CYTKERATIN FILAMENT EXPRESSION DURING IN VITRO TERATOCARCINOMA CELL DIFFERENTIATION AS DETECTED BY A MONOCLONAL ANTIBODY

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ABSTRACT

Undifferentiated F9 teratocarcinoma cells were induced to differentiate in culture using retinoic acid and cAMP. As a result, the morphology of the cultures changes dramatically. Using a monoclonal antibody directed against cytokeratin polypeptide 18 (RGE 53) in the indirect immunofluorescence technique we could show that this cytokeratin subunit is synthesized and assembled into a filamentous network upon differentiation in about 50% of the cells. Immunoblotting studies confirm these results.

INTRODUCTION

Intermediate filaments are cytoskeletal structures that show considerable morphology homology, but can be subdivided into five groups on the basis of biochemical and immunochemical studies. Different types of intermediate filament proteins occur in different tissue types. Epithelial tissues contain (cyto)keratin intermediate filaments, while, for example, cells of mesenchymal origin contain vimentin as their intermediate filament constituent (Osborn et al., 1981). Recent studies by Jackson et al (1980, 1981) have shown that early trophectoderm- and endoderm-differentiation are accompanied by the expression of cytokeratins as the first intermediate-sized filament proteins synthesized during embryogenesis. On the basis of two-dimensional gel electrophoretic experiments cytokeratin polypeptides 8 and 18 (for nomenclature see Moll et al., 1982) most likely are the constituents of the first intermediate-sized filaments.

F9 teratocarcinoma cells in culture can be induced to differentiate into endoderm cells when treated with retinoic acid (Strickland, 1981). This makes the system a suitable model for the study of some
aspects of early mammalian development. Cytokeratins have not been detected in undifferentiated embryonal carcinoma cell cultures, but appear upon differentiation of these cells (Paulin et al., 1982). Cytokeratin antisera that have been used to study the expression of tonofilaments in embryos or cultured teratocarcinoma cells are of diverse sources. Polyclonal antibodies directed against epidermal keratins have been used by Paulin et al. (1982), Jackson et al. (1980, 1981), Franke et al. (1982) and Lehtonen et al. (1983). These antibodies are directed against a variety of cytokeratin polypeptides. The use of monoclonal antibodies in such studies has so far only been described by Kamer et al. (1981). These antibodies (Troms 1 and 2) seem to react with a restricted number of keratin polypeptides and epithelial tissues. Recently we have developed a monoclonal cytokeratin antibody (RGE 53) which reacts specifically with glandular (simple) epithelial tissues but not with squamous epithelia (Ramaekers et al., 1983a). Here this monoclonal antibody is further characterized and used to monitor the expression of cytokeratin filaments in differentiating F9 teratocarcinoma cells.

MATERIALS AND METHODS

F9 teratocarcinoma cells, kindly provided by Dr. H. Jakob (Paris) were cultured in plastic tissue culture flasks coated with gelatin in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin. For induction of differentiation, cells were treated with $10^{-7}$M retinoic acid (RA, Sigma) together with $10^{-3}$M dibutyryl-cAMP (Boehringer) with a change of growth medium every two days. For immunofluorescence, cells were cultured in 3 cm culture dishes containing gelatinized polystyrene coverslips (Lux Scientific Corporation).

Preparation and specificity of rabbit antibodies to human callus keratin and calf lens vimentin, as well as their use in the indirect immunofluorescence technique have been described before (Ramaekers et al., 1983b). The monoclonal antibody to cytokeratin 18 used in this study (RGE 53) was prepared as described earlier (Ramaekers et al., 1983a).

Cytoskeletal preparations from human heptocellular carcinoma cells (PLC/PFR/5) and F9 cells were made essentially by extracting cell pellets with 1% Triton-X-100 in phosphate buffered saline (PBS) containing 0.4 mM phenylmethylsulfonylfluoride (PMSF). The insoluble fraction was washed with PBS and subjected to gel electrophoresis in sodium dodecylsulfate containing 13% polyacrylamide slab gels as described by Laemmli (1970). For immunoblotting experiments the electrophoretically separated polypeptides were transferred to a nitrocellulose sheet using an electrophoresis buffer containing 25 mM Tris.HCl, 192 mM glycine and 20% methanol (pH 8.3), essentially as described by Towbin et al. (1979). The sheets were then, at room temperature and under constant shaking, exposed consecutively to the following incubation steps: 60 min. in
2 M urea; 2 times 30 min. in PBS containing 0.5% gelatine and 0.5% Triton-X-100 (GTP-buffer); 60 min. in undiluted RGE 53 culture supernatant; 3 times 20 min. in PBS containing 0.5% Triton-X-100 (TP-buffer); 60 min. in rabbit anti-mouse IgG antibodies conjugated with peroxidase (nordic) diluted 1:200 in GTP-buffer; 3 times 40 min. in TP-buffer and 20 min. in PBS. The peroxidase reaction was developed for 5 min. in a fresh solution of 0.02% 3,3′-diaminobenzidine tetrahydrochloride in PBS with 0.12% \( \text{H}_2\text{O}_2 \). The sheets were then rinsed exhaustively.

