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Differentiation of a Ewing's Sarcoma Cell Line towards Neural and Mesenchymal Cell Lineages

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Two different pathways of differentiation were investigated in Ewing's sarcoma (ES) cell line, designated CADO-ES1, which has been established in our laboratory. This cell line was induced to differentiate and display a neural phenotype when treated with dibutyryl cyclic adenosine monophosphate or when cultured in serum-free medium (HB101). In these *in vitro* differentiation studies, two different phenotypes were demonstrated by light and electron microscopy. One phenotype, present in a major portion of the cell population, had long neurites in which microtubules were ultrastructurally demonstrated. The other one, present in a minor portion of the cell population, consisted of flat cells with many short processes. After differentiation in serum-free medium, tumorigenicity in nude mice or colony-forming efficiency in soft agar was strongly depressed. In the cells, N-*myc*, c-*fos* and c-*src* genes were not amplified, and although c-*myc* was amplified by up to 2-fold, depending on the culture conditions, this appeared to be unrelated to the changes of phenotype. When tumor cells were transplanted into nude mice, cartilage was formed. The cartilage was immunoreactive with the antibody for HLA-ABC, indicating that it was derived from the tumor cells, not from mouse tissue.

Key words: Ewing's sarcoma cell line — Neural differentiation — Chondrocytic differentiation

Ewing's sarcoma (ES) cells have been described as either very early pluripotential cells[1] or primitive neuroectodermal cells[2,3] that can differentiate along neuronal, glial, Schwannian, melanocytic, neuroendocrine, or even ectomesenchymal pathways.[4] We previously reported the establishment and characterization of an ES cell line (CADO-ES1) with a unique karyotype, derived from the malignant pleural effusion of a 19-year-old woman.[5] Briefly, these cells grew partly suspended and partly attached to tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere with 5% CO_2 at 37°C. In karyotype analysis, the chromosome number was 47. An isodicentric chromosome 8, idic[8][p11.2], was observed in every cell analyzed. In the present studies, we examined the pathways of differentiation of this cell line.

After cloning of the cell line, chemical differentiation was induced by addition of 0.1 m*M* dibutyryl cyclic adenosine monophosphate (dBcAMP). However, serum-free medium, HB101 (Hana Media, Inc., USA) was used to observe the effect of culture conditions on differentiation. The HB101 medium includes bovine serum albumin, bovine insulin, human transferrin and ethanolamine in proper concentrations. Colony-forming efficiency in soft agar, tumorigenicity in athymic mice (CD1 nu/nu, Charles River Japan, Inc., Atsugi), and c-*myc*, N-*myc*, c-*fos* and c-*src* gene amplifications were examined before and after treatment with dBcAMP or HB101 medium.

For transmission electron microscopy, cultured cells were fixed in 2.5% sodium phosphate-buffered glutaraldehyde solution containing 0.2% tannic acid (0.1 *M*, pH 7.4) and postfixed in 1% sodium phosphate-buffered osmium tetroxide solution (0.1 *M*, pH 7.4) prior to Epon embedding. Thin sections were contrasted with uranyl acetate and lead citrate. Electron photomicrographs were taken with a JEM-100CX (JEOL) transmission electron microscope at 80 kV.

A suspension of 4×10^6 CADO-ES1 cells in 0.2 ml of medium was injected subcutaneously in the backs of six-week-old male athymic mice (CD1 nu/nu). The cell inocula were allowed to grow until palpable tumors were formed. The animals were then killed. A part of the tumor tissue was processed for routine light microscopic examinations. To prepare frozen sections for immunohistochemical examination, the xenotransplanted tumor tissue was immediately frozen in isopentane and liquid nitrogen, and stored at −80°C. Five-μm-thick cryostat sections were air-dried and fixed in acetone at 4°C for 10 min. Monoclonal antibody to HLA class 1 antigen (working dilution 1/25 to 1/50; Immunotech. S.A., France) was used. The frozen section was incubated with biotinylated anti-HLA class 1 antibody. After three

washes with phosphate-buffered saline (PBS), the section was incubated with avidin-peroxidase complex. The chromogen was 3,3′-diaminobenzidine.

DNA was isolated from CADO-ES1 cells before and after treatment with dBcAMP or HB101 medium, and from normal human placenta. DNA was digested with *Eco*RI restriction endonuclease, and fractionated on a 0.7% agarose gel. The numbers of copies of c-*myc* and N-*myc* genes were determined by Southern blot analysis as reported previously.[6] Hybridization was performed using a DNA Labelling Kit (Boehringer, Germany). The intensities of c-*myc* and N-*myc* amplification were quantified by a nonradioactive digoxigenin (DIG) Luminescent Detection Kit (Boehringer). c-*fos* and c-*src* gene probes were labeled with ^{32}P, and hybridized with samples which were fixed by the slot blotting method. Hybridization signals were determined after 12–24 h of exposure to Kodak X-ray film.

