Immunofluorescence studies on deoxyribonuclease from mouse teratocarcinoma cells during cell differentiation

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SUMMARY

Specific anti-DNase-I IgG have been used to detect deoxyribonuclease in teratocarcinoma cells by an indirect immunofluorescence method. All the cells studied show fluorescence staining. However, the patterns are quite different in embryonal carcinoma cells (amorphous cytoplasmic fluorescence and absence of nuclear staining) as compared to differentiated cell lines (diffuse, bright granular nuclear and fibrillar cytoplasmic fluorescence). It is possible by this method to distinguish different cell types derived from the same origin. Deoxyribonuclease from teratocarcinoma cells can therefore be considered as a marker of cell differentiation in this system.

INTRODUCTION

Many of the problems related to early embryonic development are difficult to analyse because of the scarcity of material and its heterogeneity. Today it is possible to overcome at least some of these difficulties by using the mouse teratocarcinoma as a model system for studying some aspects of cell differentiation (Jacob, 1975, 1977, 1978; Martin, 1975). Similarities between embryonal carcinoma cells (EC cells) derived from the teratocarcinoma and uncommitted cells of the early embryo have been shown by different criteria, morphological (Pierce & Beals, 1964), biochemical (Bernstine, Hooper, Grandchamp & Ephrussi, 1973), biological (Kleinsmith & Pierce, 1964) and serological (Artz et al. 1973).

Markers of embryonic development have been described, such as LETS protein, which is absent in the earlier stages of embryonic development but begins to be expressed in the inner cell mass of the later blastocyst (Zetter & Martin, 1978), and a family of high-molecular-weight glycopeptides that are present in the EC cells and absent in differentiated cells derived from the EC cells (Muramatsu et al. 1978a). A similar observation has been made during post-implantation embryogenesis of the mouse: the amount of large molecular-

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weight glycopeptides decreases during embryonic development (Muramatsu, Condamine, Gachelin & Jacob, 1978). We have shown that alkaline DNase activity can be easily detected in EC cells when extracted at low ionic strength (0-14 m-NaCl), whereas it is undetectable in differentiated derivatives under the same conditions (Soriano & Paulin, 1978). In differentiated cells, low levels of DNase activity could be extracted at high ionic strength (1 m-NaCl) only from chromatin (our unpublished results). A similar observation has been made by O'Connor (1969), who found an alkaline deoxyribonuclease activity in chromatin from normal and regenerating rat liver. The enzyme that we have isolated from EC cells shows similarities with deoxyribonuclease I since it is inhibited by G-actin, is Mg$^{2+}$-dependent, stimulated by Ca$^{2+}$, and its pH optimum is 8.2. This result suggests that alkaline deoxyribonuclease could be used as a marker of cyto-differentiation, DNase-I has the ability to form a 1:1 complex with actin, but whether it is involved in the regulation of actin pools in cells is not known at present (Lazarides & Lindberg, 1974). Besides, immunofluorescence studies with antibodies against actin and electron microscopy observations have shown that actin organization is different in differentiated cells and in EC cells. EC cells do not show actin cables whereas the differentiated lines (fibroblast, myocardial or myoblast cells) do (Paulin et al. 1978).

On the other hand, measurement of the pool of G and F actin in EC cells, by using a selective assay for monomeric and filamentous actin (Blikstad et al. 1978) have shown that the monomeric form of the actin represents about 75–80 % of the total actin in the cell (Markey, Soriano & Paulin, unpublished results). For all these reasons we have used specific antibodies against pancreatic DNase to visualize DNase in EC cells. Our results show that DNase is present in all the cells studied, including those that show no DNase activity but the immunofluorescence patterns are different in each cell type.

**MATERIALS AND METHODS**

(1) **Cell lines**

The cell lines employed in this study and the culture conditions have been described previously (Jacob et al. 1973; Nicolas et al. 1975; Nicolas et al. 1976). Two kinds of cells have been used: (A) PCC3/A/1, PCC4/Aza and the parietal yolk-sac endoderm derivative PYS-2, which show alkaline DNase activity; (B) the differentiated derivatives PCD2 (myoblast), PCD1 (myocardial) PCD3 (fibroblast-like) and PCC3/A/1-D-1 (mesenchyme-like), which show no DNase activity.

