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5-Azacytidine is able to induce the conversion of teratocarcinoma-derived mesenchymal cells into epithelial cells

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The inhibitor of DNA-methylation, 5-azacytidine (5-AzaC) induced the appearance of cytokeratin-containing cells in several mesenchymal cell lines such as teratocarcinoma-derived fibroblasts, preadipocytes and myoblasts, NIH-3T3 fibroblasts and human embryonic fibroblasts. At optimal 5-AzaC concentrations the proportion of such cells was in the range of 10^{-1} compared with $10^{-6}-10^{-4}$ in non-treated cultures. Dose-response curves indicated that the induction of cytokeratin was the result of an interaction of the drug with few targets. Stable, mature, keratinocyte cell lines, as well as lines of myoblasts and astrocytes, could be isolated from a teratocarcinoma-derived preadipocyte line, showing that 5-AzaC is able to provoke a wide range of complete phenotypic conversions. In these cell lines, the intermediate filaments corresponded to the morphological phenotype. Altogether, the results suggest that 5-AzaC preferentially activates certain genes.

Key words: cytokeratins/differentiation/intermediate filaments/methylation/TROMA I

Introduction

The inhibitor of DNA-methylation, 5-azacytidine (5-AzaC) has proved to be a valuable tool to study the role played by methylation in differentiation (Constantinides *et al.*, 1977, 1978; Taylor and Jones, 1979; Jones and Taylor, 1980; Venolia *et al.*, 1982). Mouse embryo fibroblasts can be converted by 5-AzaC into striated muscle cells, adipocytes and chondrocytes, and both the extent of induction of muscle cells and of inhibition of DNA methylation depend on the concentration of 5-AzaC (Jones and Taylor, 1980). It was also found that teratocarcinoma-derived myogenic cells (Jakob *et al.*, 1978), which had spontaneously drifted towards a fibroblastic or an adipogenic phenotype, reverted to a muscular phenotype after treatment with 5-AzaC (Darmon *et al.* 1981).

In those previous reports, the conversion of fibroblasts appeared to be restricted to the formation of other mesenchymal tissues. The aim of the work reported here was to determine whether the conversions of mesenchymal cells treated by 5-AzaC could extend to cell types of germ layers other than mesoderm. Intermediate filaments (reviewed by Lazarides, 1980; Osborn *et al.*, 1982) appeared to be suitable markers for such a study. (i) They are cell-type specific: cytokeratins are found only in epithelial cells, whatever their embryological origin; desmin only in muscle cells; the glial fibrillary acidic protein (GFAP) only in astrocytes; the neurofilament proteins only in neurones, while vimentin is found in mesenchymal cells. (ii) Polyclonal antibodies (PAb) or monoclonal antibodies (MAb) directed against individual intermediate filament proteins are available and make possible the detection of changes at the single-cell level (Franke *et al.*, 1978a, 1978b; Sun and Green, 1978, Brûlet *et al.*, 1980; Kemler *et al.*, 1981; Oshima, 1981; Debus *et al.*, 1982; Dellagi *et al.*, 1982; Maunoury *et al.*, 1977; Lane, 1982). Results described here show that 5-AzaC induces in a large proportion of cells of various mesenchymal lines the synthesis of a cytokeratin recognized by the Mab TROMA I, while induction of other intermediate filament proteins is rarely or not at all observed. Moreover, this drug was found to provoke, in a preadipocyte line, the emergence of rare but stable epidermal, glial and myoblastic cell lines.

Results

Previously (Darmon *et al.*, 1981) we found that adipogenic clones could be isolated from the teratocarcinoma-derived myogenic cell line 984 (Figure 1). One of these clones, 1246, was particularly studied because its biochemical differentiation and its hormonal responses were very similar to those of normal preadipocytes (Serrero and Khoo, 1982). Under conditions favoring adipose differentiation nearly all 1246 cells were able to form functional adipocytes, while under other conditions this cell line could segregate 45% fibroblastic

