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Original Paper

Newly Established MST-1 Tumour Cell Line and Tumour-infiltrating Lymphocyte Culture from a Patient with Soft Tissue Melanoma (Clear Cell Sarcoma) and Their Potential Applications to Patient Immunotherapy

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The establishment and characterisation of paired autologous tumour cell line (MST-1) and tumour-infiltrating lymphocyte (TIL) culture from a tumour mass of a 14-year-old Taiwanese girl with soft tissue melanoma are described. MST-1 cells grown *in vitro* were heterogeneous in morphology, ranging from floating round cells, loosely attached round/oval or elongated cells with prominent pseudopod-like processes, to well-attached spindle and elongated dendritic cells without obvious pseudopods. Immunostaining revealed that major melanoma-associated antigens, such as S100 protein, HMB-45, melanotransferrin, chondroitin sulphate proteoglycan, and the gangliosides GD2 and GD3, were consistently expressed by the tumour tissue, severe combined immunodeficiency (SCID) mouse xenograft and derived cell lines. Flow cytometric analysis of the tumour DNA content showed an index of 1.8 relative to normal peripheral blood lymphocyte DNA. Chromosome analysis revealed all cells at a hypotetraploid level with several clonal chromosome aberrations, including deletions at 10p and 12q, an addition at 12q, translocations t(1;14) and t(5;6). Electron microscopy showed melanosome structures. This observation and the expression of the major melanoma-associated antigens were all indicative of the melanocytic origin of MST-1 tumour. Interleukin-2 (IL-2) expanded TILs had the predominant $CD8^+$ phenotype and the capacity to lyse cells of the cultured autologous tumour. The availability of the soft tissue melanoma cell line, the SCID mouse xenograft tumour system as well as autologous TILs described herein would provide useful materials for identifying T-cell-defined antigens as well as a model system for devising individualised cancer biotherapeutic strategies. This cell line can also be used for further studies aimed at uncovering the histogenesis of this rare cancer.

Key words: soft tissue melanoma, clear cell sarcoma, cell culture, ultrastructure, tumour-associated antigens, tumour-infiltrating lymphocytes, SCID mouse xenograft, immunotherapy
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INTRODUCTION

PRIMARY MELANOMA of soft tissues was originally described by Enzinger as "clear cell sarcoma of tendons and aponeuroses" in 1965 [1], as its histogenesis was not certain at that time. Melanin granules were later noted only in a minority of cases [2–4], and the tumour has also been referred to as malignant melanoma of soft parts [5, 6]. This neoplasm characteristically displays compartmentalised growth in the deep soft tissues, and shows round and polygonal cells with prominant nucleoli and optically translucent cytoplasm [2, 3, 5, 7–10]. The antigenic profile of soft tissue melanoma, as determined by immunohistology, strongly suggests its close relationship to cutaneous malignant melanoma with respect to their common

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melanocytic lineage. For example, the antigens, S100 protein and HMB-45, have been found to be associated with this tumour [5, 9, 11], aside from very occasional melanin pigmentation. Soft tissue melanoma has been shown to have a translocation of chromosomes 12 and 22, t(12;22) which is absent in cutaneous melanoma or other types of sarcoma [12, 13]. The nucleotide sequence involved in this translocation has been cloned and its deduced amino acid sequence represents a chimeric protein [14]. Furthermore, prognostic factors associated with clear cell sarcoma of soft tissues has not received much attention until recently [10].

Whether this tumour behaves clinically more like a cutaneous melanoma or a soft tissue sarcoma has often been debated [5, 6,11], but from a therapeutic point of view, this tumour has been managed as a sarcoma [5, 8, 10]. Currently, radical surgical excision is the treatment of choice for soft tissue melanoma. However, the tumour often recurs, sometimes after a few years, and approximately 50% of patients eventually die of recurrent tumours [5]. Regional lymph nodes and the lung are preferred sites of tumour metastases. Chemotherapy and radiotherapy are considered to be only useful for palliation [8]. In recent years, systemic administration of cytokines, adoptive immunotherapy with lymphokine-activated killer (LAK) cells/tumour-infiltrating lymphocytes (TILs), specific immunisation with autologous tumour cell vaccines and several other immunotherapeutic approaches have met with some success in the treatment of cancer, notably melanoma and renal cell carcinoma [15–17]. Accordingly, control of recurrence and micrometastasis by immunological interventions may provide alternative therapeutic modalities for soft tissue melanoma. In order to gain a better understanding of the histogenesis, genetics, prognostic factors and susceptibility of this neoplasm to a variety of biological response modifiers, it is important to study, at both the cellular and molecular levels, short-term cultures and continuous cell lines derived from this neoplasm.

Here we describe the establishment and characterisation of a continuous cell tumour line from a patient with soft tissue melanoma. The cell line was analysed with respect to *in vitro* growth pattern, cytogenetics, tumorigenicity in SCID mice, ultrastructures, expression of various tumour-associated antigens and flow cytometric DNA profiles. The tumour specimen contained TILs [17], which proliferated in cultures containing human recombinant interleukin-2 (IL-2). The expanded lymphocytes were activated T cells which had the capacity to lyse autologous tumour cells grown *in vitro*.

MATERIALS AND METHODS

Tumour biopsy, processing and cell culture

A tumour biopsy was obtained in February 1993 from a 14-year-old Taiwanese female patient presenting with a soft tissue mass over her right-lateral knee at Chang Gung Memorial Hospital in Lin-Kou, Taoyuan, Taiwan, ROC. The tumour sections were strongly positive with a rabbit antiserum to S100 protein and a monoclonal antibody (MAb) HMB-45. In March 1993, a wide excision of the primary tumour lesion, measuring $3 \times 3 \times 4$ cm, followed by skin graft was performed. In the operation room, a portion of the tumour tissue was sent for routine pathology, and the remaining tumour mass was sent to our laboratory for further study. The skin over the tumour showed red–purple discolouration with no evidence of ulceration. The pathological diagnosis of the tumour was clear cell sarcoma of soft part (melanoma of soft tissue).

The specimen was processed for tumour cell culture, TIL culture and cryopreservation of both cell types. To control for microbial contamination, the tumour was pretreated for 30 min at room temperature with antibiotic-rich medium, i.e. RPMI-1640 (GIBCO, Grand Island, New York, U.S.A.) containing 200 U/ml penicillin, 200 μg/ml streptomycin, 200 μg/ml mycostatin and 10 μg/ml fungizone. Small portions of the tumour were embedded in OCT (polyvinyl alcohol, benzalkonium chloride, polyethylene glycol, distilled H_2O; Miles Labs, Elkart, Indiana, U.S.A.), and snap-frozen at −80°C for immunohistology. The growth medium was composed of RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). The tumour was finely minced with scalpels and a pair of curved scissors. The mince and dispersed cells were suspended in growth medium. Mechanically released cells were collected from the supernatant in the test tube after tumour fragments settled out. After one cycle of washing in the medium, mechanically dispersed cells were seeded in T25 plastic flasks (approximately 1.5×10^5 larger nucleated cells/5 ml medium/flask). Portions of the cell suspension and mince preparations were used for TIL cultures (see below). The remaining cell suspensions, mince tissues and, intermittently, cells from short-term and long-term cultures were prepared in aliquots in freezing medium containing 20% FBS and 10% dimethyl sulphoxide and stored frozen in liquid nitrogen. Cell counts were carried out microscopically using a haemocytometer. Cell viability was determined by the capacity to exclude trypan blue.