(2) **Sera**

(a) **Preparation of specific antibodies to DNase**

Electrophoretically purified pancreatic DNase-I at 300 μg in Freund's complete adjuvant was injected into a rabbit. Booster doses were given after 2, 4 and 5 weeks. The serum was positive in Outcherlony tests after the second
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booster dose. Blood was collected and the serum was clarified by centrifugation, and heat-inactivated at 57 °C for 20 min. The Ig fraction from serum was precipitated with 1 vol. saturated ammonium sulphate, and the precipitate washed with 45% saturated ammonium sulphate. The protein was then dissolved in a small volume of phosphate buffer saline (PBS) and dialysed against PBS overnight. The preparation (227 OD_{280nm} units) was run on a DEAE-Sephadex A-50 column. The Ig fraction was eluted with PBS, pooled and precipitated with ammonium sulphate as before. The precipitate was dissolved in 5 mM Tris-HCl, pH 7.6, 0.15 M-NaCl and dialysed against the same buffer for 6 h. The preparation containing 70 OD_{280nm} units was then incubated on a DNase-I Sepharose column containing 30 mg coupled DNase-I for 30 min at room temperature and then overnight at 4 °C. The column was then washed with the same buffer as above until the OD_{280nm} in the eluate reached about 0.02. The antibodies were then eluted with 5 M guanidine HCl, 10 mM potassium phosphate pH 6.5, 150 mM-NaCl dialysed and then precipitated with saturated ammonium sulphate. The precipitate was dissolved in 5 mM Tris-HCl, pH 7.6, 150 mM-NaCl and frozen in small aliquots at —70 °C.

(b) Fluorescein-coupled sheep anti-rabbit serum

It was provided by Institut Pasteur Production, Paris, France, and was absorbed before use on F-9 embryonal carcinoma cell, PYS-2, PCC3/A/1 and 129 mouse lymphocytes (1 vol. cell: 5 vol. serum) (a gift of D. Morello).

(3) Indirect immunofluorescence staining of fixed cells

Cells (1 × 10⁴) were grown on coverslips in small Falcon dishes (3 cm diameter) in Dulbecco’s modified Eagle’s medium supplemented with 15% foetal calf serum. After 24 h of culture, unless otherwise stated, cells were washed with PBS and fixed either in formaldehyde (3.7%) for 10 min at room temperature and then for 10 min in acetone-methanol (7:3) at -20 °C or directly in acetone-methanol (7:3) 10 min at -20 °C. In both cases the structure of cells was well preserved and no differences in the quality of the staining was observed. After a quick air-drying, the cells were washed with PBS and with Hank’s medium containing 4% foetal calf serum (HFCS), followed by incubation with DNase-I IgG antibodies (2.7 mg/ml) diluted 1:10 with HFCS for 30 min at 5 °C. The cells were washed three times with HFCS and fluorescein-coupled sheep anti-rabbit serum diluted 1:40 with HFCS was applied and incubated 30 min at 5 °C. The coverslips were washed three times with HFCS and once with PBS and then mounted in glycerol on a glass slide. Slides were viewed in a Zeiss PM III microscope with ultraviolet optics. Photographs were taken using either Tri X pan film (Kodak) or HP5 (Ilford). In all cases the specificity of the staining was determined by control experiments in which anti-DNase I rabbit IgG had been previously absorbed in batch experiments with either DNase I coupled or actin coupled with activated Sepharose.
(4) (a) DNase I coupled with CNBr-activated Sepharose

(Pharmacia, Sweden)

Sepharose gel, 1 g, was swollen and washed 15 min on a glass filter with 1 mM-HCl. 10 mg of DNase I were dissolved in 0.1 M-NaHCO₃ buffer containing 0.5 M-NaCl mixed with the gel and the mixture was gently shaken overnight at 4 °C. Unbound material was washed away with coupling buffer and any remaining active groups were reacted with 1 M ethanolamine at pH 8.0 for 2 h. Three washing cycles were used to remove non-covalently absorbed protein, each cycle consisting of a wash at pH 4.0 (0.1 M acetate buffer containing 1 M-NaCl) followed by a wash at pH 8.0 (0.1 M borate buffer containing 1 M-NaCl. The final concentration of DNase I was 1.8 mg/ml.

