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Teratocarcinoma stem cells as a model for differentiation in the mouse embryo

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ABSTRACT. Embryonal carcinoma (EC) cells, which are the malignant stem cells of teratocarcinomas, are considered similar to early embryo cells. The EC cells can be grown *in vitro*, and many of them can be experimentally induced to differentiate; upon differentiation, the cells become benign. Here we review some of the changes that take place in the cellular and molecular characteristics of murine F9 EC cells as they differentiate into endodermal cells. Upon differentiation of F9 cells, distinct changes occur in their cell surface molecules, cytoskeleton-associated proteins and cell adhesion properties. Simultaneously, the rate of cell proliferation decreases due to a dramatic increase in duration of G1 and S phases of the cell cycle. The changes in gene expression and cell behavior occurring during endodermal differentiation of EC cells closely resemble those occurring when the endoderm differentiates in the embryo. Teratocarcinoma stem cell lines may thus be exploited to enhance understanding of both teratoma-type neoplasms and embryonic development.

KEY WORDS: *Embryonal carcinoma, teratocarcinoma, differentiation, proliferation, cytoskeleton, surface markers, adhesion, lectins, cell cycle*

Malignant teratomas, or teratocarcinomas, are rare tumors reported in a variety of vertebrates. The stem cells of these tumors, the embryonal carcinoma (EC) cells, are thought of as equivalent to germ cells or early embryonic cells. The grounds for so believing, thoroughly discussed elsewhere (e.g., Graham, 1977; Hogan, 1977; Solter and Knowles, 1979; Gardner, 1983a; Silver *et al.* , 1983), fall into the following categories. First, the morphological, biochemical and immunochemical properties of EC cells are very similar to those of the inner cell mass (ICM) cells or the primitive ectoderm cells of the blastocyst-stage embryo. Second, EC cells can be derived, *in vivo* and *in vitro*, from early embryonic cells or germ cells. Third, the mode of differentiation of EC cells, both *in vitro* and in tumors *in vivo*, is very similar to that of the apparently corresponding cells in the embryo: EC cells can give rise to a variety of cell types, and the first progeny to differentiate in most cases is endodermal-type (END) cells. Fourth, the EC cells, introduced back into the early embryo, can take part in normal development and give rise to all differentiated types of cells in the embryo.

Most studies on the differentiation of EC cells *in vitro* have concerned their differentiation into endodermal cells. In the embryo, the first endoderm cells are called primitive endoderm; these then give rise to visceral and parietal endoderm. The conditions required for maintaining EC cells undifferentiated and, on the other hand, for inducing them to differentiate, vary from one EC cell line to another. Depending on the experiment, the endodermal cells differentiating from EC cells closely resemble one or the other type of endoderm (Graham, 1977; Martin *et al.*, 1977; Gardner, 1983a). As endodermal differentiation is characteristic of EC cells, they can be used to mimic this step in the development of the embryo. Furthermore, the differentiation is accompanied by a dramatic decrease in the tumorigenicity and rate of proliferation of these cells (Solter *et al.*, 1979; Strickland and Sawey, 1980; Sherman *et al.*, 1981, Rayner and Graham, 1982); the EC-to-END differentiation thus resembles the mirror image of malignant transformation.

The EC cell line most widely used for studies on *in vitro* differentiation is the murine F9 cell line. These cells are derived from the transplantable, experimentally induced OTT6050 teratocarcinoma tumor (Bernstine *et al.*, 1973). The F9 cells originally gave rise to various differentiated types of cells, but the rate of spontaneous differentiation of these cells now seems to be very low. However, upon treatment with retinoic acid (RA), the F9 cells can be induced to give rise to endodermal-type cells in monolayer cultures (Strickland and Mahdavi, 1978). By various criteria, these cells have been shown to correspond to parietal endoderm cells. The differentiation of parietal endoderm-like cells can be intensified by adding dibutyryl cyclic AMP (dbc-AMP) to the culture medium (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980). Furthermore, F9 cells can be induced to differentiate into visceral endoderm-type cells by culturing them, in the presence of RA, on bacteriological grade dishes, on which the cells are not capable of adhering (Fig. 1, Hogan *et al.*, 1981, 1983; Strickland, 1981).

In the present paper we review the changes that occur in the lectin binding sites, cell adhesion characteristics, and organization of cytoskeleton-associated proteins when F9 cells undergo differentiation into endoderm-like cells *in vitro*. Furthermore, we review the data on the relationship between this differentiation and the rate of proliferation of F9 cells.

Expression of lectin binding sites in differentiating F9 cells

During differentiation of many teratocarcinoma cells, distinct changes occur in cell surface glycosylation and lectin binding sites (Gachelin *et al.*, 1976; Reisner *et al.*, 1977; Muramatsu *et al.*, 1979; Muramatsu and Muramatsu, 1982; Cummings and Mattox, 1988). Alterations in lectin binding similarly accompany embryonic development. Consequently, attempts have been made to find lectin conjugates that would specifically

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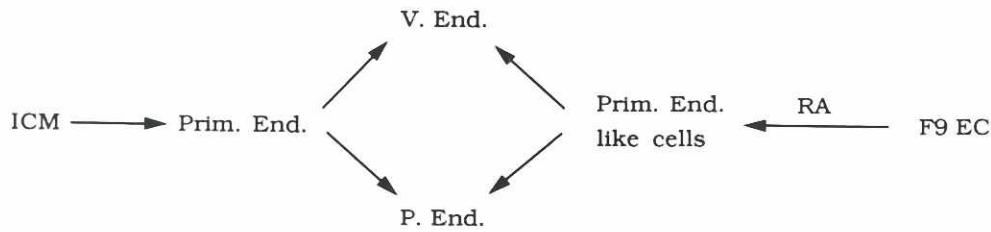
Fig. 1. *The differentiation of extraembryonic endoderm in the mouse embryo and in the F9 embryonal carcinoma (EC) cell system. In the embryo, the inner cell mass (ICM) gives rise to the primitive endoderm (Prim. End.), which then forms the visceral (V. End.) and the parietal (P. End.) endoderm. Monolayer cultures of F9 EC cells treated with retinoic acid (RA) give rise to primitive endoderm-like cells. These cells can be converted, by allowing them to aggregate, to form V. End. and, by elevating their intracellular cAMP, to form P. End.*

label undifferentiated or differentiated teratocarcinoma cells and the corresponding embryonic tissues (for ref. see Muramatsu, 1988). Such proposed developmentally regulated cell surface markers include *Dolichos biflorus* agglutinin (DBA) conjugates (Muramatsu *et al.*, 1981, 1985), *Sophora japonica* agglutinin conjugates (Sato and Muramatsu, 1985) and *Lotus tetragonolobus* agglutinin conjugates (Hamada *et al.*, 1983; Sato *et al.*, 1986). Binding of DBA conjugates by some EC cells, including F9 cells, on the other hand, has been suggested as being associated with the restricted differentiation capacity of these cells (Muramatsu, 1988).

