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Scanning electron microscopic evidence for neural differentiation in Ewing's sarcoma cell lines*

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Summary. A number of recent studies have suggested a relationship between Ewing's sarcoma (ES) and other small round cell tumours of childhood such as peripheral neuroepithelioma (PN). We report scanning electron microscopic studies on the character of induced neural differentiation in ES, neuroblastoma, PN, osteosarcoma and colon carcinoma. We found evidence of neural differentiation in both neural lines and in one of two Ewing's lines before treatment. After differentiation, both Ewing's and neural lines developed neuritic processes with varicosities and little arborization, except for the initially undifferentiated Ewing's line (A4573) which displayed extensive lateral sprouting from neuritic processes after differentiation. Neither treated nor untreated osteosarcoma or colon carcinoma displayed any evidence of neural differentiation. Further, neuroblastoma cells are easily distinguished from ES and PN by virtue of their single, unbranched neurites and lack of lateral sprouting or filopodia. These results provide further evidence for the neural character and close relationship between ES and PN.

Key words: Ewing's sarcoma – Peripheral neuroepithelioma – Scanning electron microscopy – Cell lines – Cyclic AMP

Introduction

Small round blue cell tumours of childhood consist of a heterogeneous group of neoplasms, including Ewing's sarcoma (ES), neuroblastoma (NB), peripheral neuroepithelioma (PN), as well as other neoplasms of non-neural origin [rhabdomyosarcoma (RMS), lymphoma (Lym)] (Triche and Askin 1983; Triche et al. 1986; Yunis 1986). The neuroectodermal character of NB and PN is well known and established, but a similar neural phenotype of ES remains controversial.

Recently it has been postulated that ES is an undifferentiated tumour, a member to a large extent of the "neuroectodermal" family based upon tissue culture (Jaffe et al. 1984; Cavazzana et al. 1987a), morphological (Schmidt et al. 1982; Llombart-Bosch et al. 1987), immunohistochemical (Llombart-Bosch et al. 1986) and biochemical studies (Lipinsky et al. 1987). Cavazzana et al. (1987a) have presented experimental evidence for the neural character of this tumour using ES cell lines. They demonstrated the presence of neural features such as neurosecretory granules and neural antigen expression by immunofluorescence techniques in ES cell lines treated with Bt_2cAMP. Furthermore it is well known that the neural differentiation induced by Bt_2cAMP in NB cell lines can be identified not only morphologically but also by biochemical or biological techniques (Prasad and Kuman 1975; Tsokos et al. 1987a; Gross et al. 1987; Rupniak et al. 1984; Prasad and Resie 1971). The effect of Bt2cAMP on PN has not been clearly established (Jaffe et al. 1984; Tsokos et al. 1987a).

In all these assays, the cytomorphology of cell differentiation has been mainly described by phase contrast microscopy. Therefore, the fine structural analysis of cell surfaces as well as the dendritic cell projections which characterize the neural phenotype in these tumours is lacking. Transmission electron microscopy (TEM) fails to provide a complete view of the cells and does not allow an analysis of cell projections or cell to cell attachments

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Table 1. Characteristics of tumour cell lines

Cell line	Age/sex	Site	Diagnosis	Origin
KCNR	11/M	Bone marrow, primary adrenal	Neuroblastoma	C.P. Reynolds and Naval Hospital. Bethesda MD
Tc 135	14/M	Thigh	Peripheral neuroepithelioma	NIH/NCI
A-4573	17/F	Clavicle	Ewing's sarcoma	Grace Cannon PHD Litton Biogenetics Co.
Tc 106	19/M	Sacroiliac	Ewing's sarcoma	NIH/NCI
HOS	15/F	Tibia	Osteogenic sarcoma	ATCC
CC	56/F	Colon	Colon adenocarcinoma	NIH/NCI

(Gonda et al. 1976). As is well known, scanning electron microscopy (SEM) is a powerful technique that allows three-dimensional study of the cell and readily visualizes cell surface morphology (Allen 1983).

In this paper, we communicate the results of Bt_2cAMP-induced differentiation experiments on a panel of cell lines grown on plastic substrate representing tumours of neuroectodermal origin (2), ES (2), and control tumour lines such as osteosarcoma (HOS) and colon carcinoma (CC). We have compared the fine structural topographic appearance of these tumour systems before and after differentiation, and have especially compared peripheral neural tumours to ES, in an effort to further substantiate a relationship between these two tumours, on the assumption that similarities would be more manifest after treatment.