Human cutaneous melanoma cell lines, CaCL 85-1, M-21 and MeWo [18] were obtained from S.-K. Liao (first author), Dr R.O. Dilman (Hoag Cancer Center, Newport Beach, California, U.S.A.) and Dr H. Pross (Queen's University, Kingston, Ontario, Canada), respectively. All these cells were maintained as monolayer cultures in the medium and under the same conditions as for soft tissue melanoma cells described above. Two human haemopoietic cell lines, K562 and Daudi, maintained in RPMI-1640 medium plus 10% FBS were used as targets in ^{51}Cr-release assays. The cell lines were free of mycoplasma as determined by monthly monitoring by a hybridisation method (Gene-Probe, San Diego, California, U.S.A.) and a Hoechst DNA staining technique [19].

Population doubling time and saturation density

Growth curves were constructed according to the method previously described [20]. Population doubling time and saturation density were calculated, respectively, from the exponential and saturation phases of the growth curve.

Tumorigenicity in SCID mice

Female mice with severe combined immunodeficiency (SCID) (CB.17) (6–8 weeks of age) were purchased from the Central Animal Facility of the National Taiwan University Hospital, Taipei. The mice were housed in microisolator cages under specific pathogen-free conditions, and were fed autoclaved pellets and water. Two animals were injected subcutaneously with 5×10^6 tumour cells/animal, and an additional four animals were similarly injected with 1×10^7 tumour cells/animal at one site just above the right hind leg. Animals were examined every 2 days for any tumour growth at the injection site. The volume of palpable tumour nodules

was estimated according to the formula of Attia and Weiss [21]: volume (mm^3) = 0.4 $a^2 \times b$, where a is the major tumour diameter and b is minor diameter perpendicular to the major one.

Electron microscopy

Pieces of fresh tissues from SCID mouse xenografts or cell pellets were placed in 4% glutaraldehyde (in 0.1 N sodium cacodylate buffer, pH 7.2), washed in this buffer, fixed in 1% osmium tetroxide, and embedded in epoxy resin. Sections (1 μm) were stained with toluidine blue. For monolayer cultures, the cells were washed once in PBS, fixed with 4% glutaraldehyde for 2 h and removed from culture flasks by scraping with a rubber policeman before cell pellets were made. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a Jeol-1200C electron microscope.

Immunohistochemical procedures

Frozen tissues were cut at 5 mm and placed on gelatin-coated slides, air-dried and fixed in cold (4–5°C) acetone for 10 min. Staining was carried out at room temperature using the avidin–biotin–peroxidase complex (ABC) method provided in the Vectastain ABC kit (Vector Laboratories, Burlingame, California, U.S.A.), according to the manufacturer's directions. Murine monoclonal antibodies at a concentration of 5 μg/ml, or at the dilutions recommended by the manufacturer, were incubated with the tissue sections for 1 h. Endogenous peroxidase activity in the tissues was inactivated following the biotinylated horse anti-mouse IgG step by placing the slide into methanol and 0.6% hydrogen peroxide solution for 15 min. The chromogenic substrate, 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, U.S.A.), containing 0.002% hydrogen peroxide was used to detect the peroxidase conjugate. Gill's haematoxylin #3 (Fisher Scientific, Norcross, California, U.S.A.) was used as a counterstain.

Positive (anti-HLA-ABC, MAb W6/32; purchased from DAKO Japan Co. Ltd, Kyoto, Japan) and negative (normal mouse IgG and PBS) controls were included in each test [22]. Monoclonal antibodies (MAbs) to epithelial membrane antigen, EMA (E29), HLA-DR (DK22), ICAM-1 (CD54, 65B5), HMB-45, desmin and vimentin were purchased from DAKO Japan Co. Ltd; MAb R24 (also known as Mel-1) to GD3 ganglioside [23] was obtained from Signet Laboratories, Inc., Delham, Massachusetts; MAb (14Ga) to GD2 ganglioside [24] was a gift from Dr A.L. Yu, University of California, San Diego, California, U.S.A. Another anti-GD3 MAb known as Mel 22 (IgG3) was raised in this laboratory and had a similar specificity as R24 (Liao, unpublished observations). MAb (225.28S) to high-molecular-weight melanoma-associated antigen (HMW-MAA; chondroitin sulphate proteoglycan, 250 kD + > 440 kD) [25] was kindly supplied by Dr S. Ferrone, New York Medical College, Vahalla, New York, U.S.A. MAb (140.240) to p97 melanotransferrin (140.240) [26, 27], MAb (140.72) to carcinoembryonic antigen (CEA)-crossreacting 95–150-kD glycoprotein(s) [28], and MAb (MAC-CO1) to 40-kD folate-binding protein were generated against human colonic cancer in our laboratory [29].

For testing the reactivity for S100 protein, rabbit anti-S100 antiserum (a gift from Dr A.J. Cochran, UCLA School of Medicine, Los Angeles, California, U.S.A.) [30] was used. For positive control, we used the rabbit polyclonal anti-human normal tissue antiserum, which was raised in this laboratory. Preimmune normal rabbit serum and PBS were used as negative controls. The immunostaining procedure was essentially the same as that described above for murine MAbs, except that the second antibody used was biotinylated goat anti-rabbit IgG provided in the anti-rabbit IgG vectastain ABC kit.

Chamber slide culture and immunocytology

Cells (5×10^4/chamber) were grown on Lab-Tek chamber slides (Nunc, Naperville, Illinois, U.S.A.) overnight (15–18 h). Cultured cells were washed once in PBS, air dried and fixed in cold acetone (4–5°C) for 5 min. Bound antibodies were detected by the ABC system as above for tissue sections.

Chromosome preparation

Chromosomes were prepared from cultured cells by a standard technique, and G-banding were performed by trypsin treatment and Giemsa stain according to Seabright [31].

Measurement of DNA content by flow cytometry

DNA content of nuclei from cultured cells or xenografts was analysed by flow cytometry. In brief, cells were divided 72 h prior to harvesting and seeded into T25 flasks at a concentration of 2×10^5 cells/ml in 5 ml of RPMI-1640 medium plus 10% FBS. Cells were trypsinised and the resulting suspension transferred to a 10-ml centrifuge tube, centrifuged, resuspended in 10 ml PBS and pelleted once before being resuspended in 1.5 ml of PBS. Propidium iodide (0.25 mg/ml), ribonuclease (5 mg/ml) and triton X-100 (1%) were added 30 min prior to analysis. Cells were analysed using a FACScan machine (Becton-Dickinson) and the analysis program CellFIT. The DNA index was calculated relative to normal adult peripheral blood lymphocytes.

