Virchows Arch (1995) 426:385–391

ORIGINAL ARTICLE

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In vitro differentiation and proliferation in a newly established human rhabdomyosarcoma cell line

Received: 21 September 1994 / Accepted: 25 January 1995

Abstract A newly established cell line, designated NRS-1, was derived from an alveolar rhabdomyosarcoma that developed in the left forearm of a 7-year-old girl. The cell line had a t(2; 13) chromosomal translocation. Reverse transcription-polymerase chain reaction demonstrated that 5′ PAX3–3′ FKHR chimeric transcript was expressed in NRS-1 cells. NRS-1 cells showed myogenic differentiation without any particular stimulus in vitro and exhibited various kinds of muscle markers. All-*trans* retinoic acid promoted cell differentiation in the myogenic direction. Transforming growth factor-β (TGF-β) inhibited myogenic differentiation of those cells and promoted cell proliferation.

Key words Alveolar rhabdomyosarcoma · Cultured cell line · Myogenic differentiation · Retinoic acid · Transforming growth factor

Introduction

Rhabdomyosarcoma is the most common soft tissue sarcoma of children [4], and modern intense multi-modal therapy has improved the outcome considerably [11], though the prognosis is still far from satisfactory. We know little about the factors causing growth or differentiation of human rhabdomyosarcoma cells. Recently, we have successfully established a new cultured cell line designated NRS-1, derived from a human alveolar rhabdomyosarcoma. In this paper, we describe the characteristics of the new cell line with reference to the effects of growth factors and differentiation factors.

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Materials and methods

A cell line, designated NRS-1, was derived from a patient with alveolar rhabdomyosarcoma. A 7-year-old Japanese girl was admitted to Niigata University Hospital because of a soft tissue tumour of the left forearm. Open biopsy revealed a small round-cell sarcoma. Although the patient was treated with preoperative chemotherapy (cisplatin and adriamycin) and radiotherapy (50 Gy), followed by radical surgery and postoperative chemotherapy (cisplatin, adriamycin and vincristine), she died of multiple metastases 22 months after surgery. Tumour tissue was obtained from a metastatic lesion in the left calf muscle during surgical resection and was minced with scissors. Several tumour fragments, about 2×2×2 mm in size, were transplanted into the subcutaneous tissue on the back of athymic nude mice (nu/nu) with a BALB/c genetic background (Nihon Clea, Tokyo, Japan). The source of primary culture was a heterotransplanted tumour of the first passage. Alpha-modification of Eagle's minimal essential medium (α-MEM; Cosmo Bio Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, Md.) and 200 μg/ml of kanamycin sulfate (Meiji Seika, Tokyo, Japan) was used for the culture. The tumour developed in a nude mouse was minced with scissors, and the tumour tissue fragments, approximately 1 mm in diameter, were cultured in plastic culture dishes. All the cultures were maintained at 37°C in a humidified incubator with a constant flow of 5% CO_2 in air. The medium was renewed every 4 days.

Cultured cells were observed with an inverted phase-contrast microscope. The analysis of contractile movement was done using a video camera (MV-S30; Mitsubishi, Tokyo, Japan). Pieces of both the parental tumour and the transplanted tumour were fixed in 10% formaldehyde and embedded in paraffin. The cut sections were stained with haematoxylin and eosin. For transmission electron microscopy, cultured cells and tissue cubes were fixed in 2.5% phosphate-buffered glutaraldehyde and post-fixed in 1% osmium tetroxide. Ultra-thin sections were stained with uranyl acetate and lead citrate. The formalin-fixed, paraffin-embedded sections of the parental tumour and the xenografted tumour, and ethanol-fixed cultured cells on the Lab-Tek chamber slides (22×22 mm; Nunc, Naperville, Ill.) were stained by an immunoperoxidase method using a biotin–streptavidin–peroxidase system (BSA; Nichirei, Tokyo, Japan). Mouse monoclonal antibodies to human desmin (D33; Dako, Glostrup, Denmark), muscle actin (HHF35; Enzo, New York, N.Y.), vimentin (V9; Dako), and Ki-67 (MIB-1; Immunotech, France), and a rabbit polyclonal anti-human myoglobin (Dako) were used as primary antibodies.

For chromosomal analysis, the cultured cells were treated with colchicine (0.2 μg/ml). Chromosomal preparations were made using a trypsin-Giemsa banding method.

