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Characterization of Intermediate Filaments Expressed by Ewing Tumor Cell Lines

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ABSTRACT

The histogenesis of Ewing sarcoma is still controversial; we therefore studied the expression of intermediate filaments (IF) in cell lines derived from Ewing tumors since identification of IF in tumor cells is considered a reliable marker of tissue origin and differentiation. All nine lines studied expressed vimentin IF; in addition, a small number of Ewing cells from three lines expressed keratin filaments. After treatment with phorbol esters, a high percentage of cells from these three lines synthesize keratin IF identified by immunoblotting as keratin 8 and 18 polypeptides, which are expressed by single epithelia and epithelial cells in early embryonic development. Furthermore cells from a fourth line synthesize keratins after transplantation in nude mice. These data indicate that, under certain conditions, undifferentiated Ewing cells may acquire an IF phenotype related to that of epithelial cells.

INTRODUCTION

Ewing sarcoma is a rather rare, small round-cell undifferentiated tumor of bone and occasionally soft tissues which occurs in children and young adults. Because of the lack of unequivocal lineage marker, the histogenesis of Ewing sarcoma remains controversial (1). Since identification of IF[2] in tumor cells is now considered as a very useful marker of tissue differentiation for both normal and neoplastic cells, we study here the expression of IF polypeptides in cells from 9 malignant ETCL. Consistent with data obtained on fresh tumor cells (2), cells from each ETCL expressed vimentin IF, the polypeptide characteristic of mesenchymal cells *in vivo*. However, cells from 3 cell lines coexpressed keratin polypeptides; the expression of keratin was restricted to a small percentage of cells. After phorbol ester treatment, a striking number of cells expressed both keratin and vimentin. In addition, cells from a fourth cell line which lacked keratin synthesize this type of IF after transplantation and growth in nude mice. Altogether, these data show that, in certain ETCL, tumor cells have the capability of differentiation along an epithelial pathway.

MATERIALS AND METHODS

Cell Lines

ETCL were established at the International Agency for Cancer Research (EW1, EW3, EW7, EW12, EW13, EW16, EW17) and the Centre Léon Bérard (EW11), Lyon, France, from metastatic cells of different patients. Cytogenetic studies disclosed in all cases but EW3 a t(11,22)(q24;q12) translocation found to be specific of Ewing cells (Refs. 3 and 4; Footnote 3). These cell lines did not express neuron-specific enolase as assessed with a specific rabbit antiserum [kindly provided by F. de Vitry (5)], used in indirect peroxidase staining on cells fixed for 5 min in formaldehyde and permeabilized with acetone; positive control included a neuroblastoma cell line (IMR 32).

The cell lines were grown in RPMI medium supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics. Cultures were split at confluence by trypsinization. The EW3 cell line grew as tightly packed floating clusters, whereas the remaining cell lines grew as adherent monolayers. In some experiments, cell lines were exposed to 10 to 160 nM TPA for up to 6 days.

The EW1 cell line was cloned by limiting dilution: cells were thoroughly dissociated by trypsinization and filtration through a sharp needle and cultured at 0.3 cell per well in culture plates without feeder cells. The EW7 cell line (2×10^7 cells) was transplanted s.c. into nude mice, and 2 mo later the growing tumor was snap frozen before further studies.

Detection and Characterization of Intermediate Filaments

Immunoperoxidase and Immunofluorescence Staining. Adherent cells were cultured for 48 h on plastic tissue culture dishes before testing. The EW3 cell line, which grows in suspension, was studied after trypsinization of the clusters and smearing the cells on slides. Cells were thereafter fixed with 2% formaldehyde in PBS (pH 7) for 20 min and then permeabilized for 10 min in absolute methanol cooled at −20°C. In some experiments cells were simply fixed for 15 min in absolute ethanol and then studied for IF expression. Both fixations yielded similar results. Cryostat section of frozen EW7 tumor grown in nude mice was fixed in formaldehyde before study.

Vimentin IF were detected using a monoclonal human IgM_{DUV} the specificity of which had been previously reported (6). MAbs reactive with keratins included the Troma 1 antibody, which reacts with keratin 8 (Moll's classification) (7) of single epithelia (8–10); the KL1 antibody, which reacts with various keratins of simple or stratified epithelia (11); and A28-1 and D-21 antibodies, a kind gift of Dr. J. Kaddouche, Hôpital Saint-Louis, Paris, which react with stratified epithelia except the basal layer. MAbs to GFAP were produced by one of us (D. P.); they reacted with human neurofilaments or GFAP by immunofluorescence, immunoblotting, and immunoenzymatic tests.