Culture and expansion of TILs with IL-2

Lymphocytes isolated from single cell suspensions were made from the tumour biopsy specimen. Lymphocytes at the concentration of 5×10^5–7×10^5 in T25 flasks were grown in AIM-V medium (GIBCO) containing human AB serum and 2000 U/ml IL-2 (Chiron, Inc., Emeryville, California, U.S.A.). After 6 days, they were fed with fresh AIM-V medium plus supplements as above. The cultures were expanded using new T75 flasks and fed on a weekly basis for 2–3 weeks. After an additional 2 weeks, purified OKT-3 MAb (10 μg/ml in growth medium) was immobilised on T75 flasks (by coating 0.5 mg/ml of OKT-3 MAb at 37°C overnight, followed by washing with RPMI-1640 medium + 10% FBS) to expand TIL cultures further. Intermittently, TIL cultures were primed with MST-1 cells and expanded further. A 4-h ^{51}Cr release assay was used to test for TIL cytotoxicity against cultured tumour targets including the MST-1 cell line and its SCID mouse passaged cell line, "MST-1-XS".

Immunofluorescence/flow cytometry

Lymphoid cells harvested from freshly prepared tumour cells (see above) followed by removal of red cells and tumour cells by Ficoll-Paque (Pharmacia) gradient sedimentation, and from IL-2 (+ OKT-3 MAb) expanded TIL cultures were dispensed into test tubes (5×10^5 cells/200 μl PBS + 2% FBS/tube) and were centrifuged at 500g for 10 min to pellet the cells. MAbs to various lymphocyte markers (as listed in Table 3) were purchased from DAKO Japan Co. Ltd, Kyoto. Test MAbs (5 μg/ml) were added (100 μl/tube) and incubated

for 30 min at 4°C. After a brief washing, cells were incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG antiserum (Coulter Immunology, Hialeah, Florida, U.S.A.). For two-colour analysis, FITC-labelled CD25, FITC-labelled HLA-DR and R-phycoerythrin (RPE)-labelled CD3 MAbs (DAKO) were incubated with target TILs directly without a second antiserum in the immunofluorescent staining procedure. The phenotypes of these lymphoid cells were analysed with a FACSvantage flow cytometer (Becton-Dickinson).

Chromium-release cytotoxicity assay

A 4-h chromium-51 (^{51}Cr) release cytotoxicity assay was performed by using MST-1, MST-1-XS, K562 and Daudi cell lines as targets. Single cell suspensions were prepared from monolayers of solid tumour cells by brief incubation with trypsin at 37°C, and washed twice in medium, then 200 μCi $Na_2O_2{}^{51}Cr$ was added to each target cell preparation. Incubation was performed for 1 h on a rocker platform at 37°C. The cells were washed three times in medium. ^{51}Cr-labelled target cells (2×10^3) were incubated with varying numbers of TILs in a final volume of 150 μl of medium plus 5% FBS. The mixtures of cells were incubated in V-bottom microtitre plates at 37°C. After 4 h, 100 μl of culture supernatant was removed and counted for ^{51}Cr radioactivity using a Gamma Counter LKB (Gaithersburg, Maryland, U.S.A.). Maximal ^{51}Cr release was determined in the presence of 2% Triton X-100. The per cent specific ^{51}Cr release was calculated from the following formula:

$$\frac{\text{(experimental cpm)} - \text{(spontaneous cpm released)}}{\text{(maximal cpm released)} - \text{(spontaneous cpm released)}} \times 100.$$

All determinations were made in triplicate. In all experiments, the spontaneous ^{51}Cr release did not exceed 25%.

RESULTS

Pathology of the primary tumour tissue

The tumour was well circumscribed and solid. Microscopically, the tumour tissue exhibited an organoid growth pattern, composed of irregularly shaped nests and cords of mainly oval/round or polygonal cells, separated by dense fibrous septa (Figure 1a). The tumour cells showed mild pleomorphism, irregular nuclear borders and clear cytoplasms. Mitotic figures were occasionally seen.

Establishment of a cell line

During the first 3 weeks of the primary culture, proliferating tumour cells formed small colonies, some of which were surrounded by a fair number of fibroblasts. About 60% of tumour cells appeared to be round and loosely attached to the flask, while the remaining cells appeared to be irregular, elongated and spindle-shaped with better attachment. A stepwise elimination of fibroblasts was carried out by leaving them behind in the flasks. This was accomplished by differential detachment of tumour cells through gentle shaking of the flask. Since some tumour cells still remained on the floor of flasks, a brief rinse of cells with EDTA followed by a short treatment with 0.02% trypsin–EDTA was conducted as previously described [20]. The tumour cells collected from two T25 flasks were pooled, washed in growth medium and transferred to four new T25 flasks containing fresh growth medium. Enrichment of tumour cells was achieved after three consecutive cell passages at a weekly interval using the same procedure above. At the end of the process, fibroblastic cells were rarely seen in the culture. Subsequently, the tumour cells proliferated slowly but steadily, and have currently been passaged more than 68 times (at time of writing). This cell line was designated MST-1.

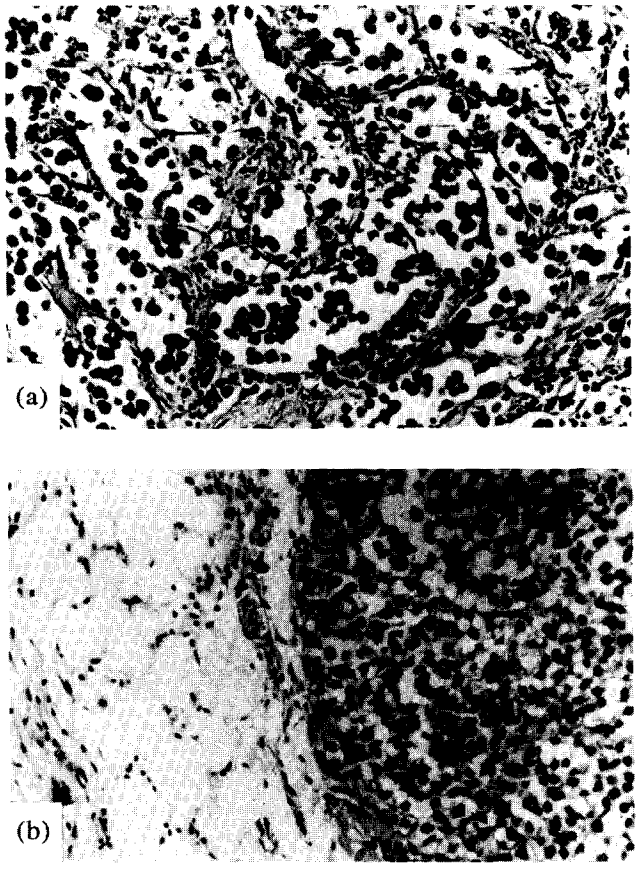

Figure 1. Photomicrographs of H & E stained tissue sections of a melanoma of soft tissue (a) from which the MST-1 was derived, and of a xenograft of MST-1 cells grown in a SCID mouse (b). × 400.