Cell surface expression of lectin binding sites

Expression of specific cellular glycoconjugates may not be associated uniformly with different phenotypes. For example, a diversity of cultured malignant or transformed cells, including F9 cells, show population heterogeneity in binding of fluorochrome-coupled *Ulex europaeus* I-lectin (UEA-I) (Virtanen *et al.*, 1985). F9 cells are similarly distinctly heterogeneous in their binding of DBA conjugates, whereas they bind another lectin specific to GalNAc, *Helix pomatia* agglutinin (HPA), homogeneously (Figs. 2-4; Tienari *et al.*, 1989b). Upon endodermal differentiation the proportion of cells binding DBA conjugates increases, whereas the proportion of cells binding TRITC-UEA-I remains low or decreases (Figs. 4, 5a). This is in line with the observation that appearance of DBA-binding sites is associated with endodermal differentiation in mouse embryos (Noguchi *et al.*, 1982). Yet, a distinct heterogeneity in binding DBA conjugates remains among the parietal endoderm-like F9 derivatives. They also bind HPA conjugates heterogeneously (Fig. 5; Tienari *et al.*, 1989b).

It thus appears that though differentiation of F9 cells leads to changes in the binding sites for DBA, HPA and UEA-I conjugates, these lectins do not label the cells selectively at specific stages of differentiation, but reveal distinct population heterogeneities among both undifferentiated and differentiated F9 cells. This suggests that some of the differentiation-associated changes in F9 cell surface glycoconjugates may involve only subpopulations of the cells.

Developmental changes in glycosylated proteins

Differentiation of F9 cells leads to diverse changes in protein expression. Several matrix components, such as laminin, entactin, type IV collagen, chondroitin sulphate, and heparan sulphate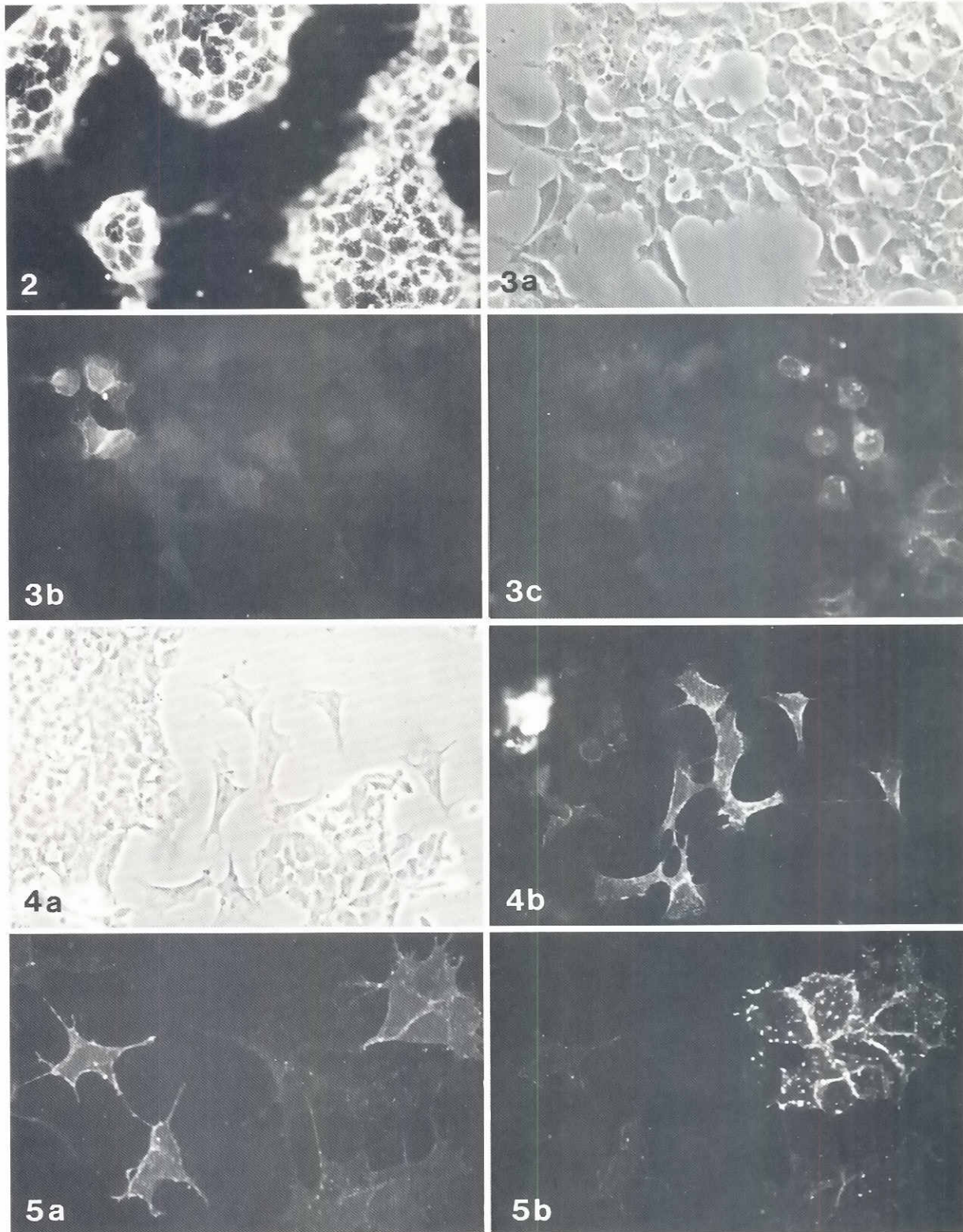 become more abundant, while synthesis of fibronectin is reduced (Carlin *et al.*, 1983; Cooper *et al.*, 1983; Kapoor and Prehm, 1983; Grover and Adamson, 1985). Lactoperoxidase surface labeling experiments have revealed changes in the expression of cell surface proteins upon differentiation of teratocarcinoma cells (Howe and Solter, 1981; Joukoff *et al.*, 1986). Virtually all cell surface and matrix proteins as well as secreted proteins are glycosylated. Therefore, the polypeptides of such proteins can be analyzed by using immobilized lectins.