All these monoclonal antibodies were studied by indirect staining using a second layer of fluorescein- or peroxidase-conjugated antibodies to human mouse or rat immunoglobulin (Institut Pasteur Production, Dacko Laboratories). For immunoperoxidase staining, the reaction was revealed by the diaminobenzidine method.

Characterization of IF Cytoskeletal Proteins by Immunoblotting. The IF cytoskeleton from the EW1 cell line was purified by Triton X-100 extraction. Briefly, confluent monolayers of EW1 grown in the presence or absence of TPA were washed in PBS, scraped with a rubber policeman, and centrifuged 5 min at 600 × *g*. The cells were extracted for 4 min with PBS-1% Triton X-100 containing 1 mM phenylmethysulfonyl fluoride and 3 mM EDTA and centrifuged 20 min at 3500 × *g*. The insoluble pellet was further extracted for 30 min in PBS-1% Triton X-100 containing 1.5 M KCl and centrifuged. Finally, after 2 washes in PBS-1% Triton X-100, the insoluble material was solubilized in 1% sodium dodecyl sulfate buffer and submitted to electrophoresis in 10% polyacrylamide gel according to the method of Laemmli (12). The proteins were thereafter transferred onto nitrocellulose sheets as described by Towbin (13) and revealed with monoclonal antibodies to vimentin or keratin (KL1 and Troma 1) using an indirect immunoperoxidase technique.

RESULTS

Expression of IF Cytoskeletal Protein by ETCL. Cells from each ETCL exhibited a distinct vimentin network (Fig. 1*A*). No

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[2] The abbreviations used are: IF, intermediate filaments, ETCL, Ewing tumor cell line(s); TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PBS, phosphate-buffered saline; MAb, monoclonal antibody; GFAP, glial (neuro)filament triplet or acidic protein.

[3] Unpublished data.

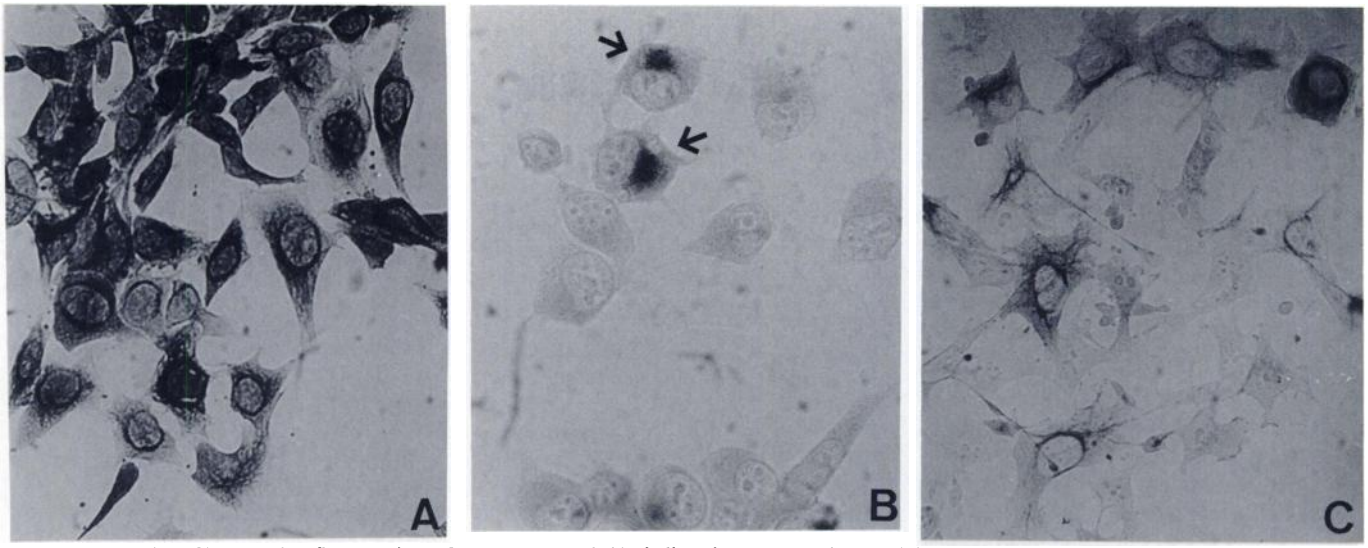

Fig. 1. Expression of intermediate filaments in ETCL. IF were revealed by indirect immunoperoxidase staining on cells fixed with formaldehyde and permeabilized in absolute methanol. *A*, vimentin expression by cells from the EW1 cell line. Note that all cells are positive. A similar pattern was observed for each Ewing cell line. *B*, keratin expression by EW1 cells. Note that a minority of cells express a small filamentous bundle (→). A similar pattern was observed in the EW3 and EW16 lines. *C*, expression of keratins by EW1 cells after treatment with phorbol esters. Note that nearly 30% of cells now synthesize keratins.