Materials and methods

Tissue culture. Six tumour cell lines were evaluated in the present study. The details of each line are listed in Table 1. All lines were established and maintained in RPMI 1640 medium (Whittaker MA Bioproducts, Walkersville, Md.) supplemented with 20% FBS (Gibco, Grand Island, NY). Cells were grown to confluence in T75 tissue culture flasks (Nunc, Roskilde, Denmark) and passaged as necessary. All the cell lines have been previously characterized and published (Cavazana et al. 1987b; Reynolds et al. 1980; Dickman et al. 1982). We used the HOS and CC cell lines (obtained from ATCC) as negative controls. Tumorogenicity of all the cell lines was also established by growth in nude mice (BALBc/nu/nu). Each cell line was tested for the presence of fungi, mycoplasma, or bacteria and was free of any detectable contamination while used in the present studies. Lines were established according to standard protocols used in one of our laboratories (Cavazzana et al. 1987b).

In differentiation experiments cells from all the lines were plated in 80 cm^2 plastic flasks at a density of 5×10^4 cells/ml with 5 ml of one of three different media: (A) RPMI 1640 medium supplemented with 20% FBS; (B) serum-free medium (DMEM + HAM F12 50:50, L-glutamine $NaHCO_3$ (1·2 g/l) 15 mM HEPES, insulin (5 μg/ml) transferrin (100 μg/ml), progesterone (6·3 ng/ml), selenium (30 nM) and putrescine (8·8 ng/ml); (C) serum-free medium with 25 nM N6-O2 dibutyryladenosine 3′:5′ cyclic monophosphate (Bt_2cAMP, Sigma) SIGMA Co St. Louis Mo.

The medium was changed every 3 days. After three changes (12 days), the cells were harvested and prepared for SEM, as described below. Throughout the time course of the experiment, the morphological characteristics were documented by phase contrast microscopy of viable unfixed cells.

For SEM in situ fixation of the cells was performed on the plastic growth surface in 2.5% glutaraldehyde in Sorensen's phosphate buffer (pH 7.2) for 1 h at 4° C, followed by three buffer washes and post-fixation in 1% OsO_4 in the same buffer for 1 h. After three further buffer washes, areas from the tissue culture flasks were removed with a hot cork borer. Dehydration was through a series of alcohols of increasing concentration. The plastic fragments of the culture flasks were transferred from absolute ethanol to isopropyl alcohol, as intermediate solvent, and critical point dried in liquid CO_2, where they were sputter coated with gold in a Polaron 5100 sputter coater and observed in a Philips PSEM 500 at 25 kV.

Results

Phase contrast morphology

The KCNR (NB) cell line, when untreated and maintained with RPMI 1640 supplemented with 10–20% FBS, showed poor substrate adherence ("tear drop cells"), spontaneous extension of cell processes, bundle branch recruitment of processes and rare varicosities (not illustrated). In the absence of serum the substrate adherence was increased, as well as the number of interconnected cell processes. At the same time, a decrease in cell growth was observed. When treated with Bt_2cAMP, KCNR displayed even greater, highly characteristic "neural" differentiation with an increase in the number of colonies formed as well as growth of long neurites associated with typical phase dense neuroblasts (Fig. 1 A).

Using RPMI 1640 supplemented with FBS, Tc-135 (PN) was composed of both "epithelioid" cells and "neuroblast-like" cells with short interconnected processes. Under serum-free conditions we detected increased cell adherence with groups of cells forming clusters. There was no increase in the number of cell processes, and the growth rate was diminished. When the cells were treated with Bt_2cAMP, short cell processes with some varicosities were occasionally detectable (Fig. 1 B).