In differentiated F9 derivatives, affinity binding to immobilized DBA, PNA, WGA and LCA reveals a doublet of polypeptides of M_r 300 000-400 000 in the detergent-soluble fraction of the cells. An additional M_r-220 000 polypeptide, bound by DBA and PNA, appears in cells treated with RA and dbc-AMP but not in those treated with RA only (Fig. 6; Tienari *et al.*, 1989b). These high-M_r polypeptides, revealed in the differentiated cells both by metabolic and surface labeling methods, are not seen in the undifferentiated F9 cells. They are likely to be distinct from laminin, since deposited laminin is not detergent-soluble. The polypeptides also fail to bind GSA-I even though terminal α-β-galactosyl residues are characteristic of laminin (Shibata *et al.*, 1982; Rao *et al.*, 1983). The polypeptides are probably distinct from heparan or chondroitin sulphate, as they are not labelled by ^{35}S-sulphate. These high-M_r polypeptides may hence represent developmentally regulated cell surface glycoproteins distinct from the matrix components previously shown to be regulated upon F9 cell differentiation.

Of the proteins secreted by differentiated F9 derivatives, the most prominent glycosylated polypeptides binding to WGA, LCA, GSA-I, and DBA comigrate with laminin A and B chains. Furthermore, a polypeptide doublet comigrating with laminin B1 and B2 subunits is bound by PNA (Fig. 7, Tienari *et al.*, 1989b). Laminin from the mouse EHS tumor contains N-glycosidic saccharides, but little or no O-glycosidic saccharides and no DBA-binding sites (Rao *et al.*, 1983; Arumugham *et al.*, 1986). Thus, laminin secreted by differentiated F9 cells may be glycosylated unlike that of the EHS tumor.

Remarks

Differentiation of F9 cells leads to changes in their binding of fluorochrome-coupled lectins. However, the lectin conjugates reveal distinct population heterogeneity among undifferentiated and differentiated F9 cells and are hence likely to be of limited value in the characterization of individual cells. At the whole cell population level, on the other hand, affinity binding to lectins can be used for analyzing developmentally regulated glycoproteins.

Figs. 2-5. *F9 cells before (Figs. 2-4) and after (Fig. 5) a 5-day treatment with RA;dbc-AMP, surface labeling with fluorochrome-coupled HPA (Fig. 2), UEA-I (Fig. 3b), DBA (Figs. 3c, 4b, 5a), and HPA (Fig. 5b). The undifferentiated cells are homogeneously stained with FITC-HPA (Fig. 2). A distinct population heterogeneity is revealed in double staining with TRITC-UEA-I (Fig. 3b) and FITC-DBA (Fig. 3c). Occasional untreated cells show morphological signs of spontaneous differentiation, and they are brightly DBA-positive (Fig. 4b). After RA;dbc-AMP treatment, the parietal endoderm-like derivatives are distinctly heterogeneous by the intensity of their binding of fluorochrome-coupled DBA (Fig. 5a) and HPA (Fig. 5b). Figs. 3a and 4a, phase contrast. (Figs. 2, 4a, 4b, 5a, from Tienari et al., 1989b).*
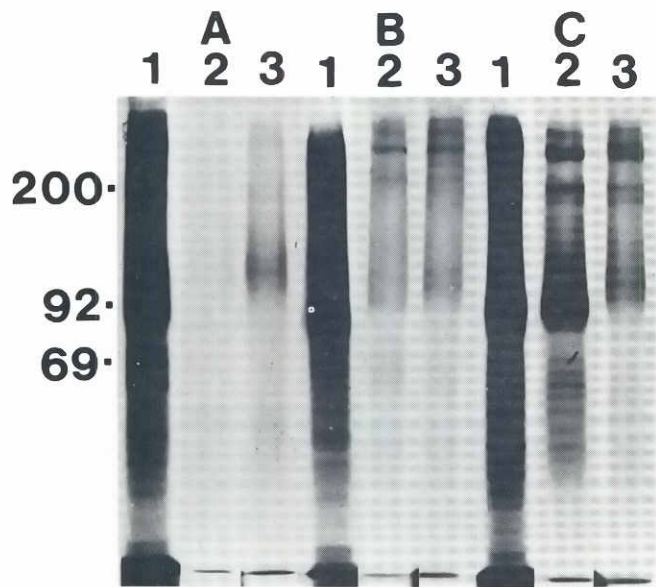
Fig. 6. *Detergent-soluble glycoproteins of untreated (lanes A), RA-treated (lanes B), and RA;dbc-AMP-treated (lanes C) F9 cells, surface labeling with the Nase;gal ox;NaBH₄-method. Total glycoproteins (A1, B1, C1), glycoproteins bound to DBA (A2, B2, C2) and glycoproteins bound to PNA (A3, B3, C3). (From Tienari et al., 1989b).*
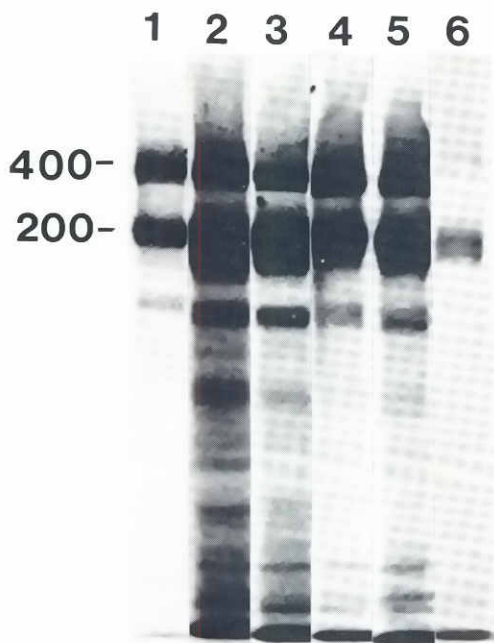

Fig. 7. *³⁵S-methionine-labeled proteins from culture medium of RA;dbc-AMP-treated F9 cells. Proteins immunoprecipitated with antibodies to laminin (lane 1), and proteins bound to WGA (lane 2), LCA (lane 3), GSA-I (lane 4), DBA (lane 5) and PNA (lane 6). (From Tienari et al., 1989b).*

Effects of F9 cell differentiation on cell adhesion

Pericellular matrix proteins influence cell attachment (Kleinman et al., 1981; Yamada et al., 1985). They also influence cell differentiation and are probably involved in the regulation of embryonic development by directing, for instance, cell migration (e.g. Hay, 1981). Fibronectin has been suggested specifically to promote the differentiation and migration of parietal the endoderm cells (Grabel and Watts, 1987). On the other hand, differentiation of cells can influence their adherence. For example, erythroid differentiation of erythroleukemia cells leads to a reduced adherence to fibronectin (Patel and Lodish, 1984).