(4) (b) Actin coupled with CNBr-activated Sepharose

Swollen Sepharose, 175 mg, was incubated with 1.75 mg of actin in coupling buffer (the same as used for coupling DNase I to Sepharose) and the mixture was shaken overnight at 4°C. All the following steps were similar to those described above (see (4a). The final concentration of actin was 0.97 mg/ml.

RESULTS

(1) Immunofluorescence patterns are characteristic of cell types

(a) EC cells and differentiated derivative cell lines

Figure 1 (A, C, D) shows the characteristic fluorescence pattern of the round EC cells. The staining is exclusively localized in the cytoplasm. There is very light fluorescence over the nuclei and this is probably due to overlying cytoplasm. A similar situation is observed in the endodermal derivative cell line PYS-2 (Fig. 1, E, F). Figure 1(B) shows a control experiment in which EC cells have been incubated with anti-DNase-I IgG previously absorbed with DNase-I-coupled Sepharose.

By contrast, in differentiated cell lines, the fluorescence patterns depend on the line considered. In myoblast cells, the pattern is very characteristic: most of the fluorescence is localized in the nuclei and a fine network in the cytoplasm is observed (Fig. 2D, E). In fibroblast cells a bright fluorescence in the nucleus and in the cytoplasm is observed (Fig. 2F).

(b) Hexamethylene bisacetamide-treated EC cells

When EC cells PCC3/A/1 are cultured in the presence of hexamethylene bisacetamide (HMBA) (5 mM, 3 days), cells change in morphology. They are flat, very large and the cytoplasm shows external protrusions, some of the cells being binucleate. The sensitivity to SV 40 and polyoma viruses appears, actin cables are formed, malignancy is lost and embryonic surface antigen F-9 disappears (Jakob, Dubois, Eisen & Jacob, 1978). The DNase
Fig. 1. Immunofluorescence patterns of EC cells and the endodermal derivative line PYS-2: (A) EC cells PCC3/A/1 (× 900); (B) the same cells in control experiments in which anti-DNase-I IgG were absorbed with DNase-I-coupled Sepharose (× 900); (C), (D) EC cells PCC4 Aza (× 900), (× 2100); (E), (F) the endodermal derivative line PYS-2 (×900), ×2100). These cell lines show the characteristic cytoplasmic localization of the staining (arrows).
immunofluorescence pattern is very characteristic: 80–90% of the cells show a bright granular nuclear fluorescence (Fig. 2C).

(c) In vitro differentiation of EC cells

The PCC3/A/1 cells were cultured in vitro for 28 days by changing the medium every 2–3 days (Nicolas et al. 1975). At 5, 9 and 17 days of culture, cells were trypsinized and plated on coverslips in the usual way. By phase-contrast microscopy one can see that the number of EC cells decreases as in vitro differentiation proceeds, whereas the differentiated derivatives increase. Figure 2(A) and (B) show cell populations of both EC and differentiated cells with their respective patterns, i.e. exclusively cytoplasmic fluorescence of EC cells and granular nuclear fluorescence and fine cytoplasmic networks of differentiated derivatives.

(2) Control experiments

(a) Absorption of DNase-I IgG antibodies with DNase-I-coupled Sepharose

Ten µl of DNase-I IgG antibodies containing 27 µg were incubated with either 50 µl of activated Sepharose alone or with DNase-I-Sepharose and 40 µl of HFCS for 60 min at 37 °C and then 48 h at 5 °C. After centrifugation the supernatant was used in the usual way. DNase-I IgG incubated with Sepharose gives the usual fluorescence patterns in both EC cells and differentiated derivatives, whereas DNase-I IgG incubated with DNase-I-Sepharose shows a very light insignificant fluorescence (Fig. 1B).