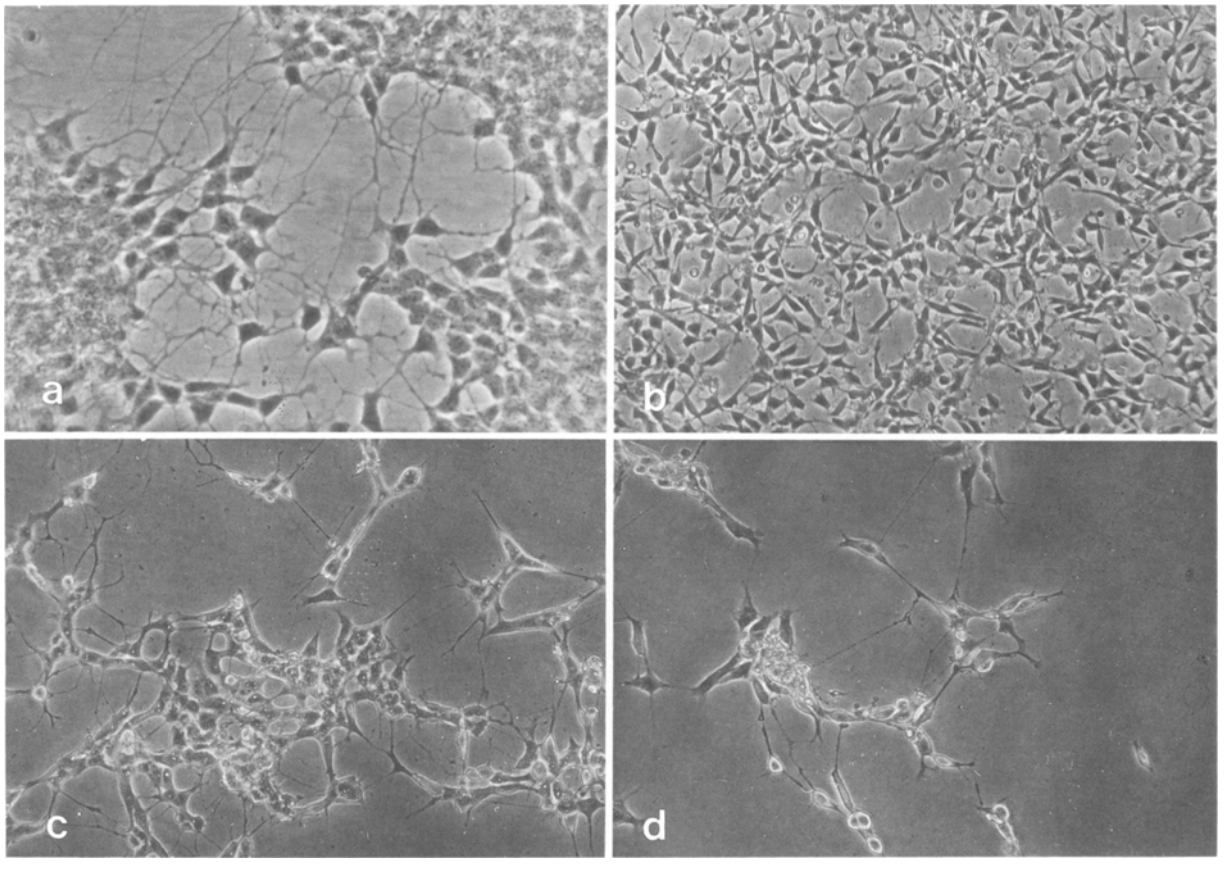

Fig. 1 A–D. Phase contrast morphology. **A** Phase contrast appearance of differentiated neuroblastoma (KCNR). Numerous neurites interconnecting cell colonies are prominent. ×100. **B** Phase contrast of peripheral neuroepithelioma (PN) (Tc-135) after treatment with Bt_2cAMP. Neuroblastic-type cells with short neuritic processes are seen. ×100. **C** Ewing's sarcoma (ES) (A4573). Phase contrast appearance after Bt_2cAMP treatment. Neural-appearing cells with short branching processes are evident. ×100. **D** ES (Tc-106). Phase contrast appearance after differentiation with Bt_2cAMP. Numerous stellate neuritic processes interconnect neural-appearing cell bodies. ×100

The first ES cell line assayed was A-4573. This line grows in a purely "epithelioid" pattern when maintained in RPMI 1640 supplemented with 20% FBS. No neurite-like processes were seen. In serum-free conditions, no neural differentiation was observed; the cells kept their epithelioid features. Increased substrate adherence was detected, as well as enlargement of the cell bodies. In contrast, when we treated the cell line with Bt_2cAMP, there was a change in morphology with the appearance of phase dense cell bodies which were less substrate adherent and displayed numerous neuritic interdigitating processes with varicosities (Fig. 1C).

The second ES cell line (Tc-106) was poorly adherent. It grew in suspension (at least initially) with standard media (RPMI 1640+20% FBS). In serum-free conditions, there was a tendency for cell adhesion to substrate associated to a decrease in cell growth. Only very few short interconnected cell processes were detected. After treatment of this cell line with Bt_2cAMP, numerous phase dense cell bodies were noted interconnected in a stellate fashion by abundant neuritic processes. These neurites were typically unbranched and displayed one or several small varicosities which terminated on other neurites with cones (Fig. 1D).

We used two other tumour cell lines as negative controls. One was of epithelial origin (CC) and the other was a tumour cell line derived from an HOS. The CC cell line was characterized by the presence of clusters of epithelial cells, with gland and lumina formation. Growth in serum-free conditions, with or without Bt_2cAMP, failed to produce neurites or any type of neural differentiation. HOS was composed of a mixed pattern of cells: spindle-shaped, multinucleated giant cells and epithelioid cells. There were no changes in morphology when we used serum-free conditions or treatment with Bt_2cAMP.