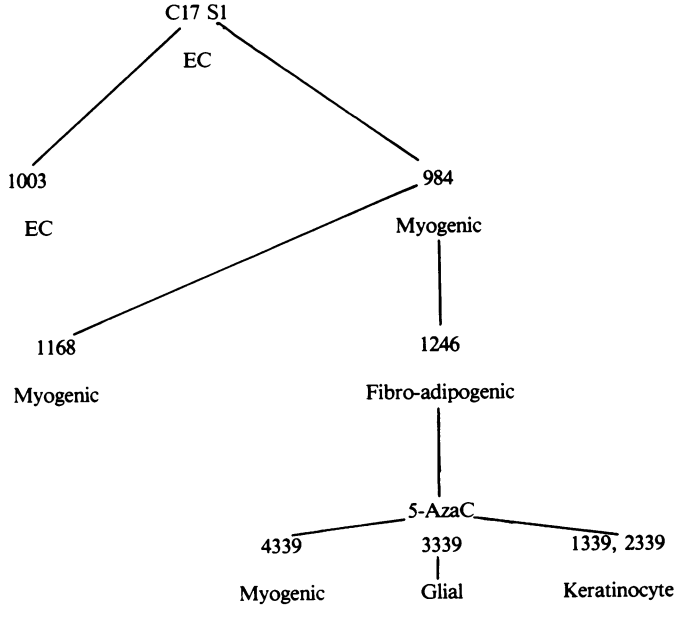
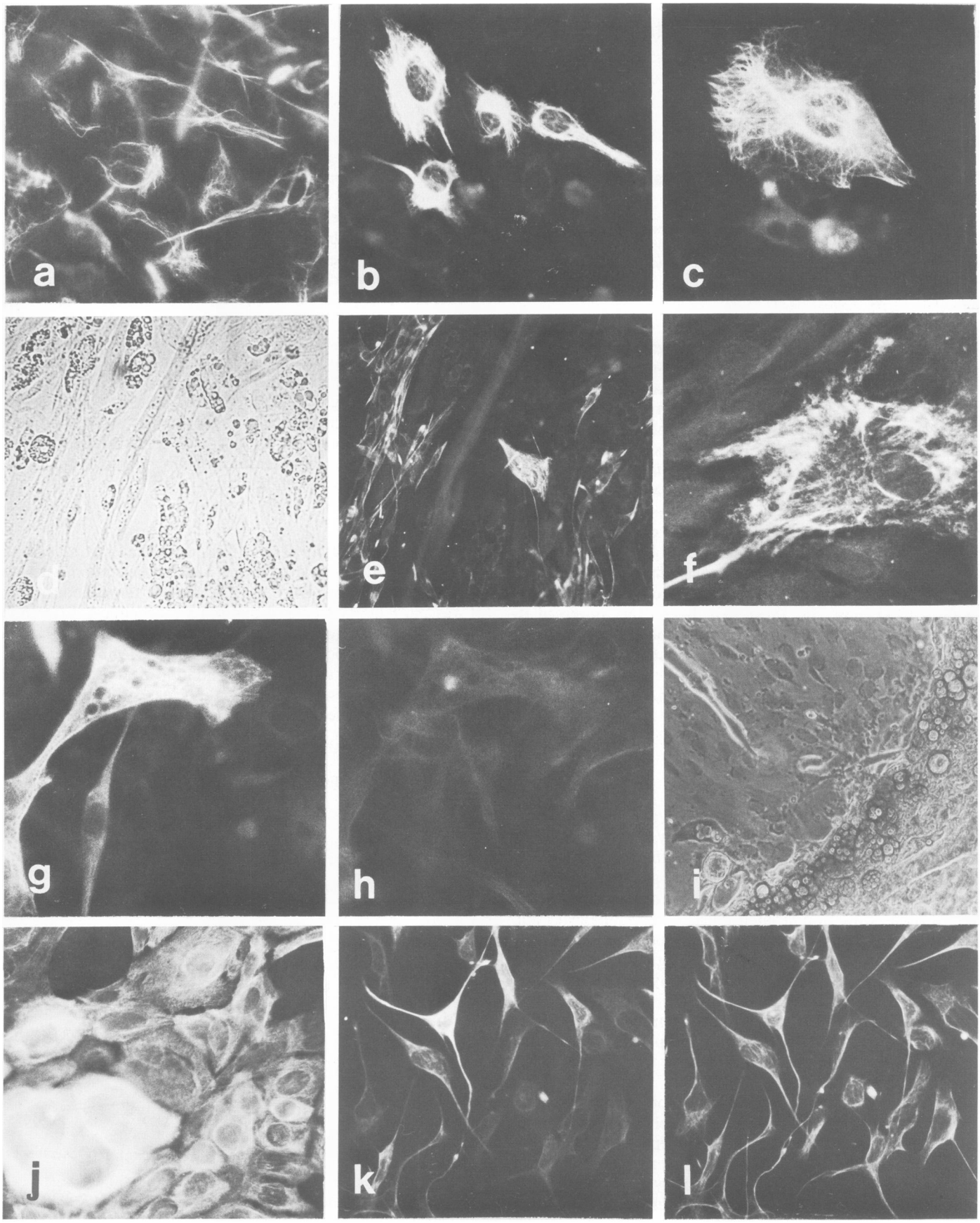