As described previously,[5] CADO-ES1 cells in control cultures displayed anchorage-dependent growth in tissue culture flasks and anchorage-independent growth in bacteriologic plates. These small, round, or spiny-shaped, poorly differentiated cells were positive for vimentin. When these cells were cultured in the presence of 0.1 m*M* dBcAMP in tissue culture flasks, most of them changed into spindle-shaped or elongated cells with very long branched neurites. Serum-free medium (HB101) also promoted morphological differentiation with very long branched neurites. These changes were observed within 24 h after treatment. In contrast, flat cells with many filiform processes were rarely observed during cultivation in HB101. Similar phenomena were observed after recloning of the cell line. Interchange between the cells with neurites and flat cells was not observed. The concentration of dBcAMP did not affect the proportion of differentiated cells.

Ultrastructurally, the small round cells had many short processes with glycogen deposits. There were very few cytoplasmic organelles and a poorly developed endoplasmic reticulum (Fig. 1A). Most differentiated cells had one or more elongated cellular processes containing a number of microtubules in orderly alignment (Fig. 1B). However, the flat cells had many short flat processes without microtubules (Fig. 1C).

The relationships between tumorigenicity or gene amplification and cell differentiation were examined. As shown in Table I, colony-forming efficiency in soft agar was markedly reduced in dBcAMP-treated cells and was diminished in cells cultured in HB101 medium. Tumori-

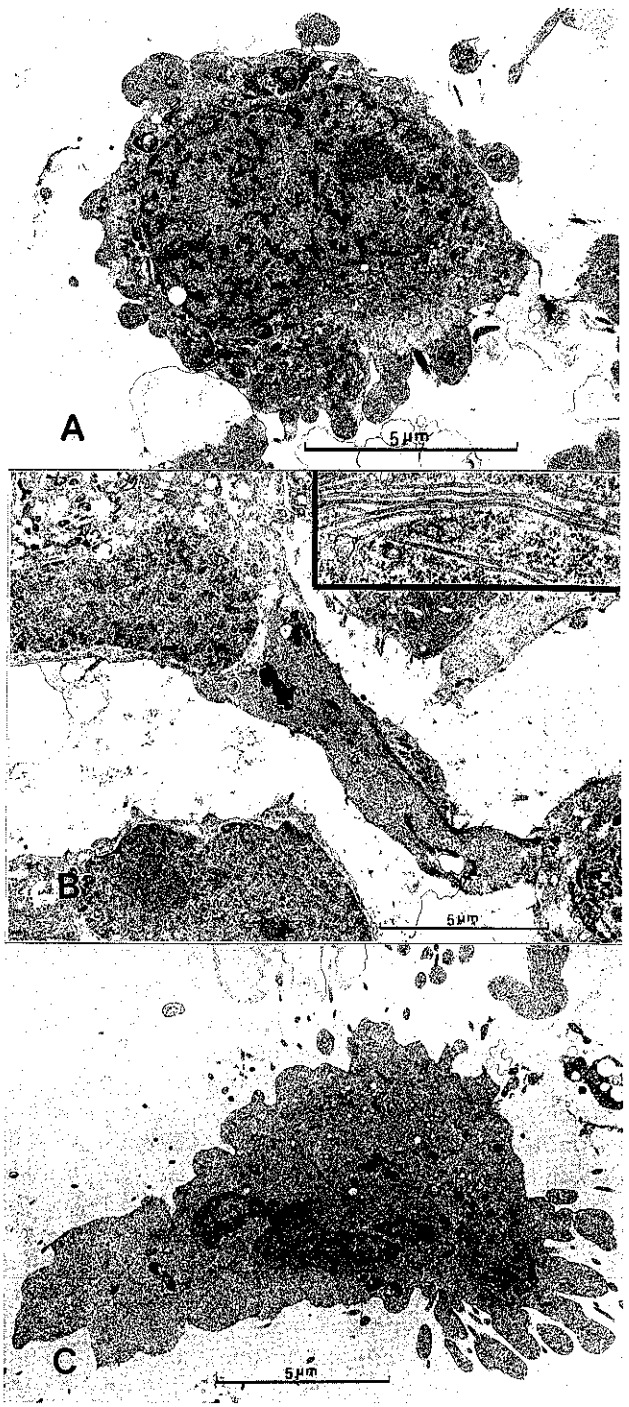

Fig. 1. Ultrastructural appearance. A: Untreated cells; B: dBcAMP-treated cells (Inset: high magnification of the long processes exhibiting abundant microtubules); C: flat cells cultured in serum-free medium.

Table I. Growth Characteristics and Gene Amplification

	Colony-forming efficiency in soft agar[a]	Tumorigenicity in nude mice[b]	Gene amplification			
			N-*myc*	c-*myc*	c-*fos*	c-*src*
Control	3.0%	9/9	(−)	×2	(−)	(−)
+dBcAMP	0.2%	1/4	(−)	(−)	(−)	(−)
Serum-free	(−)	1/4	(−)	×2	(−)	(−)

a) Number of colonies (>60 cells)/number of cells plated ×100% at 21 days (mean, 3 determination).
b) Number of tumor takes/number of mice injected.

genicity in nude mice was reduced both in dBcAMP-treated cells and in cells cultured in HB101 medium. Two-fold c-*myc* amplification compared to normal human placenta was demonstrated in the original cells and differentiated cells after cultivation in HB101 medium. However, no c-*myc* amplification was recognized in dBcAMP-treated cells. N-*myc*, c-*fos* and c-*src* genes were not amplified in any of the three groups.