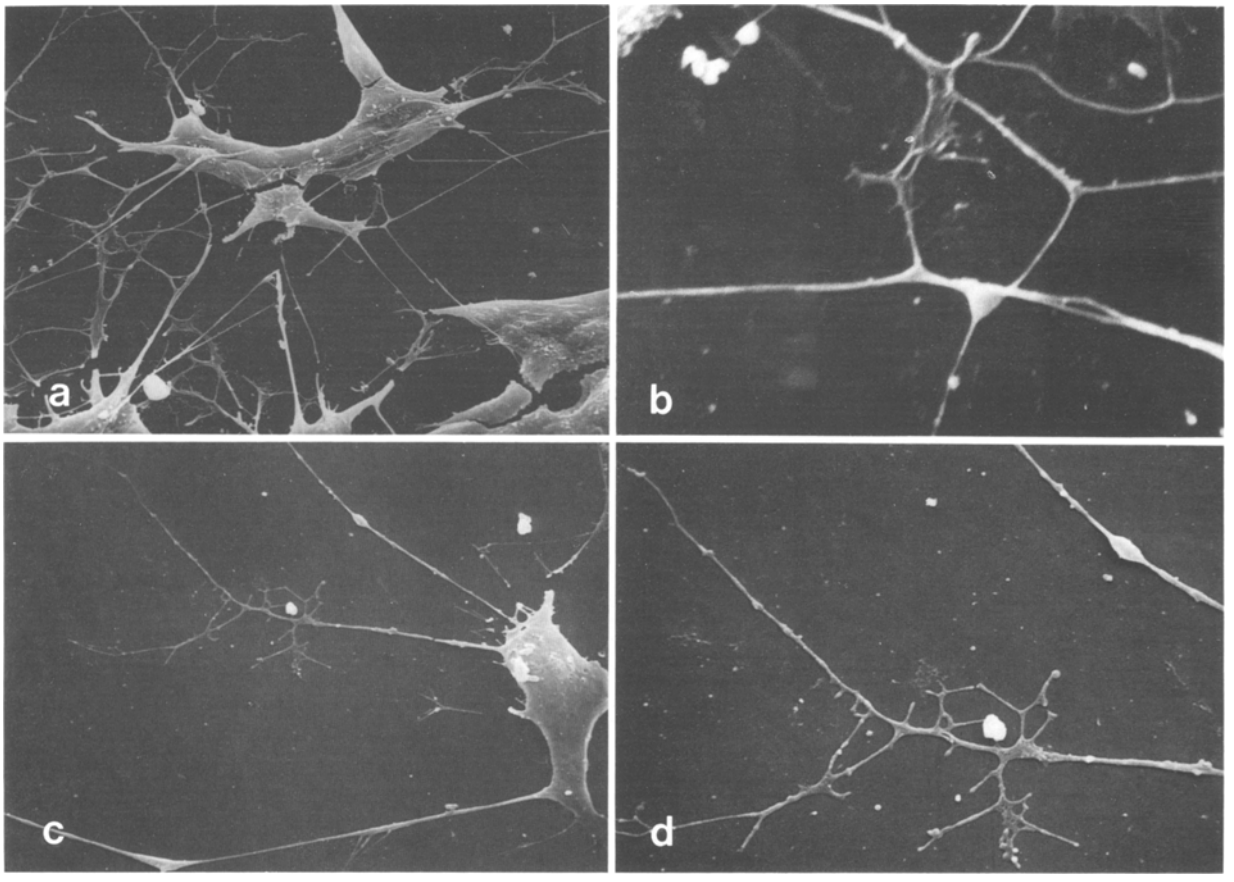

Fig. 2A–D. Scanning electron microscopy (SEM). **A** Differentiated (Bt_2cAMP) KCNR by SEM. After differentiation, innumerable unbranched neurites are seen. ×640. **B** Detail of A, upper left. Note varicosities with adherent boutons. Filopodia are not seen. ×1250. **C** SEM of differentiated PN (Bt_2cAMP). Both branched neurites with filopodia (*middle*) and unbranched neurites with varicosities (*top and bottom*) were found after differentiation. ×1250. **D** Detail of C. Both branched neurites with filopodia (*bottom*) and unbranched neurites with varicosities (*top*) are apparent. The calibre of the former is less uniform than that of the latter. ×2500

Scanning electron microscopy

The surface ultrastructural analysis of the NB cell line (KCNR), growing in standard media (RPMI 1640+20% FBS), showed groups of round to polygonal cells forming poorly adherent colonies; the cells extended isolated long, slender processes without varicosities but with occasional interconnected branches. Few interconnections between neighbouring cell colonies were found. Under serum-free conditions, we observed a change of morphology in the cells, characterized by the appearance of both substrate-adherent cells and cells with thick cytoplasmic processes; these processes were long, unbranched, with varicosities. No increase in the number or size of neurites was observed. When this cell line was treated with Bt_2cAMP, a remarkable increase in the number of cell colonies was observed, as well as a marked "fusion" (close approximation) of cell bodies (Fig. 2A). Numerous long, thin interconnected neurites were formed with widespread bundle branch recruitment, but with few varicosities. Characteristically, some neural branches emerged at right angles and interconnected haphazardly with each other (Fig. 2B), thereby forming an intricate complex of thin "mesh-like" complexes joining neighbouring cell colonies (Fig. 2A).

At the SEM level, the Tc-135 PN cell line grown in RPMI 1640+20% FBS was composed of two different cell populations: one was "epithelioid" with large bodies and short, thick filopodia. The second type of cell was "neuroblastic", characterized by dense, round to oval bodies and interconnected short, thin processes lacking varicosities.