(b) Absorption of DNase-I IgG antibodies with actin-coupled Sepharose

Ten µl of DNase-I IgG antibodies were incubated with 50 µl of actin-coupled Sepharose and 40 µl of HFCS for 60 min at 37 °C then 48 h at 5 °C. After centrifugation the supernatant was used with either EC cells or differentiated derivatives. In both cases the usual fluorescence staining is observed.

DISCUSSION

The results presented here show that deoxyribonuclease of EC cells can be detected by indirect immunofluorescence, using specific anti-DNase-I IgG antibodies. All the cells studied show fluorescence staining, but the patterns are quite different in EC cells (amorphous, cytoplasmic fluorescence and absence of nuclear staining) and in differentiated derivatives (diffuse, granular nuclear and fibrillar cytoplasmic fluorescence). Thus, since EC cells and differentiated lines display different fluorescence patterns, it should be possible to distinguish different cell types from the same origin in the EC system, and in this respect EC cell DNase detected by DNase-I antibodies can be considered as a marker of cell differentiation.

A fact which deserves some comment is the cytoplasmic fibrillar system and
Fig. 2. *In vitro* differentiation of EC cells during 28 days of culture: (A) after 5 days (×900) and (B) 9 days (×900), the arrows indicate cells which show a fluorescence differentiated pattern. (E) EC cells treated with HMBA (×900) show nuclei containing bright and irregular granules (arrows); (D), (E) myoblasts (×900), (×1600) show a bright microgranular nuclear fluorescence (arrows). In a fibroblast line (F) (×1600) one can see a dense fluorescence in both nuclei and cytoplasm.
the nuclear structures decorated by DNase antibodies in differentiated cell lines. It was not possible to establish with what kind of structures (microfilaments or 10 nm filaments) the EC cell DNase is associated, but the high affinity described between DNase-I and actin (Hitchcock, Carlsson & Lindberg, 1976) allows the assumption that EC cell DNase could be bound to the cytoplasmic actin filaments and to the nuclear actin. The fact that actin is present in the nuclei of rat liver (Comings & Harris, 1975), *Xenopus laevis* oocytes (Clark & Merriam, 1977) and that EC cell chromatin contains no actin whereas the differentiated derivatives do (Paulin et al. 1976) is consistent with our results concerning the nuclear localization of the fluorescence staining in the differentiated cell lines, and the absence of the fluorescence in the nuclei of EC cells.

In the present experiments the fluorescence staining reveals the intracellular localization of EC cell DNase since in blank experiments in which DNase-I IgG antibodies were absorbed with DNase-I-coupled Sepharose, the remaining fluorescence was negligible. On the other hand, when DNase-I IgG antibodies were incubated with actin-coupled Sepharose the fluorescence was positive and the patterns are unchanged. This second control experiment indicates that a precipitation on the actin microfibrillar network of traces of DNase-I complexed with DNase-I IgG antibodies present in small amounts in the highly purified antibody preparation is unlikely. Furthermore, the fluorescence pattern observed with actin antibodies either in EC cells or in differentiated lines are quite different from those seen in the present study. So, in EC cells the actin-specific fluorescence is found predominantly in surface protrusions and some of them resemble the structures seen in the rat glial line C6. This fluorescence pattern is very similar to that observed in the same cells by scanning electron microscopy which consists of multiple surface protrusions. The differentiated cell lines which are characterized by specialized functions (myoblasts, fibroblasts and mesenchymal-like cells) strongly develop cables similar to those seen in mouse 3T3 cells (Paulin et al. 1978).

Several markers of cell differentiation have been described, some of them being surface antigens. This is the case for F-9 and H-2 antigens (Martin, 1975), LETS protein (Zetter & Martin, 1978) and the high-molecular-weight glycopeptides (Muramatsu et al. 1978a). In contrast, EC cell DNase seems to be either an intracellular marker or a membrane bound protein since, on the one hand fluorescence staining can be revealed only in fixed cells, and on the other hand the different patterns observed indicate an intracellular localization.

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