CADO-ES1 cells injected subcutaneously in athymic mice produced tumors. At less than six passages, the resultant xenografts were completely undifferentiated, and there was no cartilage formation. Hematoxylin-eosin staining of the formalin-fixed xenotransplanted tumors revealed a highly cellular nodule of uniform, small round cells. However, xenografts of CADO-ES1 cells formed a small amount of hyaline cartilage after 7 or more passages with reproducibility (Fig. 2A). The same cartilage was formed in the xenograft from the *in vitro* dBcAMP-treated cells. Our ES cell line did not include stromal cells. The tumor cells associated with cartilage showed positive Alcian blue and periodic acid-Schiff staining. The nuclei of the chondrocytes were pleomorphic and resembled those of the tumor cells. The cells in the cartilage were immunoreactive to the antibody for HLA-ABC as were the small round cells surrounding the cartilage (Fig. 2B). However, mouse-derived cells in the connective tissue around the tumor were not stained by the antibody. Thus, cartilage in the xenograft of CADO-ES1 cells is considered to arise from the tumor cells.

The present study investigated the differentiation capability of the CADO-ES1 line. Our experiments demonstrated that differentiated ES cells morphologically reverted when the dBcAMP was removed from the medium, as described for neuroblastoma.[7,8] This phenomenon was also demonstrated when HB101 medium was replaced with serum-supplemented medium.

In CADO-ES1 cells, the degree of c-*myc* oncogene amplification appeared heterogenous, and varied with the culture conditions. Based on these results, it may be inferred that CADO-ES1 cells *in vitro* display a variable neural phenotype with variable biologic responses to diverse culture media and various differentiation agents,

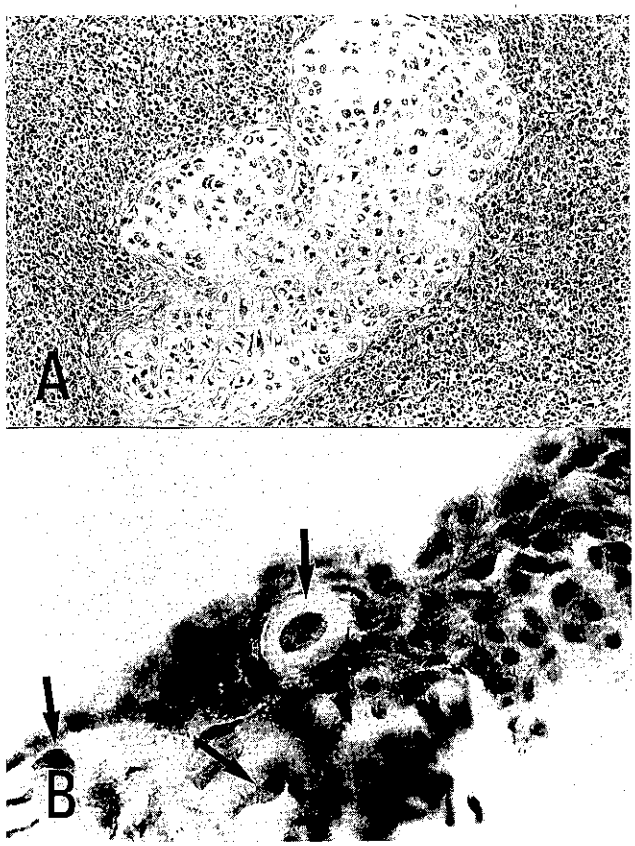

Fig. 2. A: Light microscopic appearance of xenograft in nude mouse (H-E stain, original magnification: ×40); B: immunohistochemical staining for HLA ABC of the xenograft (original magnification: ×200). Small round cells and the cells associated with hyaline cartilage (arrows) were positive.

but with no consistent relationship of c-*myc* gene amplification to the cell differentiation.

Recently, Goji *et al.*[9] described chondrocytic differentiation of the peripheral neuroectodermal tumor (PNET) cell line in nude mouse xenografts. They reported that their PNET cell line had reciprocal chromosomal translocation [t(11,22)]. Because of the absence of such

translocation in the CADO-ES1 line, there seems to be no direct relationship between the chromosomal change and chondrocytic differentiation.

In this study, it was suggested that pretreatment with dBcAMP or serum-free medium could decrease the tumorigenic ability in nude mice and/or soft agar, regardless of *myc* gene amplification. The demonstration that ES cells can be induced to differentiate and concomitantly lose their tumorigenic potential is important in determining whether differentiation therapies similar to those under investigation for other neoplasms[10] are feasible in ES.

In conclusion, CADO-ES1 cells arise from pluripotent cells capable of differentiating into both neural lineage *in vitro* and mesenchymal (chondrocytic) lineage *in vivo*. Our current observations may provide a link among the various small round cell tumors of bone, such as ES, PNET, small cell osteosarcoma, and mesenchymal chondrosarcoma.

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