After culture under serum-free conditions, we detected an increase in the "epithelioid" popula-

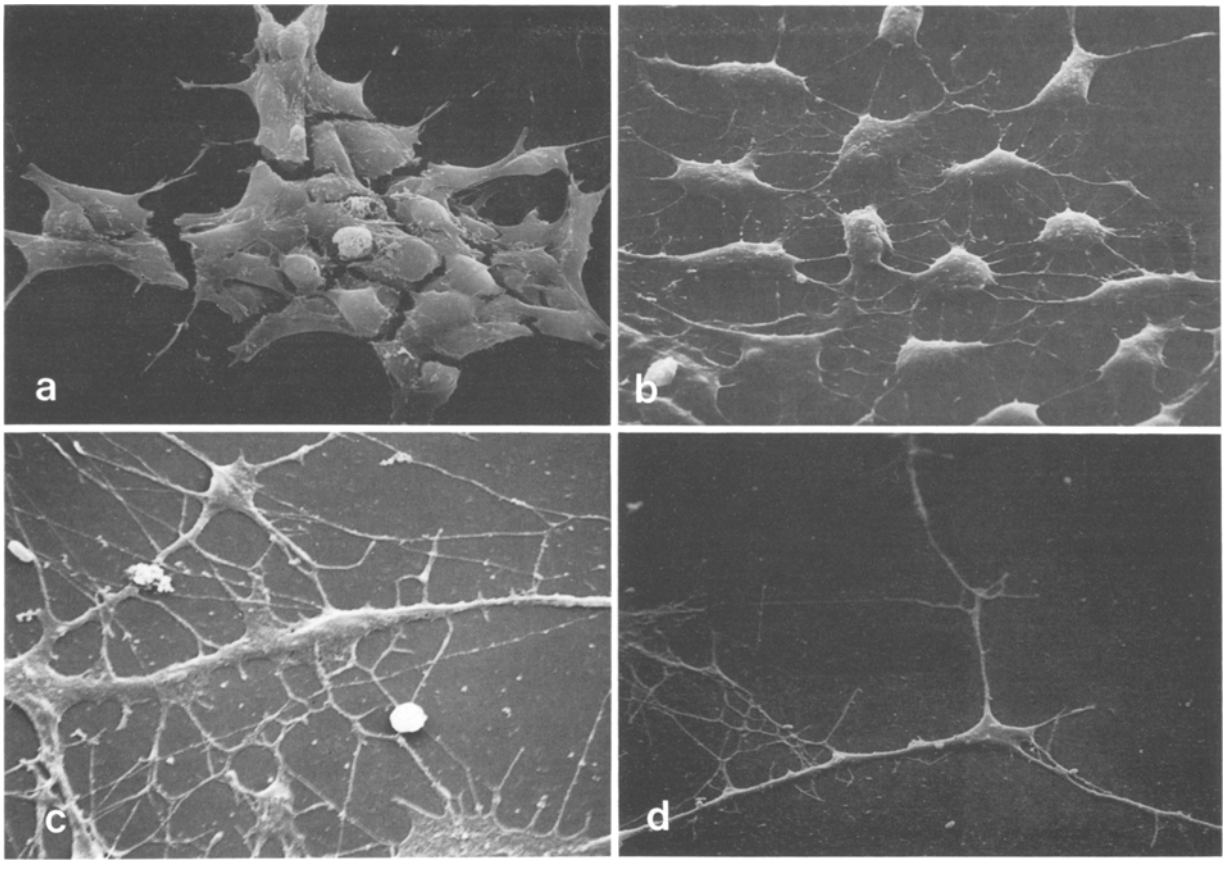

Fig. 3A–D. SEM of ES (A-4573). **A** SEM of untreated cells. Note the epithelioid appearance devoid of neural processes of any type. ×640. **B** SEM of differentiated cells. Cells are separated from one another (compare with B) and intervening spaces are filled with innumerable branching cell to cell processes. ×640. **C, D** An intricate meshwork of long and short projections, often at right angles to one another, is conspicuous. The calibre is highly variable. ×1250

tion of cells, which also displayed considerable substrate adherence by means of numerous thick filopodia. The other cell type (i.e. neuroblastic) was morphologically unchanged. After treatment with Bt_2cAMP, the neuroblastic cell type was increased. These cells showed isolated long, thin processes with some varicosities (Fig. 2C). We were able to see junctions as areas between processes of different cells. Another type of cell process was occasionally seen; this type was characterized by long, thin cytoplasmic extensions, without varicosities, showing short anastomosing branches (Fig. 2D).