Total cellular RNA of NRS-1 cells and normal skin fibroblasts were prepared by guanidine thiocyanate/cesium chloride gradient

Table 1 Polymerase chain reaction primer sequences (*β2M* β_2-microglobulin)

Primer	Sequence (5′ to 3′ orientation)	Product size (bp)	Restriction site	Restriction products (bp)
PAX3 (5′)	GCACTGTACACCAAAGCACG	409	EcoR 1	164+245
FKHR (3′)	AACTGTGATCCAGGGCTGTC			
β 2M (5′)	CCTTGAGGCTATCCAGCGTACTCC	322	EcoR 1	214+108
β 2M (3′)	CCATGATGCTGCTTACATGTCTC			

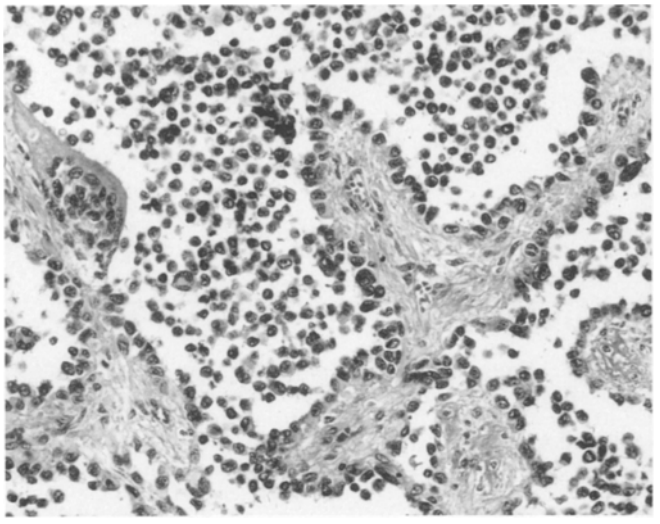

Fig. 1 Alveolar rhabdomyasarcoma. The tumour was composed of small round cells separated by varying width of fibrous septa. H&E; ×90

centrifugation. Total cellular RNA, 5 μg, was converted to cDNA by reverse transcription using the SuperScript Preamplification System (Gibco BRL, Gaitherburg, Md.), and the cDNA was amplified by PCR using a Gene Amp kit (Perkin-Elmer Cetus, Norwalk, Conn.). The oligonucleotide primers for PCR were designed according to Galili et al. [6] and Ralston [28] (Table 1). Amplification was carried out in a Program Temp Control System PC-700 (Astec, Tokyo, Japan). The cycle conditions were as follows: denaturation at 94°C for 4 min followed by 29 cycles of denaturation at 93°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min followed by 1 cycle of 93°C for 1 min, 60°C for 2 min, and 72°C for 10 min. The reaction products were analysed by electrophoresis on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide. The identity of the PCR products was confirmed both by their predicted size in agarose gel electrophoresis and by the characteristic fragments obtained after restriction enzyme digestion of the products.

The induction experiments on cell differentiation or cell proliferation were attempted using several agents including all-*trans* retinoic acid (ATRA), dimethyl sulfoxide (DMSO), dibutyryl cyclic AMP (dbcAMP; Sigma, St. Louis, Mo.), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; Hoffmann-La Roche, Switzerland), and transforming growth factor-β1(TGF-β1; King Brewing, Hyogo, Japan). ATRA and $1,25(OH)_2D_3$ were dissolved in 95% and 100% ethyl alcohol, respectively. The final concentration of ethyl alcohol was found not to have any effects on the growth and differentiation of tumour cells in preliminary tests. The other agents were dissolved directly in the culture medium. Media in both control and drug-treated cultures were replaced every 3 days. After 15 days of culture, the cells were stained by the Giemsa method, and the number of myotube-like giant cells that contained three or more nuclei were counted. Each experiment was carried out three times in duplicate. Experiments with ATRA and DMSO were performed under light protection using aluminium foil.

For the analysis of tumorigenicity, approximately 10 million cultured cells in 0.2 ml of fresh culture medium were inoculated into the subcutaneous tissue of the athymic nude mice. The animals were killed by anaesthesia either when the tumour had grown to 2 or 3 cm at its widest point or when 8 weeks had elapsed after transplantation.