Fig. 1. Origin of the teratocarcinoma-derived cell lines used. C17-S1 (McBurney, 1976) and 1003 (Darmon *et al.*, 1982) are clonal multipotential embryonal carcinoma (EC) cell lines. 984 (Jakob *et al.*, 1978) is a clonal myogenic cell line segregating myogenic and adipogenic clones (Darmon *et al.*, 1981). 1339, 2339, 3339 and 4339 were obtained after treatment of 1246 with 5-AzaC (see text).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clones. After treatment with 5-AzaC, 1246 cells were found to be able to re-express a muscular phenotype (Darmon *et al.*, 1981). In the present study the 1246 cell line was chosen to evaluate what phenotypic conversions could be provoked by 5-AzaC, using intermediate filament proteins as markers of other cell types.

Exponential cultures of 1246 apparently contained only cells which stained with an anti-vimentin MAb (Figure 2A). Other kinds of intermediate filaments could not be detected by the various antibodies described in Materials and methods with our routine immunofluorescence technique (examination of a patch of culture containing 10^3-10^4 cells). Moreover, extraction of insoluble proteins in a high-salt buffer and subsequent gel electrophoresis (see Materials and methods) revealed a single polypeptide at 56 kd apparent mol. wt. recognized by anti-vimentin MAb in immune blots (Figure 3a and b). Immune blots performed on total 1246 protein extracts with reagents having a wide specificity for epithelia such as anti-epidermal keratins PAb (Sun and Green, 1978; Franke *et al.*, 1978b) or the KL1 MAb (Viac *et al.*, 1983) did not give any specific reaction (data not shown).

To study the effect of 5-AzaC, 1246 cells ($1-5 \times 10^3/cm^2$) were treated with non-toxic concentrations of 5-AzaC ($10^{-6}-10^{-5}$ M) for 24 h, refed, and, after various times, fixed and stained with the various antibodies.

5-AzaC induces the appearance of cytokeratin-positive cells in cultures of 1246

Striking results were obtained with the anticytokeratin MAb TROMA I. Cells staining with this antibody appeared one day after the end of the 24 h treatment with 5-AzaC (5×10^{-6} M) and, at day 3, ~10% of the cells were positive (Figure 2b). This finding was particularly interesting since TROMA I is known to recognize specifically epithelial cells (Brûlet *et al.*, 1980; Kemler *et al.*, 1981). Immune blots show that it reacts exclusively in a variety of cell lines (R. Kemler, personal communication) with a cytokeratin of 55 kd called EndoA (Oshima, 1981) or cytokeratin A (Jackson *et al.*, 1981) in the mouse, and corresponding to the 52.5-kd human component 8 (Moll *et al.*, 1982). Anti-EndoA PAb (Oshima, 1981) gave results similar to TROMA I but the staining was weaker. The absence of staining of non-treated 1246 cells with anti-EndoA PAb excluded the possibility that EndoA was present in those cells, but with the epitope recognized by TROMA I masked in some way. Such a masking phenomenon was reported with other anti-cytokeratin MAb (see for example Franke *et al.*, 1983). Moreover, immune blots performed with TROMA I MAb after gel electrophoresis (see Materials and methods) of high-salt-insoluble extracts revealed a band of 55 kd apparent mol. wt. in treated but not in control cells (Figure 3c and d). No reaction was observed by using a myeloma culture medium instead of TROMA I MAb (data not shown).