We have analysed two ES cell lines by SEM: the first (A 4573) was composed of groups of homogeneous cells adherent to the substrate by means of short filopodia. The cells were round to ovoid, showing smooth surfaces and occasional cell to cell attachments (Fig. 3A). No obvious neuritic processes were observed under these conditions, though careful examination of numerous scanning electron micrographs revealed rare examples of such processes. It should be emphasized that these were not detected by phase contrast microscopy, and were exceedingly rare by SEM. Under serum-free conditions considerable enlargement (secondary to attenuation) and flattening of cell bodies with more apparent cell to cell attachment were noted, but no cell processes were detected (not illustrated). Under serum-free conditions and after treatment with Bt_2cAMP, a spectacular change in morphology was found. The cells appeared flattened with innumerable cell to cell processes (Fig. 3B). These processes formed an intricate system of long and short projections often emerging at right angles. These mesh-like complexes interconnected individual cells (Fig. 3C). In fact, clusters of cells forming colonies became inapparent and were replaced by individual cells separated by these intricate mesh works of neuritic processes. Furthermore, isolated thick processes tended to form varicosities especially at sites of attachment between two or more processes (Fig. 3D).

The second ES cell line (Tc-106), using stan-

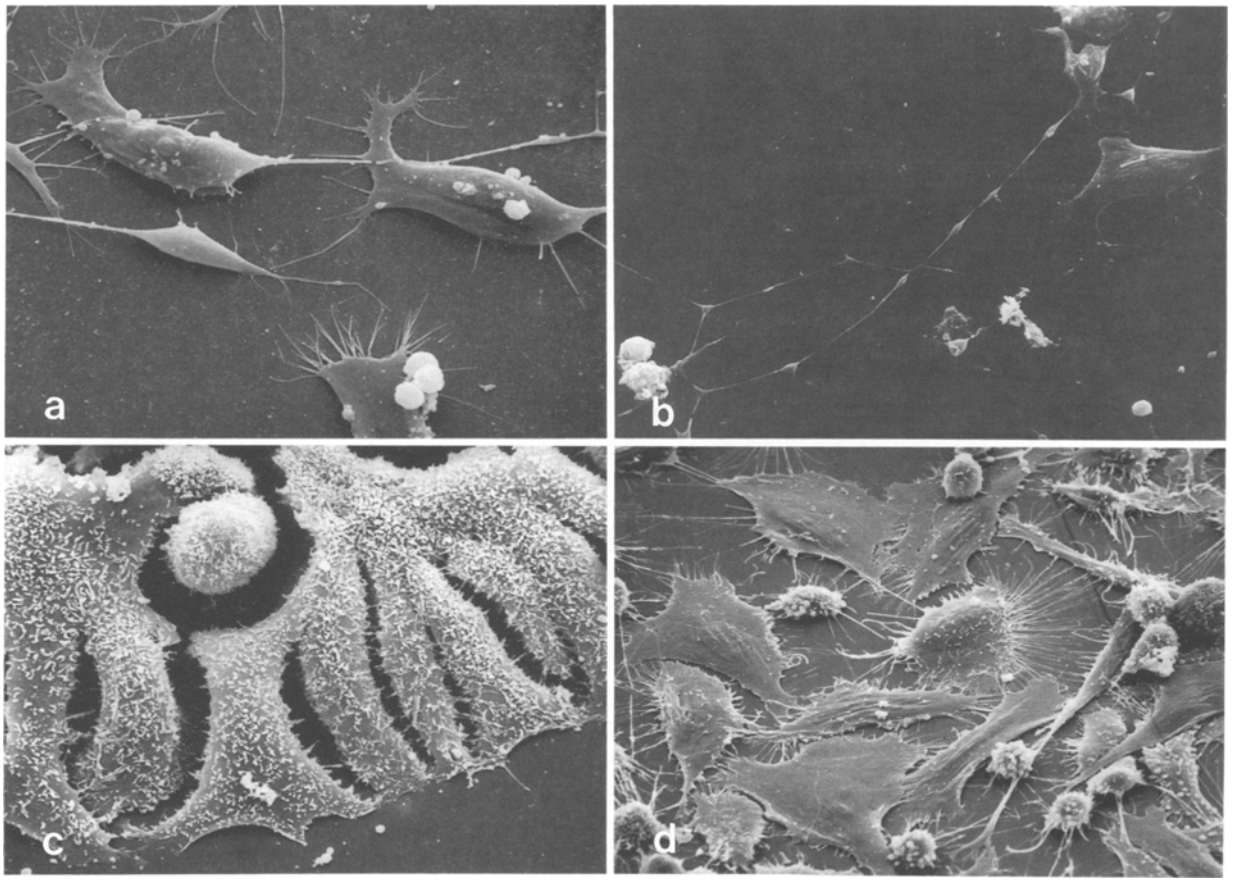

Fig. 4A–D. SEM of ES (Tc-106) compared with negative control. **A** SEM appearance of undifferentiated Tc-106. Although not evident by phase contrast (not shown), undifferentiated cells displayed occasional neuritic processes and numerous filopodia. ×640. **B** SEM appearance after Bt_2cAMP-induced differentiation. Long, thin neuritic processes extending at right angles from cell bodies have replaced the formerly numerous filopodia, now absent; only one or two per cell were observed, often at opposite poles. Numerous varicosities along each neurite were routine. ×640. **C** Colon carcinoma. Clusters of epithelioid cells with innumerable microvilli and cell to cell attachments, arranged in gland-like structures, were uniformly present, regardless of Bt_2cAMP treatment. ×640. **D** Osteosarcoma. Heterogeneous, fibroblastic appearing cells with innumerable filopodia were uniformly present, before and after differentiation. Bt_2cAMP had no apparent effect. ×640