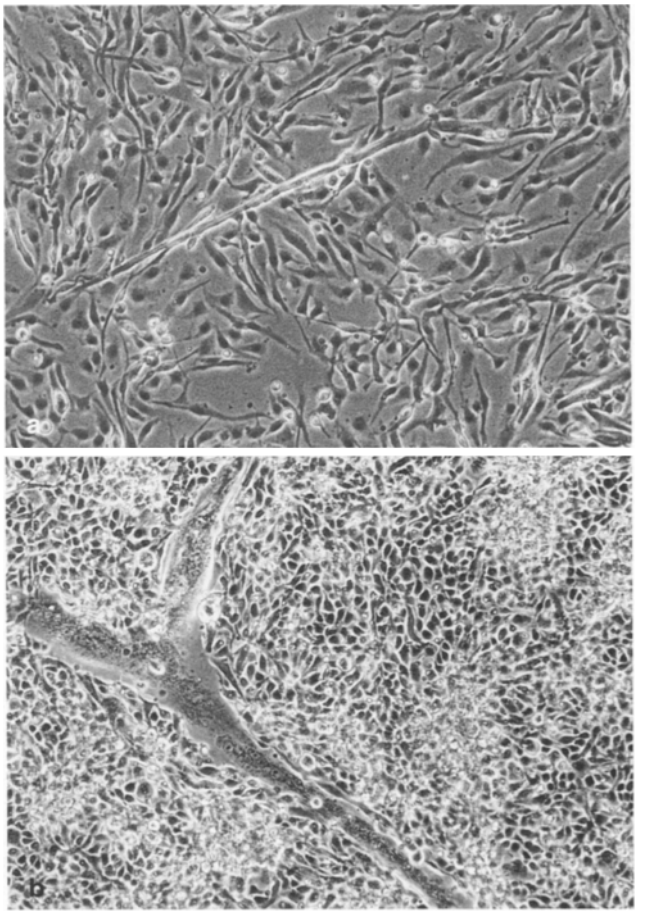

Fig. 2a, b Phase-contrast micrograph of NRS-1 cells. Elongated multinucleated cells were scattered among small round mononuclear or spindle-shaped cells (**a**) and giant cells with over 20 nuclei (**b**)

Results

Histological examination revealed that the parental tumour was an alveolar rhabdomyosarcoma showing loosely arranged aggregates of tumour cells separated by irregularly shaped fibrous trabeculae (Fig. 1). Although there were some multinucleated cells, neither myotube-like elongated cells nor rhabdomyoblasts with cross-striation were found. Ultrastructurally, the tumour cells showed features of immaturity with varying amounts of