Treated cultures of 1246 cells also contained some cells (approximately one-tenth of the TROMA I-positive cells) which were labelled with the MAb TROMA III, also previously found to decorate exclusively the cytoskeleton of epithelial tissues (Kemler *et al.*, 1981), and rare cells labelled by the anti-keratin MAb LE 61 (Lane, 1982). No cells were labelled with KL1 MAb. Double-immunofluorescence experiments showed that most TROMA I-positive cells were also vimentin-positive, but in a substantial proportion of them (~20%) vimentin was absent (Figure 2g and h). TROMA I-negative cells all appeared to be vimentin-positive.

Finally, although most cells labelled by TROMA I retained a fibroblastic morphology with a weak, diffuse cytoplasmic staining or few filaments, a few percent of them exhibited an epithelial shape with a characteristic network of filaments (Figure 2c). In older cultures they formed typical epithelial sheets. Attempts were made to identify the nature of these epithelial cells. They were not labelled by PAb directed against villin, a protein specific for intestinal and renal microvilli (Reggio *et al.*, 1982) or PAb directed against membrane proteins of kidney microvilli. However, some cells were labelled with PAb directed against human epidermal keratins. These results were only indicative since anti-epidermal keratin antibodies are not specific for epidermal cells and cross-react with keratins found in most epithelia (Sun and Green, 1978; Franke *et al.*, 1978b). Cells containing other classes of intermediate filaments such as neurofilaments or GFAP were not detected in these experiments.

In the light of these results non-treated cultures were carefully re-examined. The scanning of whole culture dishes revealed rare TROMA I-positive cells (~10^{-4}). However, the possibility that 5-AzaC would act by selecting these pre-existing cells could be ruled out since the increase in the number of TROMA I-positive cells was observed at concentrations of the drug which have no toxic effect. Knowing the instability of the 1246 cell line it was necessary to determine whether all cells in the population were equally sensitive to 5-AzaC. Five subclones were isolated from 1246 and studied. All had a lower background of TROMA I-positive cells (~10^{-6}). Three of them, D1, D3, and D6, had properties similar to 1246, but two, C4 and D5, were unable to differentiate into adipocytes or to form myotubes after treatment with 5-AzaC. Nevertheless the drug induced them to form many TROMA I-positive cells. Thus, the ability to be converted by 5-AzaC into other mesenchymal cell types is not required for the formation of cytokeratin-containing cells.

Dose-response curves

The total number of cells and the number of TROMA I-positive cells were measured for concentrations of 5-AzaC ranging from 10^{-7} to 10^{-5} M. At day 1 the treatment by 5-AzaC did not cause cell death up to a concentration of 8×10^{-6} M; at 10^{-5} M, 80% of the inoculum had survived. At day 3 the number of cells was greater than the inoculum for all concentrations although a dose-dependent reduction in growth was observed.

Dose-response curves obtained with 1246 or 1246 D5 had similar shapes but, because of a lower background of TROMA I-positive cells, 1246 D5 seemed more appropriate to perform measurements at low concentrations of the drug. The dose-response curve obtained with this cell line at day 3 is reported in Figure 4. It reveals that: (i) for small concentrations of 5-AzaC ($10^{-7}-10^{-6}$) the frequency of TROMA