Changes in the capacity to adhere to specific matrix components accompany F9 cell differentiation as well (Tienari et al., 1989a). Undifferentiated F9 cells avidly adhere to substrata precoated with fibronectin or laminin. Upon endodermal differentiation, the cells retain their capacity to adhere rapidly to fibronectin, while their capacity to adhere to laminin distinctly decreases. Concomitantly, the cells increase their synthesis of laminin, while the synthesis of fibronectin is decreased (Carlin et al., 1983). The VE- and PE- like derivatives of F9 cells do not seem to differ markedly in their capacity to adhere to fibronectin or laminin (Fig. 8; Tienari et al., 1989a).

The standard growth substratum used for culturing F9 cells is gelatin. As compared with laminin and fibronectin, F9 cells adhere distinctly less efficiently to this substratum. The adhesion to gelatin is completely inhibited if the protein synthesis is blocked with cycloheximide. However, if the medium is supplemented with soluble fibronectin, the cells adhere to gelatin even in the presence of cycloheximide. This drug does not markedly affect the adhesion of F9 cells to fibronectin or laminin. These observations (Tienari et al., 1989a) suggest that F9 cells adhere to fibronectin and laminin by specific mechanisms, whereas the adherence to gelatin depends on secreted mediators such as fibronectin.

Remarks

A clue for understanding the development of the extraembryonic endoderm *in vivo* (Gardner, 1983b) may be provided by the differentiation-associated reduction in the adhesion to laminin. In the blastocyst-stage embryo, fibronectin and laminin are found within the ICM. Before the migration of parietal endoderm cells, the inner surface of the trophectoderm carries fibronectin (Wartiovaara et al., 1979). The pericellular fibronectin, together with the restriction in the capacity to adhere to laminin, might regulate the distribution of the endoderm cells in the egg cylinder. The cells migrating on the trophectoderm would then differentiate into definitive parietal endoderm.

It should be noted that malignant transformation also affects cell adhesion. For example, transformed fibroblasts effectively adhere to laminin, whereas their normal counterparts do not (Kato and DeLuca, 1987). The restriction in adhesion capacity upon differentiation may thus also reflect loss of malignant potential of F9 cells (Solter et al., 1979).

Differentiation of F9 cells and the organization of the cytoskeleton

The intracellular cytoskeleton of cells consists of three fibrillar organizations, viz., microfilaments, microtubules, and intermediate filaments (IFs), and the surface lamina (membrane ske-
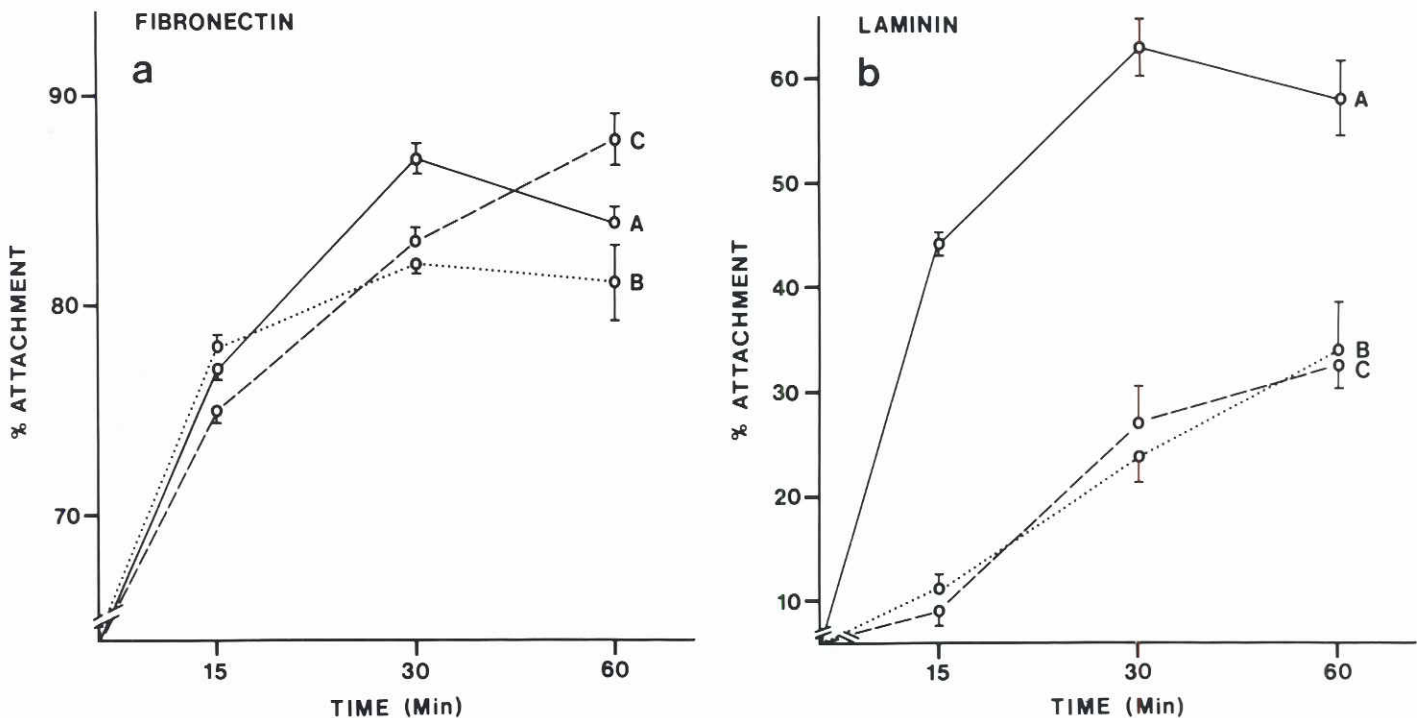
Fig. 8. *Time course of attachment of F9 cells to fibronectin-(Fig. 8a) and laminin-coated plastic (Fig. 8b). Untreated F9 cells (A), F9 cells treated with RA (B) and F9 cells treated with RA and dbc-AMP (C). The cells were labeled with ^3H- thymidine, suspended in serum-free medium containing 25 µgml^{-1} cycloheximide, and allowed to attach for 15-60 min. The proportion (%) of attached cells was then defined by counting the radioactivity in the attached and unattached cells. The values represent means from four experiments and the vertical bars standard error. (From Tienari et al., 1989a).*

leton) (cf. Lehtonen *et al.*, 1988b). Both in the embryo and in the teratocarcinoma model system, the differentiation of endoderm cells is connected with extensive modifications in the organization of cytoskeleton-associated molecules (cf. Paulin, 1982; Lehtonen *et al.*, 1983a, b; Tienari *et al.*, 1987).