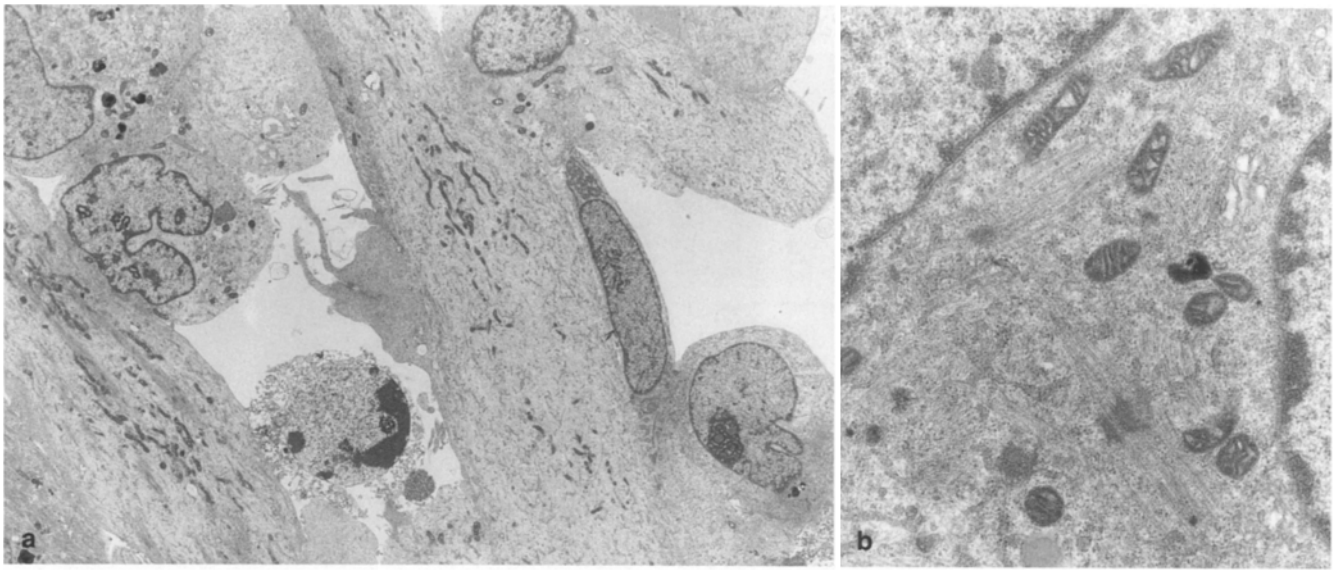

Fig. 3a, b Ultrastructure of NRS-1 cells in vitro. **a** Small round cells and elongated cells were present (×1,500). **b** The elongated myotube-like cells had large mitochondria and myofibrils with Z-band. (×20,000)

glycogen and without definite myofibrils with Z-band material.

The cultured cells formed mono- and multi-layered arrangements, and were composed of three different types of cells: small round or spindle-shaped mononuclear cells, multinucleated elongated cells resembling myotubes, and rarely occurring multinucleated giant cells (Fig. 2a, b). Ultrastructurally, the mononuclear cells frequently contained abundant glycogen particles, but few Z-bands. Myotube-like cells and multinucleate giant cells were characterized by abundant Z-bands dividing myofibrils and many elongated mitochondria (Fig. 3a, b). There were some transitional forms among these three types of cultured cells. Some myotube-like cells showed spontaneous but irregular contractions.

The doubling time of the cell population of the 15th passage was 68 h and that of the 25th passage was 64 h. The modal chromosome number derived from the 22nd passage cells was 93. Cytogenetic analysis revealed 93, XXXX, del(1)(p36), add(1)(p36), t(2; 13)(q35; q14)×2, del(3)(p11), del(4)(q31), +5, +6, –8, add(8)(p11), –13, add(14)(q32), –19, +20×2, –21, add(21)(q22), +mar (Fig. 4).

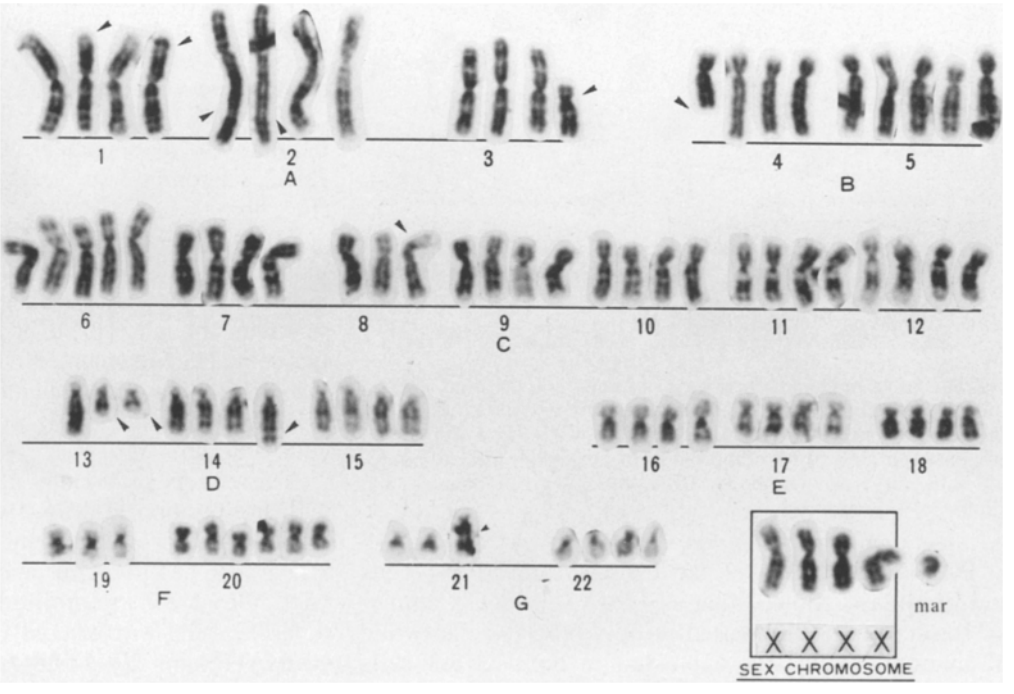

Fig. 4 The modal karyotype of NRS-1 cells. A specific translocation in human alveolar rhabdomyosarcoma t(2; 13) was confirmed