Fig. 2. Phenotypic conversions of embryonic fibroblasts. **(a)** 1246, non-treated, 5 days after plating (day 5), anti-vimentin MAb; **(b)** 1246, 10^{-6} M 5-AzaC, day 5, TROMA I MAb; **(c)** same as **(b)**, but 10^{-5} M 5-AzaC; **(d)** and **(e)**, 1246, 10^{-6} M 5-AzaC, day 18, TROMA I MAb; **(d)** direct light; **(e)** fluorescence; **(f)** GM10, 10^{-6} M 5-AzaC, day 7, anti-keratin PAb; **(g)** and **(h)** 1246, 10^{-5} M 5-AzaC, day 4, TROMA I (rat) MAb, anti-rat Ig coupled to rhodamine, anti-vimentin (human) MAb, anti-human IgM coupled to fluorescein; **(g)** rhodamine filters; **(h)** fluorescein filters; **(i)** 1246, 10^{-6} M 5-AzaC, 4 weeks, phase contrast; **(j)** 1339, day 8, anti-keratin PAb; **(k)** and **(l)**, 3339, day 6, anti-GFAP (rabbit) IgG, anti-rabbit IgG coupled to rhodamine, anti-vimentin (human) MAb, anti-human IgM coupled to fluorescein, **(k)** rhodamine filters; **(l)** fluorescein filters; **(a, b, j, k, l)**: x 380; **(c, f)**: x 580; **(d, e, i)**: x 150; **(g, h)**: x 760, **(b, c, d, e, f, g, h, i)**: cells were treated with 5-AzaC from the time of plating and for 24 h.

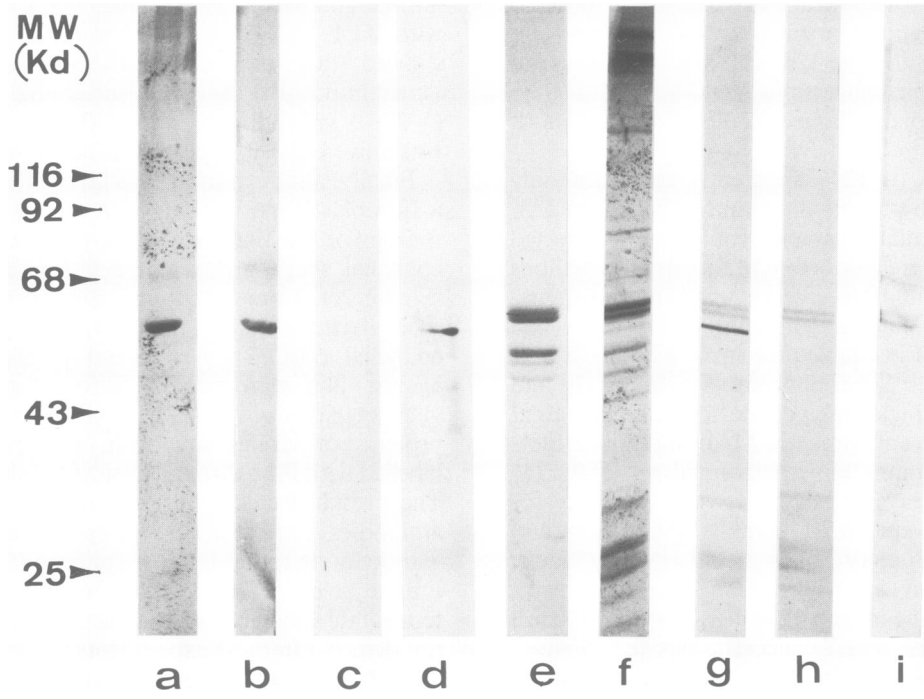

Fig. 3. 10% polyacrylamide gels and immune blots of 1246 and 1339 protein extracts. **(a)** Electrophoresis of high-salt-insoluble extract of 1246 cells; **(b)** corresponding immune blot performed with anti-vimentin MAb; **(c)** and **(d)** immune blots performed with TROMA I MAb after electrophoresis of high-salt-insoluble extracts of **(c)** 1246 untreated cells and **(d)** 1246 cells treated with 10^{-5} M 5-AzaC for 24 h and grown for 2 additional days; **(e)** and **(f)** electrophoresis of protein extracts of 1339 cells **(e)** high-salt extract, **(f)** total protein extract; **(g)** immune blot corresponding to **(f)** performed with TROMA I MAb; **(h)** same as **(g)** but with a myeloma culture supernatant instead of TROMA I; **(i)** immune blot corresponding to **(f)** performed with anti-vimentin MAb.