reactivity was observed with MAb to neurofilaments or GFAP. The antikeratin antibodies Troma 1 and KL1 stained a small percentage of cells in EW1, EW3, and EW16 cell lines. Keratin staining usually appeared as a small filamentous bundle (Fig. 1*B*) clearly distinct from the well-developed vimentin network which extended all over the cytoplasm. Immunofluorescence double-staining experiments showed that the keratin-positive cells also expressed vimentin. Of note, cells from all ETCL were unreactive with MAb to keratins specifically detected in stratified epithelia.

Keratin positive cells accounted for 1 to 5% of the cells, and this figure remained similar whether the cells were tested at low cell density or at confluence. One ETCL (EW1) was cloned by limiting dilution; 22 of the 23 subclones obtained behaved exactly like the parental cell line with regard to the keratin expression. In the last subclone, up to 40% of the cells expressed keratin IF. The number of positive cells, however, decreased progressively during culture, and after a few wk, the fraction of keratin-positive cells was reduced under 5%. Cytogenetic studies performed when nearly half the cells of the subclone expressed keratin showed the persistence in all mitoses studied of the characteristic t(11, 22) translocation of the parental line.

Effect of TPA on Keratin Expression in ETCL. Since TPA has been shown to affect keratin expression in several differentiation models (14, 15), we studied its effect on ETCL. Under TPA, the vimentin network was still present, although it appeared retracted in large bundles of filaments. Interestingly, there was a striking increase in keratin-positive cells in EW1, EW3, and EW16 cell lines. The percentage of positive cells reached 20 to 40%, and the keratin network was quite rich, extending throughout the whole cytoplasm (Fig. 1*C*). The effect of TPA was optimal at a concentration of 10 to 30 nM and detectable as soon as 24 h after TPA exposure. TPA had no effect on keratin expression in the other ETCL.

Characterization of IF Skeletal Protein in the EW1 Cell Line. The IF cytoskeletal protein of the EW1 cell line was analyzed by immunoblotting using antivimentin or antikeratin reagents. Only the M_r 56,000 vimentin polypeptide was detected in untreated EW1 cytoskeletal extracts, whereas extracts from TPA-treated cells contained both vimentin and keratin. As expected from its specificity, Troma 1 MAb stained a single band with a molecular weight of 52,000. KL1 MAb stained the same M_r 52,000 protein and also a very faint band with a molecular weight of approximately 45,000 (Fig. 2). No high-molecular-weight keratins could be detected with this antibody.

Expression of Keratin in the EW7 Cell Line Transplanted into Nude Mice. The EW7 cell line did not express detectable keratin IF either spontaneously or after TPA treatment. However, after transplantation and growth in nude mice, the tumoral cells expressed both vimentin and Troma 1 or KL1 reactive keratins

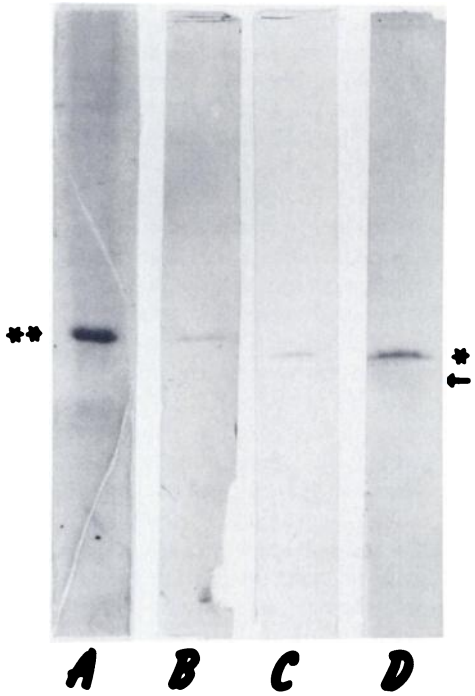

Fig. 2. Characterization of keratin intermediate filaments in the phorbol ester-treated EW1 cell line. *Lane A*, cytoskeletal extract of EW1 cells run on a 10% polyacrylamide gel and stained with Coomassie blue. Note the prominent M_r 56,000 protein (**). *Lanes B* to *D*, cytoskeletal extract of EW1 cells, transferred onto a nitrocellulose sheet. Immunostaining with antivimentin antibody reveals a M_r 56,000 polypeptide (*Lane B*); with Troma 1 MAb, a M_r 52,000 keratin band (*Lane C*); and with KL1 MAb, both M_r 52,000 (*) and M_r 45,000 (←) keratin polypeptide (*Lane D*).

as shown by immunoperoxidase staining of cryostat sections of the tumor.