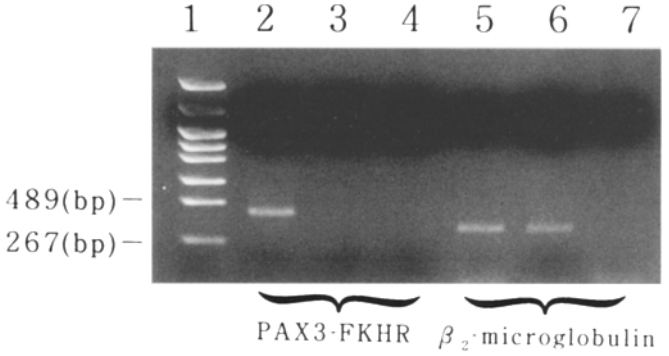

Fig. 5 Detection of PAX3-FKHR chimeric transcript by the polymerase chain reaction in NRS-1 cells. *Lane 1*, pHY size marker; *lanes 2, 5*, NRS-1 cells; *lanes 3, 6*, skin fibroblasts ; *lanes 4, 7*, blank (without cDNA)

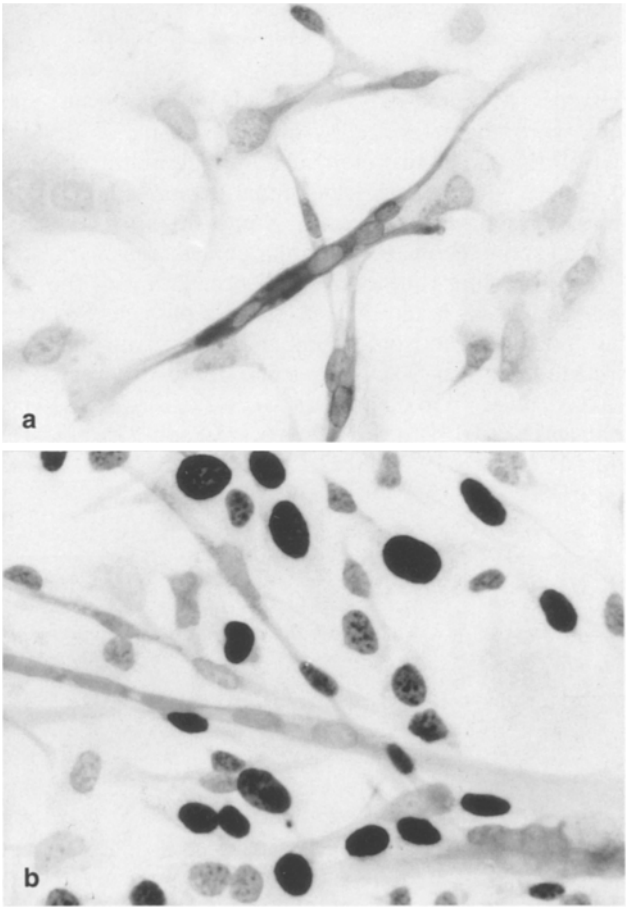

Fig. 6a, b Immunocytochemical stainings of cultured NRS-1 cells. **a** Strongly positive reaction to anti-myoglobin antibody can be seen not only in myotube-like cells but also in a number of mononuclear cells. **b** Myotube-like cells are shown that did not react with anti-Ki-67 antibody. BSA, methyl green counterstain, ×360

PCR amplification of the cDNA from NRS-1 cells and fibroblasts showed that a 5′ PAX3-3′ FKHR chimeric transcript was expressed in only NRS-1 cells, while β2-microgulobulin was expressed in both NRS-1 cells and fibroblasts (Fig. 5).