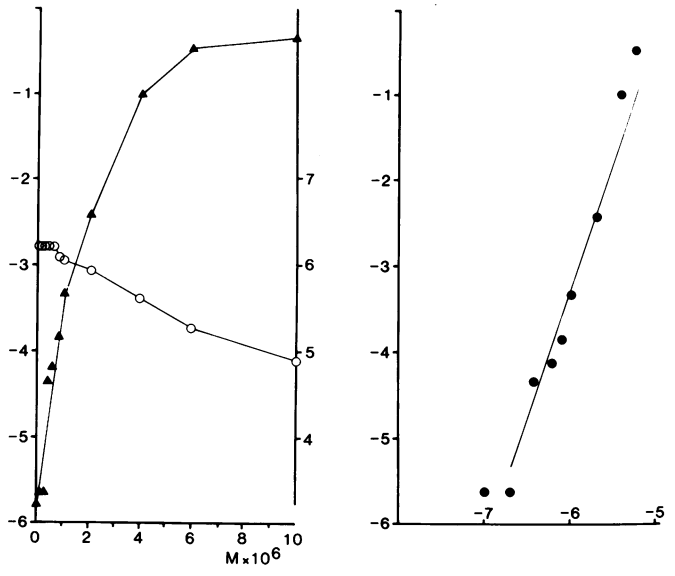

Fig. 4. Dose-response curves expressing the frequency of TROMA I-positive cells and the cell number as a function of 5-AzaC concentration. 4 x 10^4 1246 D5 cells were inoculated per 35 mm dish, and treated with 5-AzaC for 24 h. Measurements were done 48 h after the end of the treatment (day 3). The points represent the mean of duplicate samples. **Left:** (▲——▲), $\log_{10}$ of the frequency of TROMA I-positive cells (left ordinate) and (○——○), $\log_{10}$ of the cell number (right ordinate) as a function of 5-AzaC concentration. **Right:** (●——●), $\log_{10}$ of the frequency of TROMA I-positive cells as a function of the $\log_{10}$ of 5-AzaC concentration (M).

I-positive cells increases exponentially; (ii) at these concentrations the dose-dependent reduction in cell number is negligible; (iii) the slope of the log-log plot has a value of 3, indicating a small number of targets, theoretically inferior to 3.

Although at day 3 TROMA I-positive cells remained isolated or in small groups, they later formed small clusters suggesting that they were able to multiply. Nevertheless their number decreased with time as shown by dose-response curves at day 8 (data not shown) suggesting either a reduced growth of these cells or an ability to revert toward a TROMA I-negative phenotype. After 2–3 weeks some clusters of TROMA I-positive cells, frequently of epithelial morphology, were still detectable.

Myoblastic and preadipocyte cell lines are able to form TROMA I-positive cells, although myotubes and adipocytes formed in treated cultures are not labelled by the antibody

After 1 or 2 weeks, cultures of 1246 cells treated by 5-AzaC contained adipocytes and myotubes. These structures were not labelled by TROMA I (Figure 2d and c). Since preadipocytes could be induced to form cytokeratins, one could wonder whether myoblasts could behave similarly. A stable myogenic cell line (1168) derived from 984 (Figure 1), and able to give rise to 85% myogenic clones, was treated by 5-AzaC. TROMA I-positive cells were obtained at frequencies similar to those obtained with 1246 but the myotubes which eventually formed were not labelled by TROMA I. Together, these findings suggest that the induction of cytokeratins is incompatible with terminal adipocyte or muscular differentiations.