Growth pattern in vitro

Under our routine culture conditions, the growth pattern of MST-1 cells was quite stable. In the first day after subculturing, most tumour cells were round/oval and loosely attached to the flask, with a few cells showing pseudopods on the cell surface. On days 2–3, increasing number of cells attached to the plastic bottom, and morphologically became more spindle-shaped, or irregular and elongated. Some elongated cells had between six and nine pseudopod-like processes on the cell surface clearly visualised by phase microscopy (Figure 2a). In some areas of the culture, tumour cells of various shapes and sizes displayed numerous pseudopods up to 40 (Figure 3). On days 4–6 after plating, the tumour cells having pseudopod-like processes tended to attach more firmly to the flask and transformed into elongated cell shapes and spindled shapes. Subconfluency was reached at this point; a large proportion of these cells were either grown as compact colonies (Figure 2b) or presented as a scattered and less compact pattern of growth. Interestingly, pseudopod-like cytoplasmic processes became less obvious when cells grew in a greater density. It was noted that a few cultured tumour cells were not morphologically

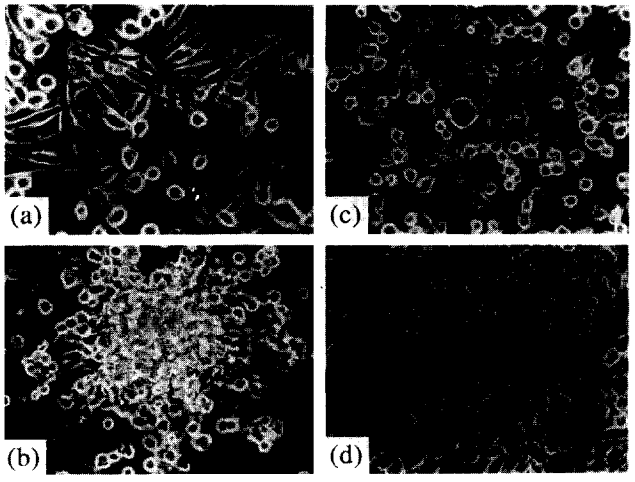

Figure 2. Photomicrographs of live cultures of MST-1 (a, b, d) and MST-1-XS (c) cells taken at the 18th and 19th passages *in vitro*, showing typical growth patterns. In (a) a few typical cutaneous melanoma-like cells can be seen along with floating cells and attached oval/round cells with pseudopods. A cell colony and a densely growing area are noted in (b) and (d), respectively. In the upper-right quarter of (c), note at least four epithelial cells among MST-1-XS cells. × 1000, phase.

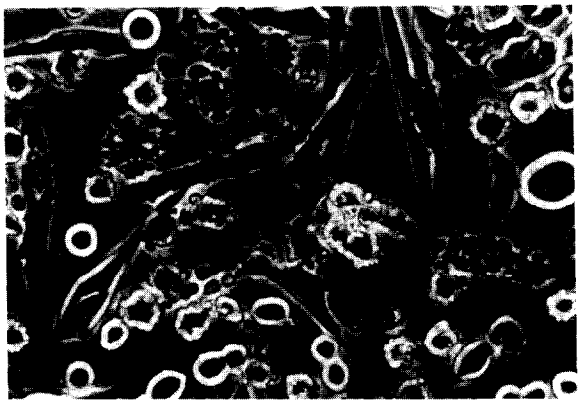

Figure 3. Photomicrograph of an area of the MST-1 live monolayer culture, showing a group of round/oval, spindle shaped and elongated cells with numerous pseudopod-like processes. [× 3200, phase].

unlike typical cutaneous melanoma cells [20, 32], exhibiting triangular dendritic, elongated dendritic or spindle cell shapes (Figures 2a and 3). By light microscopy, no obvious pigmentation was observed in these cultures.

In vitro *growth curve*

Two growth curves were established from cultures of the MST-1 line at the 15th and 20th passages with similar results obtained (not shown). This cell line grew rather slowly *in vitro* with an average population-doubling time of 69 h, and took 9 days to reach plateau levels with an average saturation density of $1.71 \times 10^5/cm^2$. The morphological characteristics and growth pattern described above persisted up to the 68th passage.

Cytogenetic studies

On 12 karyotypes analysed, the modal chromosome number for the MST-1 cell line was 77 (hypotetrapoid). A typical karyotype including two normal X-chromosomes is shown in Figure 4. Among the most consistent abnormalities were (a) deletions in chromosomes 5 ($5p^-$ in one of four chromosome 5); (b) deletions of chromosomes 10 ($10p^-$ in two of three chromsomes no. 10); (c) deletion and addition in chromosome 12 (one $12q^-$ and one $12q^+$ out of three chromosomes 12; $12q^-$: deletion of the bright band of q arm with a new (?) pale staining band present slightly away from the centromere; $12q^+$: chromosome 12 with a terminal pale staining band addition); (d) trisomies in chromosomes 1–3, 5, 13, 15, 18 and 19, and polysomies (>3) in chromosome 7, 8, 20 and 22. Additional stable chromosome markers, the origin of which could not be identified with certainty, were: translocations t(1;14) (large metacentric; chromosome 14 possibly translocated to the centromere of the q arm of chromosome 1), t(5;6) (p arm of chromosome 6 possibly translocated to the centromere of the q arm of chromosome 5), t(9;?) (chromosome 9 with terminal pale staining band), and t(21;?) (chromosome 21 with a bright chromatin addition) (Figure 4, lower frame). Deletion of one to two apparently normal chromosomes 9 was often noted. Translocation t(12;22) typified and reported for clear cell sarcoma [12, 13] was not detected in MST-1 cells.

Tumorigenicity in SCID mice

Six female adult SCID mice were tested for xenotransplantability of the MST-1 cell line. Two were injected subcutaneously with 5×10^6 cells/animal and four mice were similarly injected with 1×10^7 cells/animal in the dorsal flank above the hind leg. All animals developed solid tumours at the sites of injection with a similar latent period of approximately 75 days in both groups. The average volume of the tumours was 430 mm^3 when harvested 92 days after tumour injection. The mechanically dispersed xenograft cells were reimplanted (5×10^6 cells/animal) into two female mice, and transplantable tumours resulted at the injection sites with an average size of 450 mm^3 on day 52 after inoculation (latent period: 45 days). Histologically, the xenograft was very similar to the original tumour in their clear cell appearance and oval/round hyperchromatic nuclei (Figure 1b). There was one difference in that there were no prominent organoid arrangements consisting of nests or cords, which were seen in the original tumour biopsy, in the xenograft (Figure 1b).

The xenograft tumours were harvested and processed to produce mechanically dispersed cells, and cultured *in vitro*. A cell line was developed from this cell preparation and grown for 17 passages with no obvious murine fibroblastic cells observed (Figure 2c). Their *in vitro* growth pattern and cellular morphology and those of the MST-1 were very similar, except that a few larger epithelial cells were seen only in the tumour culture initiated from the xenograft (Figure 2c). This xenograft-derived cell line was designated MST-1-XS.