dard media, grew initially after plating in a suspension pattern, adopting a "morula-like" formation of round to oval cells without any kind of cell processes (not illustrated). After several hours of culture in the presence of fetal bovine serum, cell-substrate attachments by means of short filopodia were noted (Fig. 4A). Under serum-free conditions, the cells appeared more flattened and showed short cell to cell processes (data not shown). After the addition of Bt_2cAMP, the cells developed a strikingly neural morphology. Individual cells extended one or a few extremely elongated, fine calibre, unbranched processes (neurites) with periodic varicosities (Fig. 4B). These protruded from the cell body at right angles, forming apparent growth cones. Even rare bipolar cells were noted. Occasional lateral attachments from other neurites appeared to form structures mimicking synapses (Fig. 4B).

At the SEM level, both negative control cell lines were strikingly different from any of the above cell lines, and were in fact easily distinguishable from one another. The HOS cell line was heterogeneous, composed of at least three cell types, i.e. round cells, spindle cells, and flattened giant cells which showed a considerable number of filopodia adhered to the substrate. No effect on morphology was detected under serum-free conditions or after Bt_2cAMP treatment (Fig. 4D). The epithelial cell line (CC) was characterized by the presence of clusters of cells forming gland-like structures with occasional lumina. The tumour cells presented abundant surface microvilli and few cell to cell attachments (Fig. 4C). Under serum-free con-

ditions or after addition of Bt_2cAMP, no visible effects on morphology were detected; the cells retained their epithelial glandular appearance.

Discussion

Histologically, ES, NB, RMS and Lym together form the group of so-called small round cell tumours of childhood (Triche and Askin 1983; Triche et al. 1986). In the past decade this family has grown to include additional entities, with the widespread recognition of PN of soft tissue (PN) and the malignant tumour of thoracopulmonary region (Askin's tumour) (Askin et al. 1979).

Two main problems must be considered regarding these neoplasms, particularly in reference to the most undifferentiated variants. The first pertains to the differential diagnosis when the pathologist examines the specimens solely by routine techniques. The second problem exists because of the unknown origin of some of these tumours, in particular ES. Tissue culture (short or long term) (Cavazzana et al. 1987b; Navaro-Fos 1987), has become a technique to alleviate both these problems in selected cases, not only because of distinctive morphological results, but also because this method alone can provide enough material (potentially unlimited) to perform other investigations in molecular genetics, cytogenetics, and immunohistochemistry, for example.

The most well known and widely studied tumour within this group is NB, a unique neoplasm of neural crest origin and one of the most common solid tumours of childhood. Its capacity to differentiate and undergo maturation both in vivo and in vitro is well known, and several studies have been performed assessing the capacity for differentiation in vitro using NB tumour cell lines (Prasad and Kuman 1975; Tsokos et al. 1987a; Gross et al. 1987; Rupniak et al. 1984; Prasad and Resie 1971).

The maturation effect of serum-free medium (Bottenstein et al. 1979) or of several agents such as Bt_2cAMP and retinoic acid are well recognized. Morphological changes are readily detected by phase contrast microscopy, SEM and TEM (Tsokos et al. 1987a; Gross et al. 1987; Rupniak et al. 1984; Prasad and Resie 1971; Sidell 1981). Furthermore, several morphological analyses have demonstrated that these agents may induce neurite out-growth (Prasad and Kuman 1975; Tsokos et al. 1987a; Sidell 1981). Moreover, it has been shown that these agents simultaneously induce several biochemical changes (i.e. increased synthesis of neurotransmitter enzymes) (Rupniak et al. 1984) and biological events such as decreased expression of N-myc oncogene (Thiele et al. 1985; Amatruda et al. 1985) and immunological modifications such as the down-modulation of antigenic expression, particularly of NB-associated 5A7 or Leu 7 antigens (Gross et al. 1987). Also changes in the synthesis and expression of the extracellular matrix proteins have been described in NB and other round cell tumour lines treated with Bt_2cAMP or retinoic acid (Tsokos et al. 1987c).

Our purpose in using a well characterized NB cell line (KCNR) was to confirm already published morphological results (Reynolds et al. 1980) and therefore to use this cell line as a positive control in our assays of cell differentiation for other poorly differentiated small round cell, presumably neural, tumours (PN and ES). We showed that Bt_2cAMP induces a remarkable neurite outgrowth in these cells which is easily recognized both by phase contrast and SEM.