DISCUSSION

The cytoskeleton of nucleated cells is composed of 3 main filamentous structures: microfilaments, microtubules, and IF. This latter network is heterogeneous with regard to its constitutive proteins and can be biochemically and immunologically individualized into 5 types: keratins occur in epithelial cells, vimentin in fibroblasts and mesenchymatous cells, desmin in muscle cells, neurofilaments in neural cells and GFAP in astrocytes (reviewed in Ref. 16). Since the expression of IF proteins is retained after malignant transformations, the immunotyping of IF has proven to be a valuable tool for the diagnosis of human malignant tumors (17, 18). Since the origin of tumor cells from Ewing sarcoma is still controversial, we studied the expression of IF polypeptides in 9 ETCL. Most cells from each line expressed vimentin, a common finding for cultured cells whatever their tissue origin. Vimentin is the single IF observed in fresh Ewing tumor cells (2). Unexpectedly, in 3 of 9 lines, a small percentage of cells expressed both vimentin and keratin polypeptides. Under TPA exposure, a high percentage of cells from the same 3 lines developed a rich reseau of keratins while retaining vimentin expression. No keratin IF were detected after TPA treatment of cells from the 6 other ETCL which did not contain any keratin-positive cells. However, cells from one such line (EW7) developed a rich keratin network after transplantation and growth in nude mice. Other classes of IF such as neurofilaments of GFAP were not detected in ETCL cells even after TPA treatment.

The early appearance of numerous keratin-positive cells under TPA, the detection of keratin-expressing cells in all subclones of the EW1 cell line, and cytogenetic data rule out the possibility that some lines may be contaminated with epithelial cells and support the hypothesis that the expression of keratins follows differentiation events induced by TPA or transplantation in nude mice. With regard to IF expression, it is of interest that TPA has been previously shown to induce keratin synthesis in a human embryonic carcinomatous cell line and of keratin 8 in SV_{40}-transformed keratinocytes (14, 15). Of note, induced ETCL expressed selectively keratins having molecular weights of 52,000 and 45,000 which are detected by MAbs Troma 1 and KL1. These polypeptides correspond most likely to keratins 8 and 18, which are expressed by single epithelia and epithelial cells in early embryonic development as well as by a human embryonic carcinomatous cell line (7, 14, 19).

Our data indicate that Ewing cell lines can, under certain circumstances, acquire *in vivo* or *in vitro* an IF phenotype related to that of epithelial cells. On the other hand, the study of surface antigens of ETCL has shown their reactivity with some monoclonal antibodies directed to glycoconjugates present in the nervous system. These reagents included antibodies to the neuron cell adhesion molecule N-CAM, and the GD_2 ganglioside (20). In addition, cytogenetic study of neuroepithelioma, a neuroectodermal tumor, has revealed the t(11,22)(q24;q12) translocation found in Ewing sarcoma (21). These similarities led to the hypothesis that Ewing sarcoma and neuroepithelioma could both derive from related primitive neuroectodermal cells, the latter reaching a more differentiated stage as assessed, for instance, by the expression of neuron-specific enolase. In this respect, it is of interest that keratin IF were detected in one of 2 cases of neuroepithelioma we have tested.[4] It is therefore tempting to regard Ewing sarcoma and neuroepithelioma as tumors of related origins: both could derive from primitive neuroectodermal cells with discrete possibilities of differentiation towards more mature phenotypes having characteristics of epithelial or nervous cells. It is worth recalling here that cells from lung small cell carcinomas may express either keratin IF or neurofilaments (22) and that some neuroendocrine tumors may contain both cytokeratins and neurofilaments (23, 24).

Finally, we did not detect any keratin-expressing cell in the 2 fresh Ewing tumors that we could study. Additional data are clearly needed to establish whether expression of keratin IF can be observed or induced in fresh Ewing sarcomas that have not been adapted to *in vitro* propagation. These data may have diagnostic or prognostic significance, as in the case of other undifferentiated human tumors (25).

[4] Unpublished results.

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