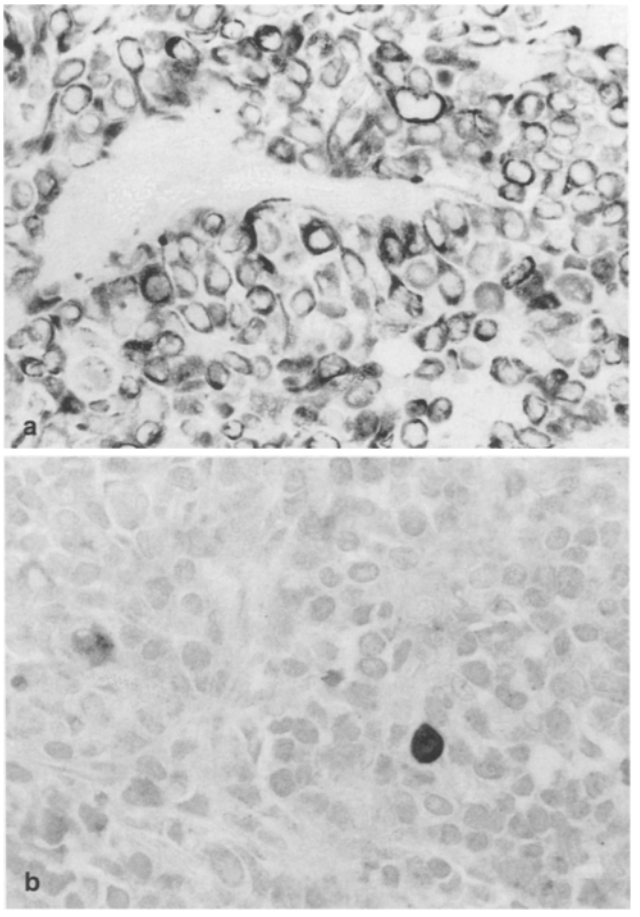

Fig. 7a, b Immunohistochemical staining of transplanted NRS-1 cell tumour. **a** The tumour cells are shown, which were frequently positive for desmin. **b** Few cells were positive for myoglobin. BSA, methyl green counterstain, ×360

The results of immunostaining are summarized in Table 2. Both mononuclear NRS-1 cells and myotube-like NRS-1 cells were frequently positive for myoglobin, desmin and vimentin (Fig. 6a). Both types of cells also occasionally reacted with anti-muscle actin antibody. Although most mononuclear NRS-1 cells were positive for Ki-67, myotube-like cells were negative for Ki-67 (Fig. 6b).

NRS-1 cells successfully formed transplanted tumours in six of the nine nude mice examined. These tumours also showed the features of alveolar rhabdomyosarcoma, though typical histological patterns were less frequent. The transplanted NRS-1 cell tumours as well as the parental tumour frequently contained desmin-positive cells (Fig. 7a), but rarely contained myoglobin-positive cells (Fig. 7b).

The effects of various agents on cell differentiation and proliferation in NRS-1 cell line are summarized in Table 3. Cell growth was inhibited by the treatment with ATRA, 10^{-3} M dbcAMP and 2% DMSO. TGF-β stimulated NRS-1 cells to proliferate. The proportion of myotube-like cells was markedly increased by the treatment with ATRA or 2% DMSO, but was decreased by the treatment with TGF-β (Fig. 8).

Table 2 Immunocytochemical phenotypes of NRS-1 cells (– negative, + a few poisitive, ++ occasionally positive, +++ frequently positive)

Antigen	Cultured cell		Parent tumor	Transplanted tumor
	Mono-nucleated cell	Myotube-like cell		
Myoglubin	+++	+++	+	+
Desmin	+++	+++	+++	+++
Muscle actin	+	++	++	++
Vimentin	+++	+++	+++	+++
Ki-67	+++	–	++	+++