Ultrastructural characteristics

Ultrastructurally, tumour cells harvested from MST-1 monolayer cultures displayed pseudopod-like processes and contained numerous mitochondria in most of the cells (Figure 5a). Although not too obvious in this micrograph, cytoplasmic vacuoles were frequently observed. Melanosomes and premelanosomes (Figure 5b) of different sizes were infrequently observed in a very small proportion of the cells examined (~8%). Variable-sized glycogens were also observed, but rarely presented as aggregates (results not shown). Cytoplasmic

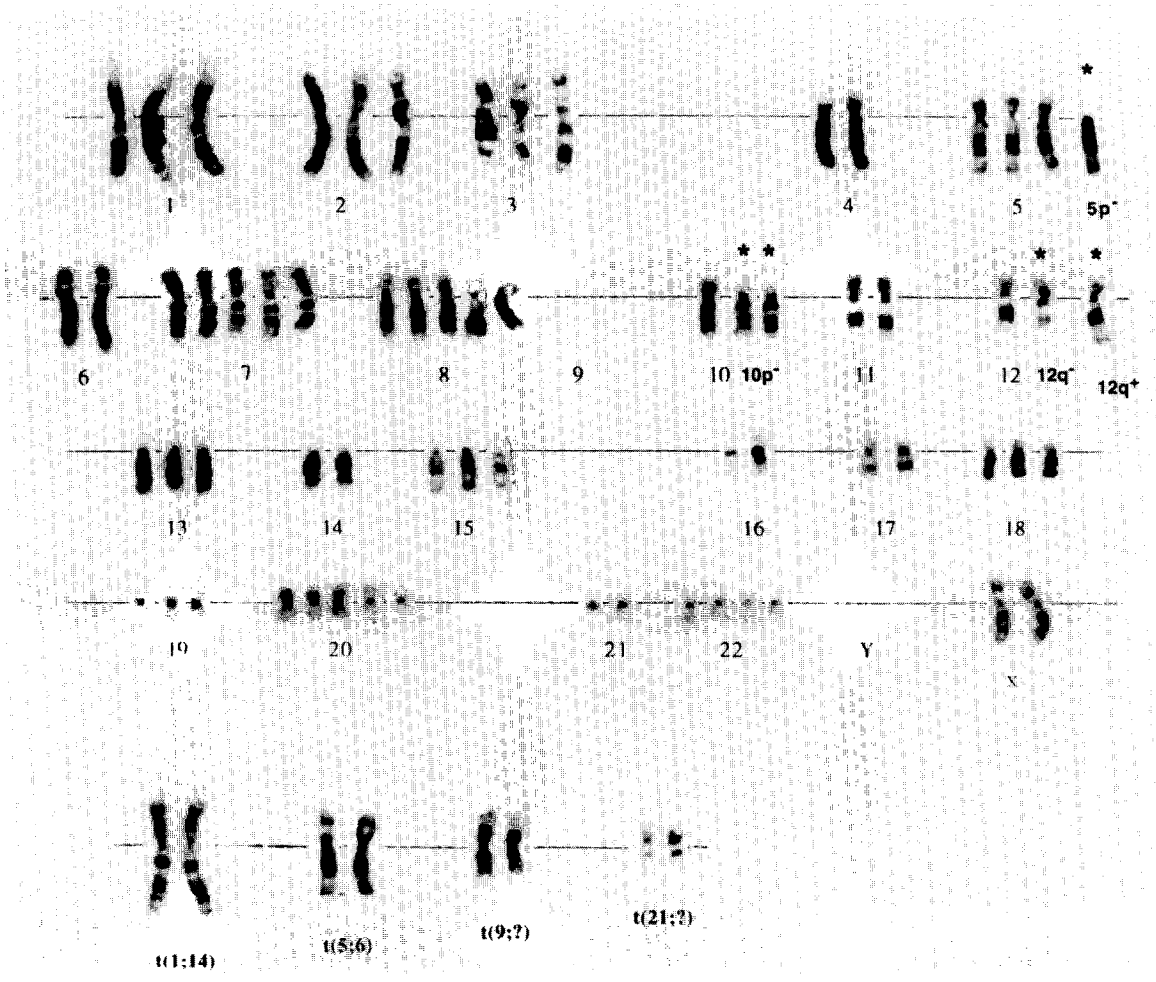

Figure 4. Representative karyotype of MST-1 cells (passage 13). For description of chromosomal abnormalities and markers, see text. An asterisk (*) indicates where the individual chromosomes with abnormalities are as compared with normal counterparts next to them.

vesicles containing heterogeneously electron-dense to granular substances, which appeared to be lysosomes, were frequently observed. Despite pseudopod formation, we did not note chromatin condensation, fragmentation of the nucleus or apoptotic bodies.

Expression of melanoma-associated antigens

Table 1 is a summary of immunocytochemical results with a panel of 14 MAbs to various tumour-associated antigens and to intermediate microfilaments. MST-1 cells grown overnight on chamber slides reacted strongly with antibodies to HLA-A,B,C, HMB-45, S100, ICAM-1, vimentin, as well as with MAbs to four well-defined melanoma-associated antigens, namely melanotransferrin (p97), chondroitin sulphate proteoglycan (250 kD + >440 kD), CEA-cross-reacting 95–150-kD glycoprotein, gangliosides GD2 and GD3. This cell line did not react with MAb to desmin nor with MAbs to epithelial and/or pancarcinoma-associated antigens, including MAK6 (cytokeratins 14, 15, 16, 18 and 19), EMA (epithelial membrane antigen) and folate-binding protein (40 kD). In fact, the reactivity spectrum with the MST-1 cell line was very similar to those obtained with three human malignant melanoma cell lines, CaCL 85-1, M-21 and MeWo, when the same panel of MAbs were tested (Table 1). MST-1 cells showed no reactivity with anti-HLA-DR antibody. Among the three melanoma cell lines tested, CaCL 85-1, which is known to express HLA-DR, was the only one reactive with MAb to HLA-DR. The MCF-7 human breast carcinoma cell line reacted with MAbs to MAK-6, EMA and folate-binding protein, as well as with MAb to HLA-A,B,C monomorphic determinant.

Immunostaining patterns of MST-1 cells varied depending on antibodies employed. Figure 6 shows representative reaction patterns. For instance, positive staining (reddish-brown colour), mostly confined to the cytoplasm, was seen with anti-vimentin MAb (Figure 6a), while highly granular staining was observed in the cytoplasm with anti-HMB45 MAb (Figure 6b). In both cases, although virtually all the cells were positively stained, a wide range of variation in staining intensity among the cells was noted. Rather diffuse staining patterns in varying intensities with less distinctive cell membrane demarcations were obtained with anti-HMW-MAA MAb (Figure 6c) and anti-GD2 MAb (Figure 6e). About 50% of cells were not positively stained with these two antibodies. Anti-GD3

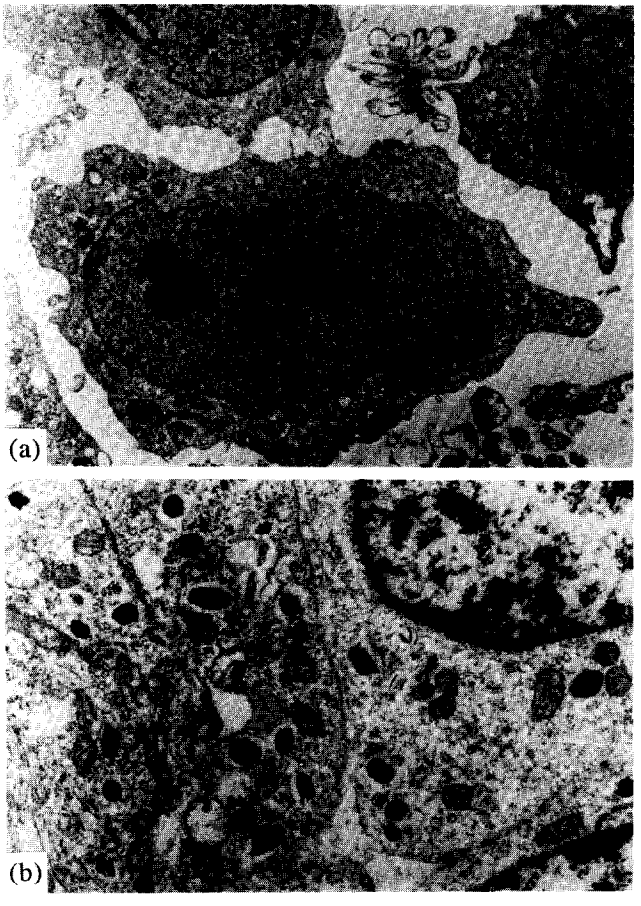

Figure 5. Electron micrographs of parts of a few different MST-1 cells, displaying pseudopod-like processes, mitochondria and small vacuoles (lower left) (a); premelanosomes and melansomes at different stages of melanisation distributed in the cytoplasm (b). (a) × 12 000; (b) × 36 000.