PN is a member of this small round cell tumour family and is presumed to be of neural crest derivation. Although this neoplasm shares some common characteristics with NB, such as neuron specific enolase (NSE) immunostaining or neurosecretory granules and rosettes by TEM (Triche and Askin 1983; Triche et al. 1986), there are several clinical features that differentiate these two entities, such as age, location and prognosis (Triche and Askin 1983; Triche et al. 1986; Yunis 1987).

Significant controversy has existed regarding whether ES and PN are closely related, if not the same tumour. The two tumours share a common cytogenetic abnormality (the chromosome 11:22 reciprocal translocation) (Whang-Peng et al. 1984; Aurias et al. 1984; Turc-Carell et al. 1984), display a similar pattern of oncogene expression (c-myc overexpression, no N-myc expression) (Israel et al. 1985) and have a virtually identical immunophenotype as determined by monoclonal antibodies (Donner et al. 1985). Conversely, ES is generally regarded as being devoid of morphological evidence of neural differentiation, and molecular genetic differences have also been described (Vecchio et al. 1989).

Contradictory results have been published regarding the action of Bt_2cAMP on PN cell lines. While Jaffe et al. (1984) could not find any morphological change in their four cell cultures of PNET Primitive Neuroectodermal Tumour of bone (possibly the nearest relative of PN), Tsokos et al. (1987) proved that Bt_2cAMP differentiated several cell lines of PN, though to a lesser degree than NB studied in parallel.

The present light and electron microscopic analysis provides further support for the differen-

tiating capacity of Bt_2cAMP on neural or potentially neural cells; it induces true neural differentiation in our cell lines of known and presumed neural character. Both PN and ES underwent striking neural differentiation when grown on plastic substrate in serum-free conditions and in the presence of Bt_2cAMP. This differentiation was in one case virtually indistinguishable from NB, though in most cases the neuritic processes tended to branch and form a meshwork of interwoven, shorter processes in PN and ES. There was no significant difference in the character of this neural differentiation between PN and ES. Generally speaking, however, the phase contrast appearance of Ewing's cells grown in normal growth medium was decidedly non-neural in appearance, unlike PN cells grown under identical conditions.

ES is the most controversial tumour in the group of small round cell tumours, because of its unclear histogenesis. This point is emphasized by the heterogeneity in the appearance of this presumed sarcoma and the presence of atypical variants which have suggested the possibility of diverse origins (Llombart-Bosch et al. 1986, 1978, 1980; Friedman and Gold 1968). Recently a number of papers have been published supporting a neuroectodermal origin for this neoplasm (Schmidt et al. 1982; Jaffe et al. 1984; Cavazzana et al. 1987c, 1988; Llombart-Bosch et al. 1986, 1987; Yunis 1986), while others have documented certain neural phenotypic traits (NFTP, NSE) while claiming heterogenetic pluripotential differentiation, based on keratin expression (Moll et al. 1987). Keratin expression, however, is well documented in known neuroectodermal tumours (Cavazzana et al. 1987c). In 1987 Cavazzana et al. reported five cell lines of ES that developed true neural differentiation after being treated with Bt_2cAMP. This neural differentiation in vitro was demonstrated by phase contrast microscopy and TEM, as well as by the expression of several neural markers (NSE, NFTP) by immunofluorescence techniques.

In this analysis we have studied two of the five Ewing's cell lines used by Cavazzana, along with analogous differentiation agents and conditions in vitro. We have further characterized the resultant neural differentiation by SEM. The use of this more discriminating morphological technique is necessary to confirm that the induced cell process outgrowth was actually neuritic in nature. The present study clearly demonstrates the neuritic character of these processes, including growth cones, varicosities, and terminal (? synaptic) boutons. Furthermore, we detected morphological diversity in the neural differentiation induced in each Ewing's cell line. The A-4573 Ewing's line presented a "neural" pattern similar to that observed in Tc-135 (PN), while Tc-106 Ewing's line more closely resembled KCNR (NB). This degree and complexity of neural differentiation has not been previously documented or reported.

Although a neural-like differentiation effect of cAMP on non-neural cells has been reported (Borman et al. 1985; Dubpernell and Gavurin 1978; Tienari et al. 1987), we found no such effect on our non-neural control cell lines, CC and HOS. In contrast, our results on ES cell lines confirmed the true neural differentiation induced by Bt_2cAMP using a sensitive, high-resolution morphological technique (i.e. SEM), in comparison with the immunocytochemical detection of neural markers already published (Cavazzana et al. 1987c, 1988). Despite the fact that molecular genetics studies (Rb gene analysis) may provide more specific information regarding the relationship between ES and other childhood tumours (Weinberg 1989), the morphological data presented here provide further evidence for a solely neural character for the ES lines studied. Marked similarity to PN was documented. These findings support other reports indicating a neural origin for ES.

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