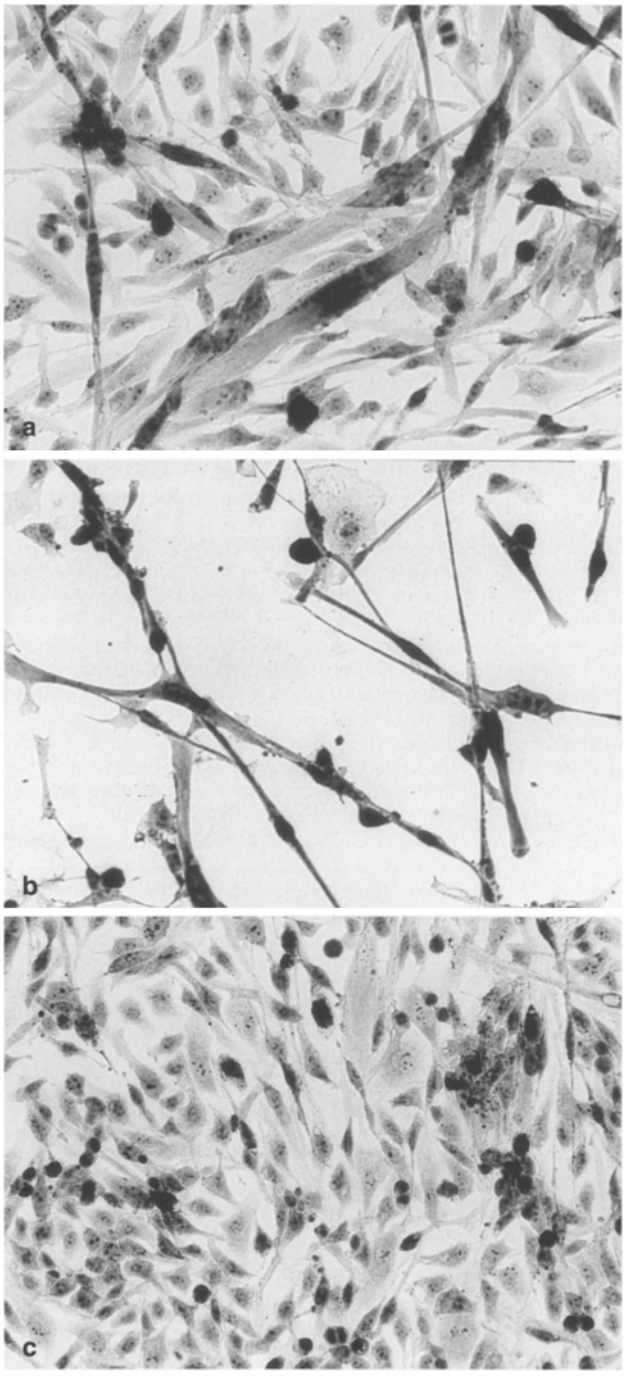

Table 3 Effefct of various agents on the differentiation of NRS cells in vitro

Incubation with		No. of cells ($X10^6$)	% of myotube-like cells
Agent	Concentration		
Control		2.3±0.7	6.1±0.9
ATRA	$5X10^{-6}$	0.8±0.3*[a]	20.3±3.9*
	$5X10^{-8}$	1.1±0.2*	10.2±2.4*
$1,25(OH)_2D_3$	$1X10^{-6}$	1.8±0.4	8.8±0.6
	$1X10^{-8}$	2.2±0.3	7.3±1.2
TGF-β1	$5X10^{-10}$	4.1±0.3*	1.6±0.5*
	$5X10^{-12}$	3.5±0.4*	2.4±0.7*
dbcAMP	$1X10^{-3}$	0.3±0.1*	4.0±1.5
	$1X10^{-4}$	2.4±0.5	3.1±0.3
DMSO	2%	0.3±0.1*	39.0±6.1*
	0.2%	1.8±0.5	4.5±1.1

[a] Statistical significance was determined by Student's *t* test
* $P<0.01$

Discussion

To the best of our knowledge, few cultured cell lines derived from human rhabdomyosarcoma of unquestionably alveolar type have been reported [12, 27]. Morphological differentiation towards myogenesis is recognized by the appearance of myotube-like cells [5, 16]. The alveolar rhabdomyosarcoma cell line RMZ, for instance, showed such myogenic differentiation under the presence of FCS in vitro [12, 27]. Some cell lines derived from human rhabdomyosarcomas with undifferentiated features, which were initially diagnosed as undifferentiated sarcomas, were also able to demonstrate myogenic differentiation in the presence of FCS in vitro [7, 13]. In general, cell lines derived from human embryonal rhabdomyosarcoma had little capacity for myogenic differentiation in vitro [1, 15, 16, 25]. Our new cell line, NRS-1, however, seems to be very similar to the RMZ cell line. The NRS-1 cell line was also composed of different types of cells; small mononuclear round or spindle-shaped cells were observed along with a few multinucleated myotube-like elongated cells, and multinucleated giant cells. Only the mononuclear cells were positive for Ki-67, which is a nuclear antigen associated with cell proliferation [8], while myotube-like cells were negative for this antigen. Moreover, myotube-like cells showed high levels of myoglobin expression. These findings agree with those previously reported by Gabbert et al. [5], although they used a rat rhabdomyosarcoma cell line. The differentiation in rhabdomyosarcomas presumably occurs in a manner similar to that in normal striated muscle, where postmitotic myotubes arise from mononuclear myoblasts by fusion [26]. The spontaneous contractile movement indicates that myotube-like cells are well differentiated in the functional aspect.