Actin and vinculin

In addition to the main structural components of the cytoskeleton, the cells contain numerous other cytoskeleton-associated proteins. These include vinculin, an M_r-130 000 protein associated with the sites of contact between actin bundles and the cytoplasmic face of the plasma membrane in cells *in vivo* and *in vitro* (cf. Geiger, 1983; Lehtonen and Reima, 1986; Geiger *et al.*, 1987). Accordingly, in adherent cells in culture, vinculin is typically found concentrated in the areas of focal contacts. These areas have a central role in the cell-substrate interactions. In addition to vinculin, the components involved in these interactions include talin, cell adhesion molecules, and integrins (cf. Geiger *et al.*, 1987; Dahl and Grabel, 1989).
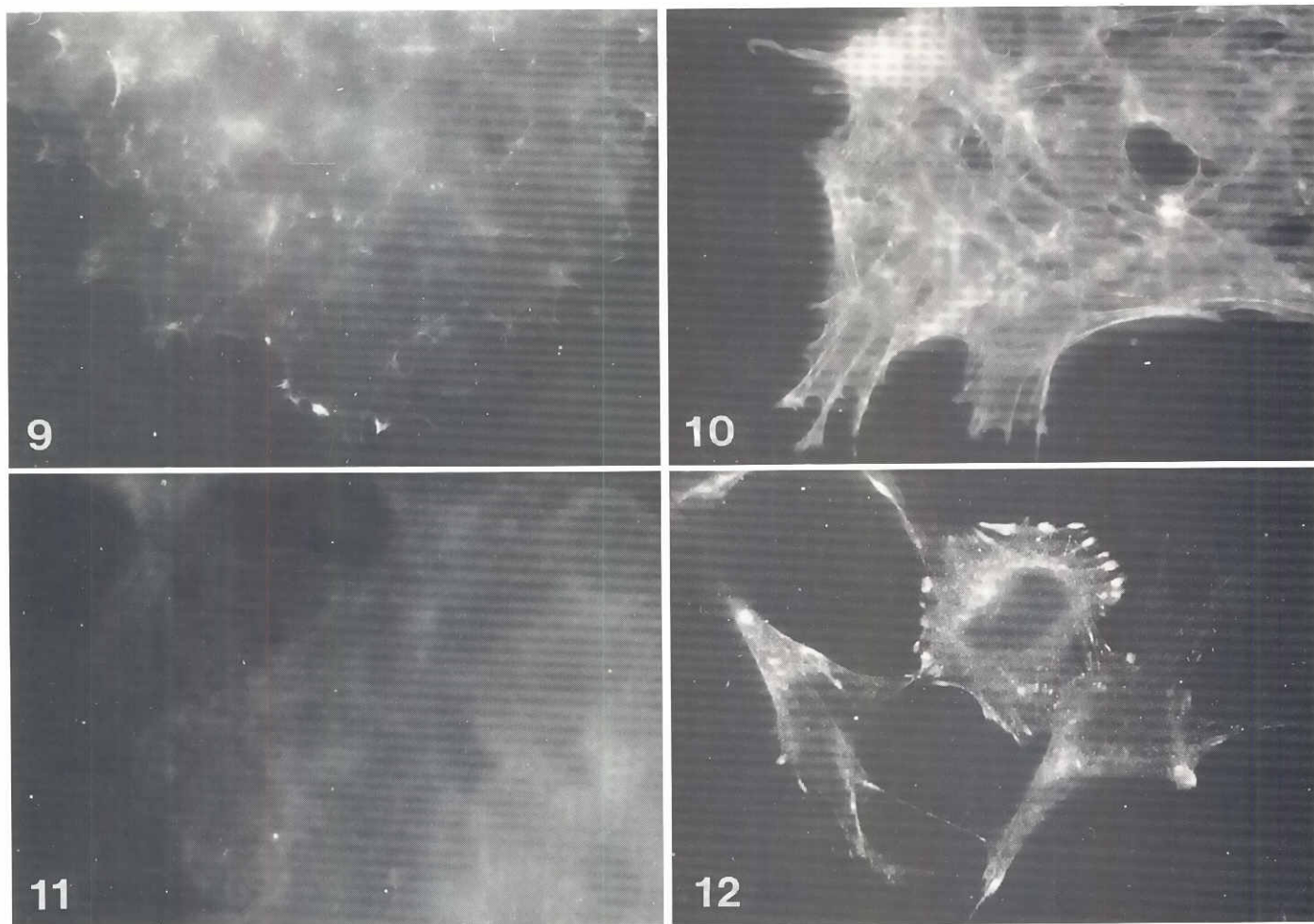
Differentiation of F9 cells is accompanied by distinct changes in their capacity to adhere to specific substrata (Fig. 8, cf. Tienari *et al.*, 1989a). Furthermore, cell interactions, involving cell adhesion and aggregation, regulate differentiation of EC cells (cf. Hogan *et al.*, 1983; Dahl and Grabel, 1989). The microfilament system plays a central role in cell adhesion and movement. Accordingly, modifications in the organization of actin and vinculin, for instance, characteristically occur in systems involving changes in cell behavior and morphology, such as the teratocarcinoma system.

In undifferentiated F9 cells, actin generally appears as short filaments and as spikes at the edges of the colonies, together with some diffuse cytoplasmic actin (Fig. 9; Lehtonen *et al.*, 1983a). Upon differentiation, actin is reorganized into typical stress fibres (Fig. 10); these appear during the second and third day of RA treatment (Table I, Lehtonen *et al.*, 1983a). Before the appearance of stress fibres, a distinct change occurs in the organization of vinculin. In the undifferentiated F9 cells, vinculin is expressed at a low level, and its distribution is diffuse. Upon differentiation, the quantity of vinculin increases, and it accumulates in focal contacts: typical vinculin plaques appear at the ventral surfaces of the cells (Fig. 12; Lehtonen *et al.*, 1983a).

Intermediate filament proteins

While the structural proteins in microfilaments and microtubules, i.e., actin and tubulin, are very similar in all cell types, the biochemical and immunochemical characteristics of IFs vary from one cell type to another. Based on their molecular composition, the IF proteins are currently divided into five categories: type I, acidic keratins; type II, basic keratins; type III, vimentin, desmin and glial fibrillary acidic protein (GFAP); type IV, neurofilament proteins; and type V, laminins. The fact that different cell types express distinct subclasses of IF proteins has been widely used to characterize cells (cf. Lehtonen *et al.*, 1985, 1988b; Virtanen *et al.*, in this issue).