RESULTS

Using the one-dimensional immunoblotting assay RGE 53 was shown to recognize a 45,000 D molecular weight protein in cytoskeletal preparations from the human hepatocellular carcinoma cell line (Figure 1), as well as in several other human cell lines (not shown). In some cell lines a small amount of staining of a 42,000 mol weight band was occasionally found in some cytoskeletal preparations. Since hepatocellular carcinoma cells are known to contain only the two cytokeratin polypeptides (8 and 18) it is evident that RGE 53 recognizes only keratin polypeptide 18 and we assume the faint lower band to be a breakdown product of keratin 18. In the hepatocellular carcinoma cell line described above (as well as in other human cell lines; not shown), RGE 53 elaborately stains filamentous networks, similar or identical to the cytoskeletal structures recognized by the polyclonal keratin antisera (Figure 2). No reaction was found in cultured bovine lens cells, meaning that RGE 53 does not react with actin filamentous, vimentin intermediate filaments or microtubules.

F9 teratocarcinoma cells were plated at two densities (10^6 and 10^5 cells/3 cm Petri dish) and cultured in the presence of 10^{-9} M RA and 10^{-3} M cAMP for 2-6 days. Morphological changes were observed already after 2 days starting with the appearance of more flattened cells, while "nerve like" cells appeared at day 4. The latter cells were most conspicuous in the low density cultures at day 6. Also some colonies with smaller round cells were seen after 4 and 6 days. Thus at all times considerable heterogeneity existed in the cultures. Both undifferentiated and differentiated cultures were tested for the presence of keratin intermediate-sized filaments in the indirect immunofluorescence technique using RGE 53 and (for comparison) a polyclonal rabbit antiserum to human callus keratins. In untreated cultures practically all cells were negative. Interestingly, however, we have found that in these cultures at day 6 some differentiated cells containing keratin filaments were present at the periphery of a few colonies. The cells in differentiated cultures contained intracellular filamentous structures, which could be decorated both by the monoclonal and the polyclonal antikeratin antibodies in a similar fashion (Figure 3). The percentage of cells positive for the cytokeratin filaments varied with the time of induction and cell density (see Table 1).
Fig. 1: Immunoblots of RGE53 and a polyclonal antivimentin antiserum against cytoskeletal proteins from cultured human hepatocellular carcinoma cells. Lane a shows the Coomassie Brilliant Blue stained polypeptide pattern of the cytoskeletal preparation with two main bands in the 45-53 kD region, representing keratins 8 and 18. Minor bands represent vimentin (Vi) and actin (Ac). Lanes b and c show immunoblots of RGE 53 and the polyclonal antivimentin antiserum (for comparison) respectively. Note the exclusive reaction of RGE 53 with the cytokeratin 18 band. The antiserum to vimentin gives a major band at 57 kD with very faint reactions in some lower bands, most probably break down products of vimentin.

Fig. 2: Immunofluorescence of the human hepatocellular carcinoma cell line stained with RGE 53 (x600).
In the low density cultures maximally about 50% of the cells were positive, while in the high density cultures positive cells first appeared in the same percentage as in the low density cultures but later their percentage decreased. The time course of appearance of the filaments as visualized by RGE 53 in the low density cultures is shown in Figure 4. Cells negative for keratin antisera included morphologically differentiated cells with a flattened appearance. The "nerve like" cells were in most cases also negative.

Antibodies to vimentin stain cells at all stages, although intensity of vimentin fluorescence seems to be dependent on the density of the culture and morphological appearance of the cells (see Figure 3c). Using the immunoblotting technique we were able to demonstrate the expression and incorporation of a protein with a molecular weight of 48 kD into the cytoskeleton of differentiated F9 cells (Figure 5). In untreated cells this polypeptide was not detected.