Fig. 8a–c NRS-1 cells treated with ATRA and TGF-β. **a** Control. **b** Myotube-like cells that appeared after the treatment of ATRA. **c** There were few myotube-like cells in the TGF-β1-containing culture

However, transplanted NRS-1 cell tumours were mainly composed of small round cells, which expressed desmin but rarely expressed myoglobin. Ultrastructural examination showed little evidence of differentiation in the small round cells in the transplanted tumours. It has been reported that transplanted embryonal rhabdomyosarcoma cell tumours exhibit more differentiated features in nude mice than in vitro [17, 24]; perhaps the alveolar and embryonal forms of the tumour behave differently in vitro and in nude mice.

Alveolar rhabdomyosarcomas also differ from embryonal rhabdomyosarcomas in other aspects. For example, alveolar rhabdomyosarcomas are frequently associated with a chromosomal translocation, t (2; 13), as observed in the NRS-1 cell line [3, 30], a cytogenetic abnormality not seen in embryonal rhabdomyosarcomas. In addition, PCR analysis has shown that 5′PAX3-3′FKHR chimeric transcript is expressed in NRS-1 cells. PAX3 encodes developmentally regulated transcription factors, and FKHR is a member of the fork head domain family, which encodes transcription factors containing a conserved DNA-binding motif related to *Drosophila* region-specific homeotic gene fork head [6]. This fusion mRNA may provide a unique target for specific detection of this tumour-specific translocation, assisting in the diagnosis and clinical management of patients with this tumour [30].

TGF-β is a multifunctional cytokine family found in normal and neoplastic tissues. Although TGF-β was initially identified as a growth factor for fibroblasts [18], this cytokine is now known to inhibit the growth of certain tumour-derived as well as normal cells [20, 29]. Different cell types may respond to TGF-β in different ways, and the same cell may even exhibit opposite responses under different experimental conditions [23]. In addition to its effects on cell proliferation, TGF-β inhibits adipogenic differentiation without altering the growth rate of mouse preadipocytes [14] and also inhibits myogenic differentiation without altering the growth rate of rat skeletal muscle myoblasts [21]. However, NRS-1 cells derived from human alveolar rhabdomyosarcoma responded to TGF-β in a different way. TGF-β inhibited myogenic differentiation in the NRS-1 cell line, but stimulated cell proliferation. It is extremely important to ascertain whether or not these phenomena are common in human alveolar rhabdomyosarcoma cells, because TGF-β may act as a growth factor for human alveolar rhabdomyosarcoma cells. Some investigators have claimed that alveolar rhabdomyosarcomas frequently express TGF-β [22], and this factor may thus have an active role in the growth of some alveolar rhabdomyosarcomas, acting in an autocrine mode.

Myogenic differentiation of human rhabdomyosarcoma cells are induced in vitro [19] and in nude mice [25] by antineoplastic agents, which are frequently used in clinical practice. However, these agents have not been successful in patients with rhabdomyosarcoma. ATRA, 1,25(OH)2 D3, dbcAMP and DMSO are well known strong inducers of differentiation in many types of cells, but only ATRA and high concentrations of DMSO induced differentiation in NRS-1 cells. However, some investigators have shown that these agents failed to induce cell differentiation in some human alveolar rhabdomyosarcoma cell lines [2, 7]. These discrepancies may be due to heterogenous response among alveolar rhabdomyosarcoma cells, and this variability was observed even in a clonal rhabdomyosarcoma cell line [9, 10]. We are now attempting serum-free culture and cell cloning of the NRS-1 cell line in order to elucidate the role of growth factors in human alveolar rhabdomyosarcoma.

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