Of the different subtypes of IF proteins, undifferentiated EC cells *in vitro* generally express vimentin. Some cell lines also express cytokeratin IFs, either alone or together with vimentin.

Figs. 9-12. *Distribution of actin (Figs. 9-10) and vinculin (Figs. 11-12) in F9 cells before (Figs. 9, 11) and after (Figs. 10, 12) RA treatment. The differentiated cells show typical actin stress fibers (Fig. 10) and vinculin plaques (Fig. 12), characteristic of adherent stationary cells.*

Upon differentiation, the cells may start to express new types of IF proteins (e.g., Jones-Villeneuve *et al.* , 1982; Paulin *et al.*, 1982; Lehtonen, 1987; Tienari *et al.*, 1987). Undifferentiated F9 EC cells express vimentin, and upon differentiation, they start to express cytokeratin as well (Table I; Figs. 13-14). Cytokeratin filaments are typical of epithelial cells, and thus it is not surprizing that endodermal differentiation is associated with cytokeratin expression. The exact polypeptide composition of the cytokeratins synthesized by the differentiated F9 cells remains to be studied, but it is clear that the cells contain cytokeratin polypeptides 8 and 18 (cf. Lehtonen, 1987; Tienari *et al.*, 1987; Kurki *et al.*, 1989). Interestingly, after polonged culture, differentiated F9 cells may lose both vimentin and cytokeratin filaments (Boller and Kemler, 1983; Tienari *et al.*, 1987).
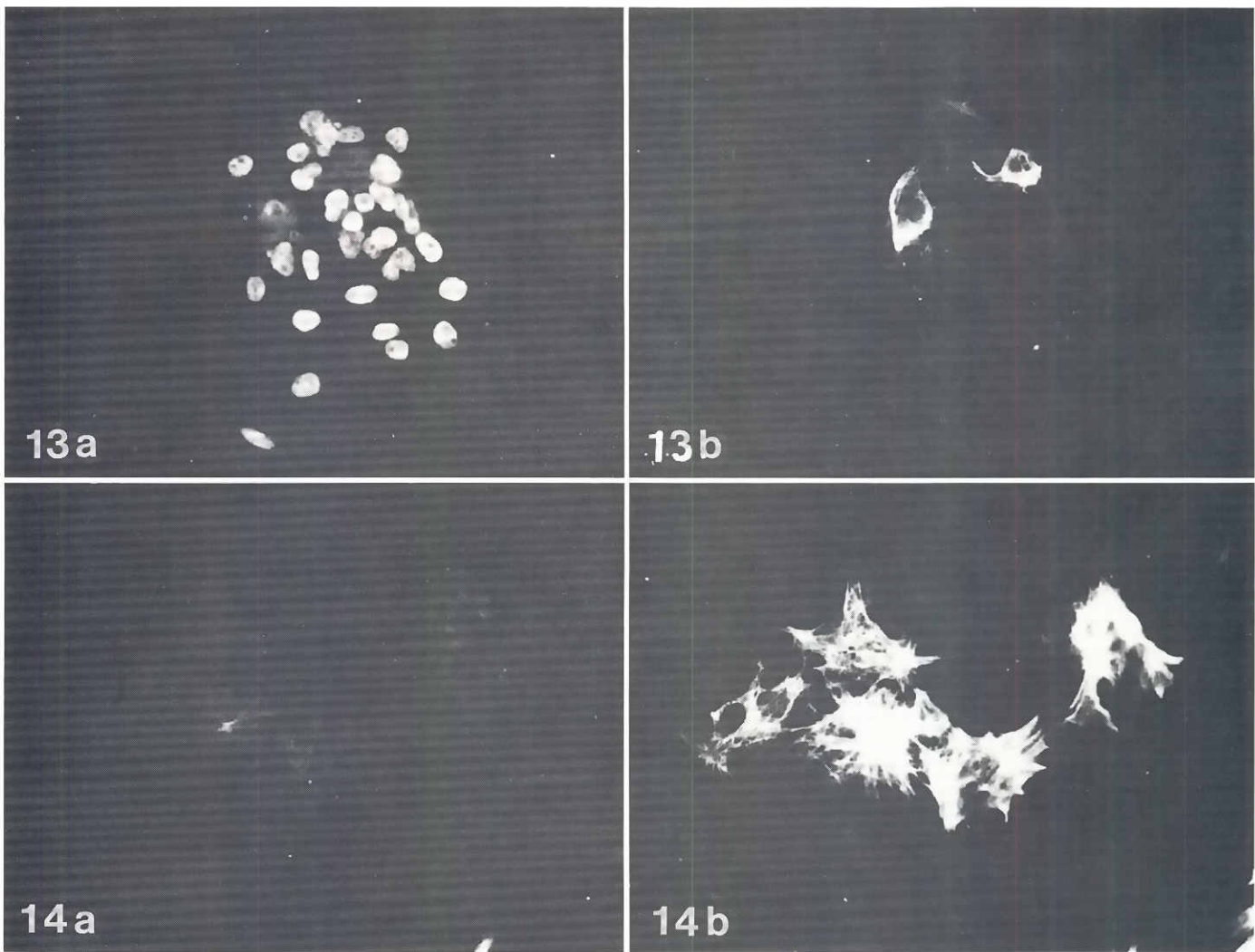
The IF composition of the primitive ectoderm cells of the early embryo has not been fully characterized. However, the presence of cytokeratin-type IF protein at the preceding stages (Chisholm and Houliston, 1987; Lehtonen, 1987; Lehtonen *et al.*, 1983c, 1988b) suggests that cytokeratin might be present in the primitive ectoderm as well. Murine EC cells are generally considered to be equivalent to the inner cell mass cells or the

TABLE 1

CYTOSKELETON-ASSOCIATED PROTEINS DURING RETINOIC ACID (RA)-INDUCED DIFFERENTIATION OF F9 CELLS

Duration of RA-treatment	Vinculin plaques	Actin stress fibres	Cytokeratin filaments	Vimentin filaments
0d	−	−	−	+ +
1d	+	−	−	+ +
2d	+ +	+	+ / −	+ +
3d	+ +	+ +	+ + / −	+ +
5d	+ +	+ +	+ + / −	+ + / −

−, not present; +, first signs of appearance; + +, distinct; + / −, some cells positive, some negative.

F9 cells start to form vinculin-containing adhesion plaques after a 1-2-day RA treatment. Slightly later, actin is reorganized into stress fibers terminating at the plaques. The number of cells containing filamentous cytokeratin (CK) sharply increases between days 2 and 3 of RA treatment. Regardless of the length of the treatment, a proportion of the cells remains CK-filament-negative. Some cells lose their vimentin filaments upon RA treatment. The Table is based on immunofluorescence microscopy observations (Lehtonen *et al.*, 1983a; Tienari *et al.*, 1987; Kurki *et al.*, 1989).