**TABLE I:**

<table>
<thead>
<tr>
<th>Days of incubation with RA + cAMP</th>
<th>Percentage of cells showing filaments</th>
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<tr>
<td></td>
<td>$10^4$ cells/dish</td>
</tr>
<tr>
<td>0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
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a) Percentages of F9 cells in culture expressing cytokeratin 18 after treatment with $10^{-7}$ M retinoic acid and $10^{-3}$ M cAMP (RA + cAMP) as detected by immunofluorescence using RGE 53.
Fig. 3: a) Differentiated F9 cells (treated for 6 days with retinoic acid and cAMP) showing elaborate filamentous networks when incubated with RGE 53 for indirect immunofluorescence staining. b) Differentiated F9 cells after 4 days treatment with retinoic acid and cAMP incubated with a polyclonal antiserum to human callus keratins (compare with Fig. 4f). c) Differentiated F9 cells (as b) incubated with the polyclonal antiserum to calf lens vimentin (a x600; b and c x300).

Fig. 4: Phase contrast (a, c, e, g) and immunofluorescence (b, d, f, h) pictures of F9 cell cultures, stained with RGE 53. The cultures were treated for various time periods with retinoic acid and cyclic AMP. (a,b) Untreated cells. (c-h) Cells incubated with retinoic acid and cAMP for 2 days (c,d), 4 days (e,f) and 6 days (g,h). (x200).
Fig. 5: Immunoblotting with RGE 53 of cytoskeleton preparations of a) F9 cells treated for 4 days with retinoic acid and subsequently grown for 2 days in the absence of inducer. b) Untreated F9 cells. Note the positive reaction of RGE with a polypeptide with an apparent molecular weight of 48 kD in the differentiated cells. No such reaction is seen in the undifferentiated cells, which show only a faint background staining.

DISCUSSION

In this study we have used a monoclonal antibody (RGE 53) to monitor cytokeratin intermediate filament (IF) expression in differentiating F9 teratocarcinoma cells.

As described elsewhere (Ramakers et al., 1983a) reactivity of RGE 53 when tested on frozen human tissues is restricted to simple (glandular) epithelia. Positive cells include glandular cells from female breast, the digestive tract, columnar cells of the respiratory tract, some cells of urogenital tracts, exocrine and endocrine tissues as well as mesothelial cells. No reaction is found in squamous epithelial or non-epithelial tissues.

The immunoblotting results described in this paper show that RGE 53, when tested on cytoskeletal preparations from cultured human cells, reacts specifically with only a 45,000 D IF protein, corresponding to cytokeratin 18. On the basis of our studies on frozen sections of human tissues (Ramakers et al., 1983a) cross-reactivity of RGE 53 with cytokeratin 17, which behaves identically on one dimensional SDS gels, can be excluded. In detail, the cells of the various zones of the pilosebaceous tract (outer root sheath of hair follicle and sebaceous gland) which contain cytokeratin 17 (Moll et al., 1982b) are negative for RGE 53. Moreover several tumor types, which have been shown to contain cytokeratin no. 17 by Moll et al (1982a), are negative after incubation with RGE 53 in the indirect immuno-fluorescence assay. Furthermore, RGE 53 has been shown not to react with epidermal keratins or cytokeratins 7, 8 and 19 in immunoblotting studies (to be published). Therefore it may be concluded that RGE 53 antibodies are specific for cytokeratin 18. The positive staining patterns of RGE 53 with filament networks in
differentiating F9 cells strongly indicates that these filaments must contain cytokeratin 18. Furthermore, the immunoblotting study reveals decoration of a 48 kD polypeptide in cytoskeletal preparations from differentiated but not from undifferentiated F9 cells. The electrophoretic mobility of this polypeptide is similar to that of cytokeratin D (= no. 18) from mouse liver. The difference in molecular weight between human cytokeratin 18 and the corresponding mouse polypeptide has already been described by Denk et al (1982). Our results show that one of the IF proteins expressed upon F9-teratocarcinoma differentiation is cytokeratin 18.

In the differentiating F9 cell cultures the number of cells positive for RGE 53 or for the polyclonal antikeratin antiserum did never exceed 60%. This finding is in agreement with an earlier report by Paulin et al (1982). It is noteworthy that Kemler et al (1981) found almost 100% positive cells in differentiated F9 cultures when using TRIMA 1 and 2. The number of RGE 53 positive cells in our experiments was independent of the induction protocol used. Treatment of the cells with $10^{-7}$ M RA + $10^{-3}$ cAMP for two days and thereafter with normal growth medium for another two days or for four days with $10^{-6}$ M RA alone gave similar percentages of positive cells (results not shown). Negative cells induced morphologically differentiated cells. Therefore, our findings demonstrate considerable heterogeneity in the cell cultures.

In conclusion, RGE 53 provides a probe to study the expression of a single cytokeratin polypeptide at the single cell level. It is therefore a powerful tool in in vitro differentiation studies using teratocarcinoma cell lines. Furthermore, this antibody may prove to be a valuable marker for studies of in vivo teratocarcinoma differentiation as well as normal embryonic development.

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