MAb showed strong cytoplasmic and membraneous staining with groups of cells, but failed to do so with cells in other areas under the same micrscopic field (Figure 6d). The positive control (Figure 6g) with anti-HLA-A,B,C MAb gave clear membranous staining. In contrast, no positive staining was observed with normal mouse Ig (Figure 6f) or PBS (not shown). The latter was used to control for the second biotinylated antibodies of the test system.

Profiles of DNA content

Analysis of DNA from the original tumour, MST cells (10th, 16th, 24th and 47th passages) and MST-1-XS (third passage) by flow cytometry indicated that the samples contained one major aneuploid population of cells with approximately 1.8 times DNA content of normal human diploid cells (histograms not shown).

Comparative studies of MST-1 and MST-1-XS cell lines

The two cell lines derived from the primary tumour (MST-1) and from xenograft (MST-1-XS) were compared with respect to *in vitro* growth and antigenic expression. Results show that there were no major differences in *in vitro* doubling time, saturation density and DNA content between these two cell lines (Table 2). Constitutive expression of various antigens of this tumour was quite stable, when the tumour biopsy, the tumour xenograft grown in a SCID mouse (results not shown) and the derived cell lines (Table 1) were compared. It should also be noted that no mouse IgG was detected in the xenograft and surrounding normal tissues, since the treatment of the sections with the biotinylated horse anti-mouse IgG (omission of the first antibody) followed by the ABC reagent as part of the negative controls gave no background staining.

Development of TILs and their cytotoxic effects on autologous and allogeneic tumour cells

Both the original knee tumour biopsy and metastatic lymph node contained TILs. These lymphocytes were grown out from mechanically prepared cell suspensions in medium containing IL-2. During the first 2 weeks of culture with added IL-2, tumour cells were gradually lost or destroyed by activated lymphocytes. This was evident by the observations that many activated lymphocytes attached on to the tumour targets which were visible in the early cultures. Eventually the tumour cells disappeared as the number of lymphocytes increased in the primary culture in which IL-2 was added. This phenomenon was absent in identical cultures without IL-2, and in these cultures tumour cells grew progressively, although the rate of growth was somewhat slow (doubling time, approximately 69 h). Activated TILs were expanded successfully in the presence of IL-2, and the later passages further activated with immobilised MAb OKT-3 (to the culture flasks) periodically, until 4.5×10^8 cells were obtained (about 2 months in culture). Formation of cell aggregates floating in culture medium, typical of TIL cell culture, was seen in both stages of initiation and expansion. Other TILs settled on the bottom of the flask, showing irregular and tadpole-like cellular morphology, which was not unlike LAK cell morphology in culture. These TILs were used for the subsequent phenotypic and functional analyses.

Flow cytometry evaluation of 9-week TIL cultures using a panel of MAbs to specific lymphocyte markers showed that about 80% of these cells were of the $CD8^+$ phenotype, while 11% had the $CD4^+$ phenotype. By two-colour analysis, 33 and 53% of the cells co-expressed the $CD3^+CD25^+$ and $CD3^+HLA\text{-}DR^+$ markers, respectively. This indicates that a significant proportion of the cells were activated T cells (Table 3). Note that some phenotypic changes occurred in the presence of IL-2-expanded lymphocyte populations from the freshly isolated lymphocytes.

These activated lymphocytes exhibited cytotoxicity against cultured autologous tumours, MST-1 and MST-1-XS, but did not kill Daudi (NK resistant and LAK sensitive) nor K562 (NK sensitive) cells efficiently under similar test conditions (Figure 7). These functional results along with phenotypic profiles indicated clearly that the TIL population did not belong to either NK or LAK cell categories.

DISCUSSION

Melanoma of soft tissues (clear cell sarcoma of soft parts) occurs in patients of different ages with a preference for adolescence and early adulthood [1, 5, 7–9]. Melanin granules are rarely seen [2, 9]. Out of 141 cases reported by Chung and Enzinger [5], most were Caucasian. However, the tumour has also been seen in black and Asian subjects. Regardless of racial origins, the tumour is almost always located on an extremity, the most common sites of occurrence being sole of the foot, heel, ankle, knee and thigh. In the present case, the

Table 1. Immunocytochemical reactivity of soft tissue melanoma MST-1 and MST-1-XS cell lines with a panel of antibodies to melanoma-associated antigens and to intermediate microfilaments. Included are also results with three cutaneous melanoma cell lines

	Soft tissue melanoma		Cutaneous melanoma		
Monoclonal of polyclonal antibody*	MST-1	MST-1-XS	CaCL 85-1	M-21	MeWo
Control					
HLA-A,B,C (W6/32)†	4+‡	4+	3+	4+	4+
Normal mouse IgG	–	–	–	–	–
PBS§	–	–	–	–	–
Anti-cell/tumour associated antigen					
Vimentin	3+	4+	4+	4+	4+
Desmin	–	–	–	–	–
S100 protein	3+	3+	4+	4+	3+
HMB-45	4+	3+	4+	4+	4+
p97 (Melanotransferrin)	3+	3+	4+	4+	3+
HMW-MAA (250 kD + >440 kD)‖	4+	4+	3+	4+	4+
GD3 (R24)	4+	4+	4+	3+	4+
GD3 (Mel-22)	4+	3+	4+	4+	3+
GD2 (14G2a)	4+	4+	–	3+	NT¶
CEA-crossreacting MAA (95–150 kD)	2+	3+	4+	4+	3+
Cytokaratins (MAK-6)**	–	–	–	–	–
EMA††	–	–	–	–	–
Folate-binding protein 40 kD	–	–	–	–	–
ICAM-1	4+	4+	4+	4+	3+
HLA-DR	–	–	3+	–	–

*Primary antibodies used were all murine MAbs except for normal mouse IgG and rabbit anti-S100 protein. See Materials and Methods for the sources of antibodies. †Anti-HLA-A,B,C monomorphic region. ‡Results are expressed by: –, negative; +, weakly positive; 2+, moderately positive; 3+, strongly positive; 4+, very strongly positive. §PBS, phosphate buffered saline. ‖HMW-MAA, high-molecular-weight melanoma-associated antigen (chondroitin sulphate proteoglycan). ¶NT, not tested. **Anti-cytokeratin cocktails including types 14, 15, 16, 18 and 19. ††Epithelial membrane antigen.

tumour studied was located on the right knee of a 14-year-old Taiwanese girl. Histopathologically, the neoplasm typically displayed the compartmentalised growth of predominantly oval/round, and polygonal cells with prominent nucleoli and optically lucent cytoplasms. Vacuolar structures were seen in the cytoplasm of most cells, and melanins presented as premelanosomes and melanosomes in a very small proportion of cells.

