were 15.3, 15.1, and 23.0 h, respectively (Table 2). The supplement of 10% FBS, and EGF or HC to 1% FBS medium did not produce significant changes to the doubling time of any of the three cell lines.

Discussion

We have established three mesothelioma cell lines obtained from ascitic fluid of spontaneous abdominal mesotheliomas that developed in F344 male rats. These cells proliferated in DMEM with 10% FBS and required no growth factor supplementation. They have been cultivated for more than 18 months through at least 60 passages. The morphological and histochemical characteristics of the original tumour cells are maintained in the cell lines even after 18 months of isolation. The cellular morphology and intermediate filament expression were similar to those previously documented in the cell lines derived from human malignant mesothelioma [24, 29, 32, 39] and asbestos-induced mesothelioma in rats [7, 18]. The malignant nature of the present three cell lines was shown by their high growth capacity in soft agar and their high tumorigenicity in F344 rats following i.p. and/or s.c. inoculation.

In human malignant mesotheliomas, three histological subtypes have been reported: (1) epithelioid, (2) sarcomatous or fibrous, and (3) biphasic or mixed [1, 36]. In F344 rats, it has been mentioned that most spontaneous abdominal mesotheliomas resemble the epithelioid type of the human counterpart [14, 36] and show a more nearly uniform histological pattern than asbestos-induced mesotheliomas in the same species [7, 8]. In the present study, all three mesotheliomas showed papillary growth of epithelioid cells, but in the original tumour of the MeET-5 line there were some sarcomatous areas composed of spindle-shaped or stellate cells. Both types of tumour cells were positive to immunohistochemical staining with keratin and vimentin, as described in previous reports [15, 26]. The different phenotypes, i.e. epithelioid, spindle-shaped or stellate, have also been reported in the cell lines isolated from human malignant mesotheliomas [24, 38] and from asbestos-induced mesotheliomas in rats [7, 18], and the phenotypic variation is said to be a unique feature of mesothelioma. Two different types of cells have been retained in MeET-5 during a long series of subcloning repeats, and it is probable that the two phenotypes are interchangeable with each other. The factors that alter phenotypic expression have not been described in rodent mesothelioma cell lines, whether spontaneous or chemically induced. However, some cell lines from human malignant mesotheliomas are known to change their phenotypes according as which species' serum is used in the medium [19]. In human benign mesothelial cells, it has been reported that different phenotypes sometimes develop in dependence on their growth state [5].

Although chromosomal abnormalities have frequently been reported in human malignant mesotheliomas, substantial similarity in the types of changes has not been elucidated. Both in spontaneous and asbestos-induced mesotheliomas in rat and in spontaneously immortalized mesothelioma cell lines, trisomy of chromosome 1 has been reported to be the major chromosomal aberration [21, 28, 29]. It has been suggested that involvement of the gene(s) located on chromosome 1 may have an important role in the transformation of rat mesothelioma cells [11]. In the present study, a large submetacentric chromosome was very frequently observed in all cell lines, but trisomy 1 was not detected. Further studies will be needed to characterize the translocation observed in these cell lines.

Our mesothelioma cell lines grew in a semi-solid medium. This property is preserved by many cell lines with high tumorigenic potential and established from malignant tumours, including malignant mesotheliomas [4, 7, 18]. Mesothelial cells obtained from normal Sprague-Dawley rats show no anchorage-independent growth [18]. A variety of cytokines, such as platelet-derived growth factor, transforming growth factor-beta (TGF-β), insulin-like growth factor and interleukin-6, are known to be produced in malignant mesotheliomas [10, 13, 25, 31, 38]. A high level of TGF-β expression in some human and murine malignant mesothelioma cell lines was found to be involved in regulation of the proliferation and anchorage-independent growth of these cells [10]. However, reduced TGF-β expression by anti-sense mRNA resulted in specific inhibition not only of anchorage-independent growth in soft agar, but also of tumorigenicity in vivo in a syngeneic mouse challenge model [10]. In the present study, the result of in vitro colony assay correlated with those of tumorigenicity in vivo, since MeET-4, which made the largest number of colonies, was the most highly tumorigenic in the recipients. These results suggest a possible involvement of TGF-β in the growth of our cell lines; this is most likely in the case of MeET-4.

FBS supplement (1%) enabled all three of these cell lines to grow in vitro. The fact that these cell lines can grow even at low serum concentrations might suggests that production of growth factors by these tumour cells is quantitatively and/or qualitatively different from that of normal mesothelial cells. Such independent proliferation by way of the formation of an autocrine loop is a common phenomenon seen in many tumour cells, including human malignant mesothelioma cells [13, 20, 38] and rat asebestos-induced mesothelioma cells [37]. EGF and HC induce rapid growth of human mesothelial cells [5], whereas EGF is not mitogenic for normal mesothelial cells of rats and may inhibit their growth in vitro [31]. EGF and HC have commonly been used to establish mesothelioma cell lines [40]. However, once a cell line is established, the cells become insensitive to these factors

[39]. Similarly, EGF and HC did not shorten the doubling times of our cell lines.

Our established mesothelioma cell lines have some morphological resemblance to human counterparts. They appear to be more adaptable to in vitro conditions than their human counterparts. They also have a high tumorigenic potential in syngeneic F344 rats. Further studies on the biological and biochemical features of these cell lines will exploit the availability of this experimental system as an appropriate model for human malignant mesotheliomas.

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