Figs. 13-14. *Double immunofluorescence micrographs of differentiating F9 cells after 2 days (13a, b) and 5 days (14a, b) of RA treatment. Figs. 13a and 14a show stainings for proliferating cell nuclear antigen (PCNA; cf. Kurki et al., 1988, 1989) and Figs. 13b and 14b for cytokeratin (CK). The undifferentiated F9 cells do not express CK filaments. As early as after 2 days of RA treatment, some of the differentiating cells show CK filaments (Fig. 13b), and after 5 days, most of the cells are CK- filament-positive (Fig. 14b). During early phases of differentiation, the cells maintain the extremely high rate of proliferation, characteristic of undifferentiated F9 cells: practically all the nuclei in Fig. 13a are PCNA-positive. As the differentiation proceeds, the rate of proliferation slows down: all the CK-positive cells in Fig 14b are PCNA- negative (Fig. 14a). (From Kurki et al., 1989).*

primitive ectoderm of the blastocyst (see Introduction). Thus, it appears that in their expression of IF proteins, F9 cells do not fully mimic their proposed embryonic counterpart. The differentiation of endoderm cells is then characterized by the formation of cytokeratin filaments both in the embryo and in the F9 cell system. It should be noted that in the embryo as well, early modulation occurs in the expression of IF proteins: the parietal extraembryonic endoderm expresses both cytokeratin and vimentin, whereas the visceral extraembryonic endoderm expresses cytokeratin only (Lane *et al.*, 1983; Lehtonen *et al.*, 1983b).

Remarks

The appearance of vinculin-containing adhesion plaques seems to be an early sign of endodermal differentiation of F9 cells, and it apparently precedes modifications in other adhesion-related molecules such as integrins and actin (Lehtonen *et al.*, 1983a; Dahl and Grabel, 1989). The reorganization of the IF cytoskeleton follows these changes (Table I). It should be noted, however, that expression of soluble cytokeratin-type IF protein has been observed in F9 cells at an early stage of differentiation, when fibrillar cytokeratin cannot yet be detected in the cells (Fig. 16; Laasonen *et al.*, submitted).

The causal relationship between the changes in the cytoskeleton-associated proteins and overt differentiation and proliferation of F9 cells remains to be studied. Interestingly, many of the changes mimic the mirror image of those occurring upon transformation of cells (cf. Lehtonen *et al.*, 1983a; Dahl and Grabel, 1989). This accords with the fact that the differentiation of EC cells involves loss of their malignant characteristics.

Relationship between differentiation and proliferation of F9 cells

Normal embryogenesis depends on spatial and temporal control of cell differentiation and proliferation. Growth kinetics studies and cell cycle analysis during normal mouse embryonic development have been carried out by several groups (Wimber and Lamerton, 1965; Gamow and Prescott, 1970; Sawicki *et al.*, 1978). During preimplantation mouse embryogenesis cells divide with a generation time of 9-12 h. At this stage the G1 phase is very short (1-2 h) or absent (Gamow and Prescott, 1970; Mukherjee, 1976; Streffer *et al.*, 1980). As the early embryogenesis proceeds, cell division rates seem to slow down concomitantly with the appearance of signs of differentiation (Mukherjee, 1976). In the blastocyst, the ICM cells retain the
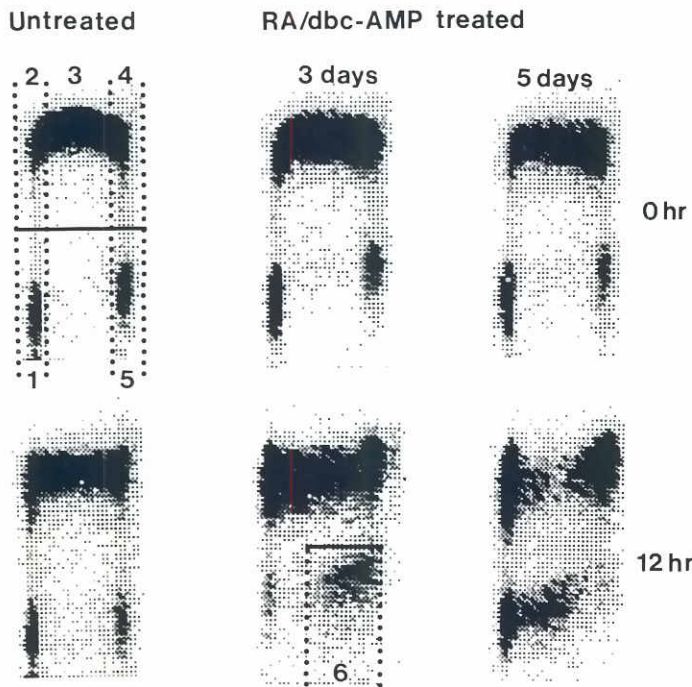

Fig. 16. *Flow cytometry analysis of cytokeratin (CK) expression of F9 cells upon RA;dbc-AMP treatment. Y-axis, the percentage of CK-positive cells from the total cell number; X-axis, the duration of the RA;dbc-AMP treatment. Whole cells (filled bars) and detergent-extracted cells (striped bars) were analyzed separately. No definite CK signal is observed in normal whole F9 cells. After a 1-day RA;dbc-AMP treatment, a small fraction of whole cells, but not of detergent-extracted cells, gives a CK signal in flow cytometry. After a 2-day treatment, the fraction of CK-expressing cells has increased clearly in the whole-cell samples. Some CK-positive cells have also appeared in the detergent-extracted samples. The most remarkable increase in CK expression, including detergent-resistant filamentous CK (striped bars), occurs between days 2 and 3 of treatment. After a 5-day treatment, the number of CK-expressing cells has further increased; the change is particularly distinct in the case of filamentous CK. (Laasonen et al., submitted).*