The observed expression of S100 protein, HMB-45, vimentin, the four well-defined melanoma-associated antigens, p97 melanotransferrin, chondroitin sulphate proteoglycan (250 kD + > 440 kD), CEA-crossreacting 95–150-kD glycoprotein(s) and two gangliosides, GD3 and GD2, and the absence of cytokeratins, EMA, 40-kD folate-binding protein and desmin in the tumour as well as in derived cultured cells (MST-1, MST-1-XS) substantiate a close relationship between the soft tissue melanoma and cutaneous malignant melanoma in their origins. Unexpected results in this study were the *in vitro* cellular morphology and growth patterns of this tumour, which were somewhat different from those of most cultured cutaneous malignant melanomas [20, 32] in that the former exhibited loose attachment to the plastics, pseudopod-like processes on the cell surface and slower growth rate. Nevertheless, a minority of MST-1 cells showed triangular dendritic and elongated dendritic cell shapes, which resemble cutaneous melanoma cells *in vitro* [20, 32]. MST-1 cells persisted in the characteristics of growth behaviour, antigenic expression, DNA content and susceptibility to autologous TIL cell killing.

Regarding the observed blebbing or pseusodopia, one may argue that this phenomenon may mean that the cell cultures were either maintained under suboptimal conditions or contaminated with mycoplasma. The latter possibility could be ruled out, since the MST-1 cultures were found to be free of mycoplasma during the course of the study as monitored by both hybridisation and DNA histochemical techniques. The former possibility resulting from technical oversights, such as allowing cells to be overgrown or the medium to become acidic, seems less likely, as the cells were fed with fresh medium and passaged to new culture flasks regularly. Although pseudopod formation may represent a sign of apoptosis, chromatin condensation, fragmentation of the nucleus and/or apoptotic bodies, characteristics of apoptosis were not seen at the ultrastructural level. It is possible, however, that the cells with pseudopodia might have resulted from a particular requirement for supplements which were absent from the culture medium employed. Of interest is that this morphology was lost at a higher cell density a few days after each subculturing. This seems to be consistent with possible production of certain autocrine growth factors by MST-1 cells. Nevertheless, further studies are needed to test this possibility.

The MST-1 cells were transplantable in SCID mice. The failure of MST-1 cells to demonstrate compartmentalised growth in SCID mice might have been related to the microenvironment of the subcutaneous injection site, which might not be suitable for the formation of fibrous septa. Since SCID mice have no functional T and B cells, antitumour effects of exogeneous human effector cells can be tested by intravenous

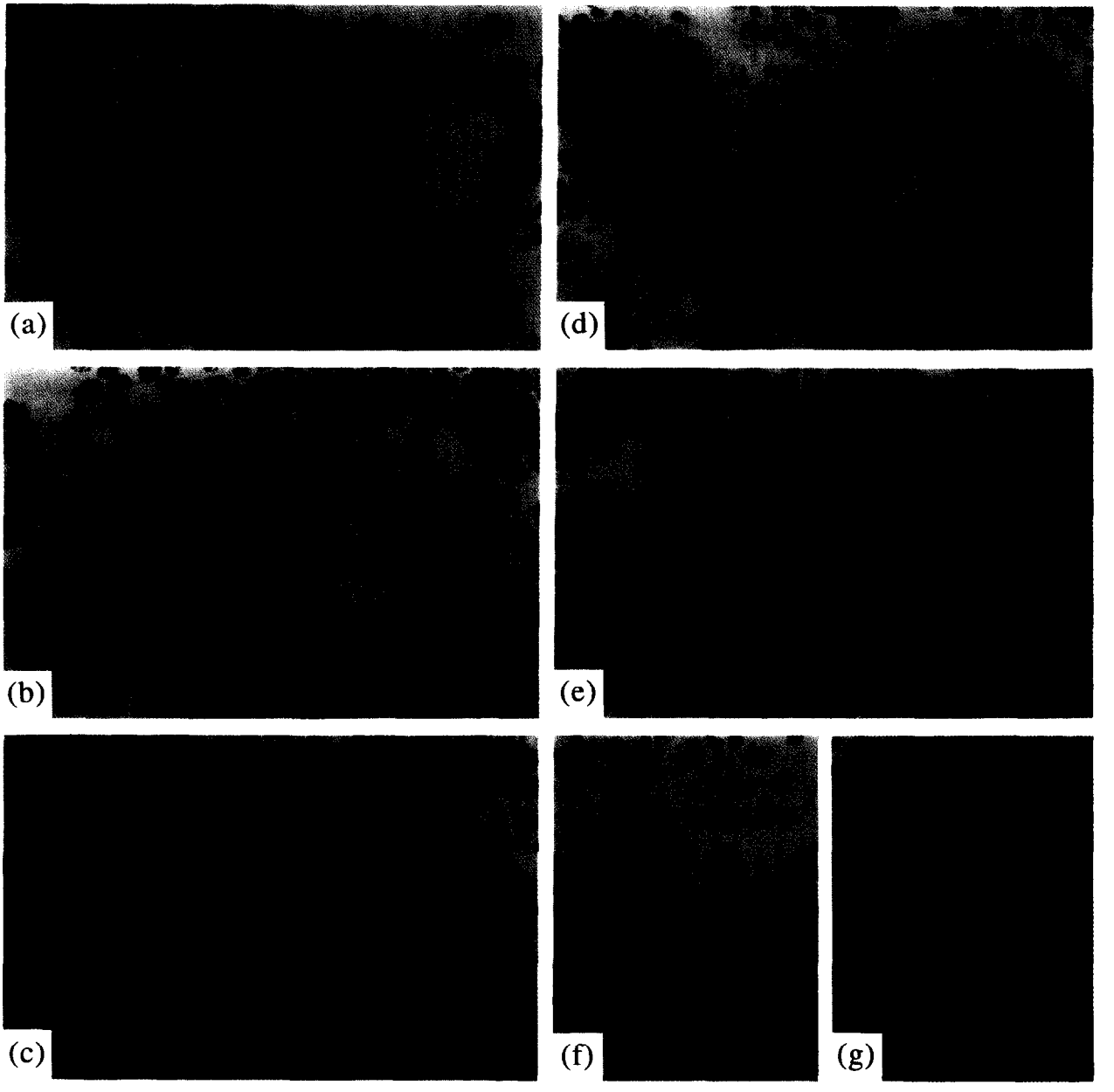

Figure 6. Immunoreactive patterns with various monoclonal antibodies of MST-1 cells grown on chamber slides overnight prior to testing. Primary antibodies or control reagent used are the following: anti-vimentin (a), anti-HMB45 (b), anti-HMW-MAA (chondroitin sulphate proteoglycan) (c), anti-GD3 ganglioside (d), anti-GD2 ganglioside (e), normal mouse Ig (f), and HLA-A,B,C (g). See text for description of immunostaining patterns. × 1000.

Table 2. Characteristics of cultured MST-1 and MST-1-XS cell lines

Parameter	MST-1 cells	MST-1-XS cells
Population doubling time	69 h	71 h
Saturation density (No. of cells/cm^2)	1.6×10^5	1.5×10^5
DNA index	1.8	1.8
Sensitivity to autologous TIL killing*	Yes	Yes

*See Figure 7.

administration to such animals bearing human tumour xenografts [9, 33, 34]. The demonstration of melanosomes, loose attachment of cells to the plastic substratum and the presence of three basic cell shapes, one of which showed numerous pseudopodia, were some of the chief attributes of MST-1 cells. Whether these characteristics can be generalised for all or most soft tissue melanomas in cultures remains to be determined. Epstein and associates [11] reported a cell line termed SU-CCS-1 derived from a 16-year-old Caucasian girl with clear cell sarcoma. SU-CCS-1 was the first cell line derived from a soft tissue melanoma. Cells of this line were found to be loosely attached to the culture flask, but remained in a round configuration. Unlike MST-1, SU-CCS-1 cells did not form pseudopodia in culture, despite the use of the same

Table 3. Phenotypic analysis of freshly isolated TILs and in vitro *IL-2-activated TILs from a soft tissue melanoma*

	% positive cells	
Lymphoid cell marker	Freshly isolated	*In vitro* IL-2 activated
CD2	72	97
CD3	65	85
CD20	NT*	0
CD4	35	11
CD8	41	79
CD56 (NKH-1)	23	2
CD25	15	36
HLA-DR	45	70
CD43	NT	95
CD3/CD25†	NT	33
CD3/HLA-DR†	NT	53

*NT = not tested. †A set of two markers tested for dual expression.

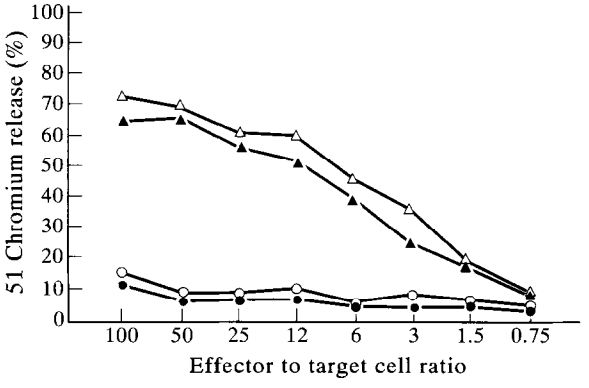

Figure 7. Lytic responses of activated TIL cells to autologous MST-1 (▲), MST-1-XS (△), allogenic K562 (●) and Daudi (○) cell lines.

kind of medium. This might be related to the fact that SU-CCS-1 cells came from malignant pleural effusion (a variant of the primary tumour cells) rather than a primary solid tumour, from which the MST-1 cell line was directly derived. To our knowledge, MST-1 represents the second soft tissue melanoma cell line reported.

A specific set of chromosome rearrangements, resulting in the fusion of genes from different chromosomes, has recently been identified for clear cell sarcoma (i.e. soft tissue melanoma) [12]. This has been characterised by a translocation between chromosomes 12 and 22: t(12,22)(q13;q12), not seen in other sarcoma types and cutaneous melanomas [12–14]. Cytogenetic analysis of MST-1 cells showed numerous abnormalities, such as $5p^-$ (deletion of the p arm of chromosome 5), $12q^-$ (deletion of the bright band of q arm of chromosome 12 with a new pale band present slightly far away from the centromere) and $12q^+$ (chromosome 12 with a terminal pale staining band). The missing region of $12q^-$ was not obvious in any of four chromosomes 22 as reported for soft tissue melanomas by others [12, 13]. In addition, $10p^-$ was consistently observed in two of the two to three chromosomes 10. The translocation t(1;14) presented in pair (Figure 4) resembles "8B", one of the marker chromosomes of the SU-CCS-1 cell line mentioned above [11]. The reason for lack of the specific translocation, t(12;22), in MST-1 cells at the karyotypic level is not clear. It is important to note that the t(12;22) chromosomal translocation was not found in SU-CCS-1 cells either, but an abnormal chromosome 12 was detected [11] which resembles the $12q^+$ marker of MST-1 cells. Interestingly, despite the absence of this typical translocation at the chromosomal level, the fusion transcript of *EWS/ATF*-1, a fusion gene created by the t(12;22) translocation was identified in SU-CCS-1 cells [14]. The gene involved on chromosome 22 is *EWS*, the same gene that is rearranged in Ewing's sarcoma and peripheral primitive neuroectodermal tumours (PNETs). The *EWS* gene in soft tissue melanoma is fused with the *ATF*-1 DNA transcription factor on chromsome 22. More work with the use of specific molecular probes, such as *EWS/ATF*-1 (coded for a specific chimeric protein) on cells of MST-1 and additional cases of soft tissue melanoma is needed to accurately pinpoint the regions of chromosomal translocations, and determine whether there are differences in specific chromosome rearrangements of soft tissue melanomas between oriental and Caucasian patients.

It is important to look for obvious and subtle differences between soft tissue melanoma and cutaneous melanoma, and between their respective normal and benign counterparts at both the cellular and molecular levels, to probe how melanocytes stray into deep soft tissues when they migrate from the neural crest to the dermal area during the early embryonic development. Since both soft tissue melanoma and cutaneous melanoma seem to be derived from a common neuroectodermal origin and share a number of phenotypic characteristics, it is possible that soft tissue melanoma may be as immunogenic as cutaneous melanoma in cancer patients in expressing T cell-defined antigens, such as a peptide of the MAGE-1 protein presented by cell surface HLA-A1 molecules and a peptide of tyrosinase by HLA-A2 molecules (for review, see [34]). The availability of soft tissue melanoma cell lines, such as MST-1 and SU-CCS-1, should contribute to elucidation of these basic immunobiological questions.

In the past decade, the laboratory measures involving tumour acquisition, processing and cryopreservation has collectively become an integral part of a comprehensive programme for cancer patients undergoing biologic therapies [15, 22, 35, 36]. The therapies include the use of specific tumour vaccines [15, 36], lymphokine-activated killer (LAK) cells [16], TILs [17] and MAb-based therapies [22, 24]. Cultured tumour cells [36], IL-2-activated and expanded LAK [16, 24, 29, 36] or TIL [17] cells, xenografts [29, 33, 34, 36, 37], MAbs [22, 24, 29] and cryopreserved materials [15, 36] of those aforementioned could be used for development and testing of biologicals designed for individual patients. Moreover, the recent development of gene therapy for cancer involves the use of appropriate target cells into which selected genes can be inserted for expression. In this context, the target cells could be either tumour cells or TILs. The ability of generating sufficient numbers of tumour cells and TILs as target-effector cells for further analysis or for transfer of cytokine genes, such as GM-CSF, IL-2, γ-interferon, as well as the ability to evaluate interactions between these gene-modified cells for antitumour effects in a SCID mouse xenograft system are important keys to success in biological and gene therapy programmes. The availability of

paired autologous tumour cell line and TIL culture, and of human tumour xenografts in SCID mice demonstrated in this report should facilitate further preclinical studies, identification of T-cell-defined tumour rejection antigens (or peptides), and development of biological therapy strategies for individual patients.

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