Fig. 15. *Flow cytometry analysis of the progression of F9 cells in the cell cycle before (untreated) and after 3 days and 5 days of RA;dbc-AMP treatment. X-axis, DNA content (propidium iodide staining) in a linear scale; Y-axis, bromodeoxyuridine (BrdUrd) content in a logarithmic scale. The analysis was performed every second hour up to 24 h (shown here: 0 hr and 12 hr) after a 10-min BrdUrd pulse. Labeled (BrdUrd-positive) cells are seen above the solid line (untreated, 0 hr). On the basis of DNA content, the following categories of cells can be distinguished (dotted lines): Box 1, unlabeled (BrdUrd-negative) G1-phase cells; Box 2, labeled early S-phase cells; Box 3, labeled mid-S-phase cells; Box 4, labeled late-S-phase cells; Box 5, unlabeled G2 M-phase cells. A good separation of labeled and unlabeled F9 cells is seen in all samples immediately after the BrdUrd pulse (0 hr). 12 hr after the BrdUrd pulse, the cells in the untreated cultures have completed one cell cycle and divided (the histogram looks like the one immediately after the pulse). The effect of RA;dbc-AMP on the progression of F9 cells is obvious in the BrdUrd-negative cells. As compared with the untreated cells, a slower proliferation rate is seen in RA;dbc-AMP-treated cells: in the culture treated for 3 days, most of the unlabeled cells are in late S and G2 M phase (box 6). In the culture treated for 5 days, most of the unlabeled cells have reached early and mid S phase, but some cells are still in G1 phase. (Laasonen et al., submitted).*
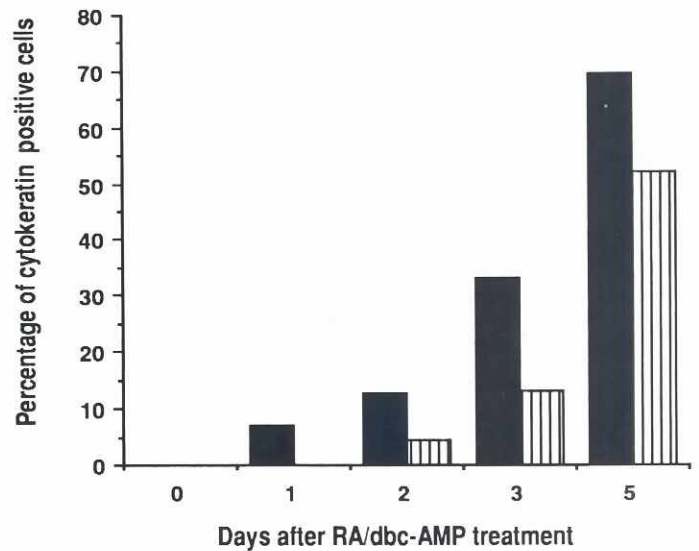
cell cycle charcteristics of their pluripotent predecessors, whereas the differentiated trophectoderm cells grow more slowly. After gastrulation, the primitive ectoderm (epiblast) still proliferates at a very high rate while the mesoderm divides relatively slowly (Snow, 1977). EC cells are considered equivalent to the ICM or primitive ectoderm cells, and therefore they can be used for studying the relationship between differentiation and proliferation during these early developmental stages.

Many EC lines, both murine and human, can be induced to differentiate *in vitro*. The results from these experiments have repeatedly confirmed that upon differentiation, the rate of proliferation of EC cells decreases (e.g., Rayner and Graham, 1982; Mummery *et al.*, 1984, 1987a, b; Griep and DeLuca, 1986; Kurki *et al.*, 1989). In the case of F9 cells, the endodermal differentiation, induced by RA or RA/dbc-AMP, is accompanied by expression of new proteins, including cytokeratin (e.g., Kemler *et al.*, 1981; Lehtonen *et al.*, 1983a; Lehtonen, 1987; Tienari *et al.*, 1987). The first cells with distinct cytokeratin filaments appear in the cultures after a 2-3-day treatment with RA or RA/dbc-AMP. At this stage, the proliferation rate of the differentiated cells is similar to or only slightly slower than that of the undifferentiated EC cells. After a 5-day treatment, the growth rate has distinctly decreased (Figs. 13-14; Lehtonen *et al.*, 1988a; Kurki *et al.*, 1989; Laasonen *et al.*, submitted). Our preliminary results show that the lengthening of the cell cycle is mainly due to the prolongation of G1 and S phases (Fig. 15). The rate of proliferation decreases simultaneously with the

appearance of cytokeratin filaments in the cells. However, a change in gene expression occurs somewhat earlier: our immunofluorescence microscopy, immunoblotting, and flow cytometry results suggest that soluble cytokeratin appears early during RA/dbc-AMP treatment and that the organization of typical nucleus-associated cytokeratin filaments occurs somewhat later (Fig. 16; Lehtonen *et al.*, 1988a; Laasonen *et al.*, submitted).

It has often been suggested that induction to differentiation can only occur in G1 or early S phase of the cell cycle (e.g. Griep and DeLuca, 1986; Tsuda *et al.*, 1986; Clegg and Hauschka, 1987; Clegg *et al.*, 1987; Mummery *et al.*, 1987a, b). In F9 cells, the G1 phase of the cell cycle is unusually short, of the order of a couple of hours (Rosenstraus *et al.*, 1982, Sennerstam and Strömberg 1984; Griep and DeLuca 1986; Laasonen *et al.*, submitted). Accordingly, a change in cell cycle phases, or a general decrease in the rate of proliferation, might be a prerequisite for F9 cell differentiation. The present data does not, however, warrant decision on whether the reduction in the rate of proliferation is a prerequisite for or a result from the differentation of F9 cells. The prolongation in the cell cycle occurs at the same time as the expression of cytokeratin filaments becomes detectable (Lehtonen *et al.*, 1988a; Kurki *et al.*, 1989; Laasonen *et al.*, submitted). The morphology of EC cells may change before detectable changes in the rate of proliferation (Linder *et al.*, 1981; Mummery *et al.*, 1984, 1987a, b), but this does not necessarily prove that the cells are irreversibly committed to differentiate.

Remarks

The appearance of cytokeratin in F9 cells occurs simultaneously with the accumulation of cells in G1/early S phase, and, in fact, the treatment with RA or RA/dbc-AMP might independently cause both the change in gene expression and the change in the rate of proliferation. It should be noted, however, that drugs which block the cells in G1 or early S phase, such as inhibitors of DNA synthesis, can induce differentiation of EC cells (Nishimune *et al.*, 1983; Griep and DeLuca, 1986). This is consistent with the hypothesis that the extremely short G1 phase of F9 cells may not allow the expression of genes necessary for differentiation.

Concluding remarks

The findings discussed here represent mere examples of the modifications in cell behaviour, gene expression, and proliferation charcteristics, which take place upon differentiation of embryonal carcinoma cells. Numerous changes occur more or less simultaneously, and, consequently, it is difficult to judge their causal relationships. Nevertheless, the cellular changes and the molecular mechanisms involved are probably far less complicated in the teratocarcinoma cell system than in the normal embryo. The embryonal carcinoma cells, easily manipulated *in vitro*, therefore provide a useful model system for the analysis of cellular and molecular features during differentiation.

Acknowledgements

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