Isolation of stable keratinocyte, astrocyte and myoblast cell lines from 1246 cultures treated with 5-AzaC

The presence of cells of epithelial morphology labelled with antibodies directed against epidermal keratins in 1246 cultures treated with 5-AzaC encouraged us to isolate keratinocyte cell lines from these cultures. We took advantage of the observation made by Rheinwald and Green (1975) that although keratinocytes are rapidly overgrown by fibroblasts in mixed

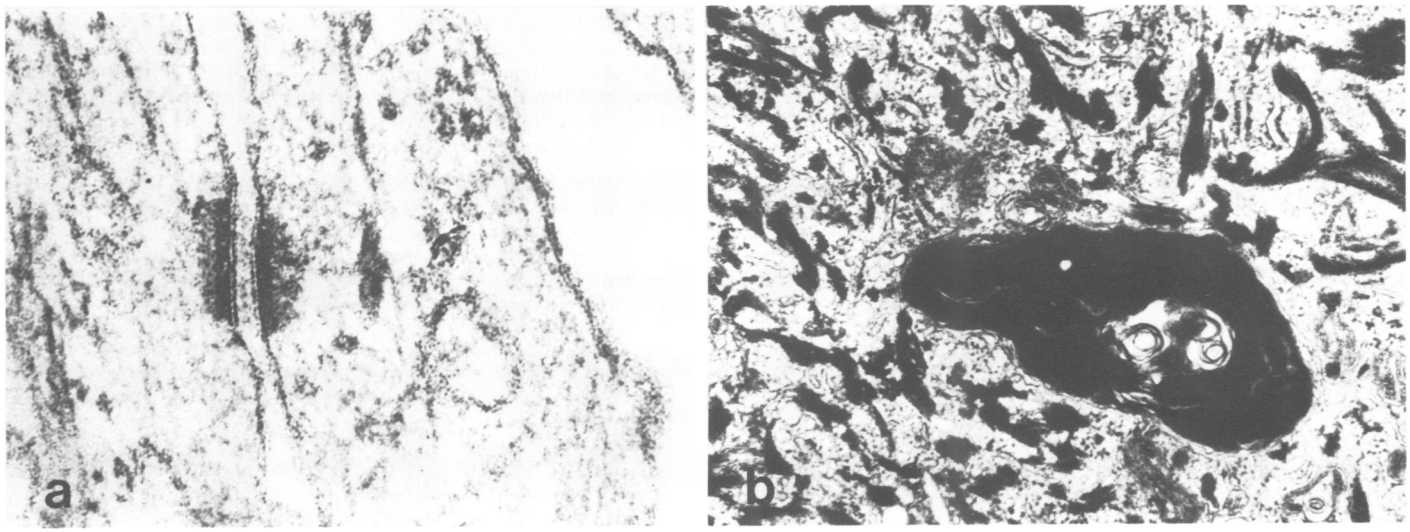

Fig. 5. Electron micrographs of 1339 keratinocytes. (**a**) desmosome, (x 130 000); (**b**) tonofilaments and keratin pearl (x 250 000).

cultures, they are able, in a second step, to push away the confluent fibroblastic monolayer and to give rise to macroscopically observable plaques. In a first experiment a total of 4 x 10^6 cells were seeded in a hundred 10 cm diameter dishes; half were treated with 10^{-6} M 5-AzaC and half used as controls. A total of 12 plaques formed by cells of epithelial morphology were observed after 4–5 weeks in the treated dishes but none in the controls. One of these plaques (Figure 2i) continued to expand and could be successfully subcultured, giving rise to a cell line, 1339. Confluent cultures of 1339 resembled primary cultures of epidermal cells; they were able to stratify and to produce rigid, vacuolated corneocytes desquamating in the medium (Figure 2j). Their keratinocyte nature was confirmed both by electron microscopy showing abundant desmosomes, tonofilaments, and keratin pearls (Figure 5a and b) and by the specific resistance to ebullition, in the presence of SDS and β-mercaptoethanol (Rice and Green, 1977), of the cornified envelopes of detaching cells. 1339 cells were all vimentin-negative; most of them were labelled with TROMA I MAb, TROMA III MAb and anti-epidermal keratins PAb (Figure 2j), but none with LE61 MAb. 1339 cells were not stained with antibodies directed against other intermediate filaments.

Immune blots performed with TROMA I MAb after gel electrophoresis of total protein extracts of 1339 revealed a strong band of 55 kd apparent mol. wt. (Figure 3g) and two non-specific (Figure 3h) faint bands of 56 and 58 kd corresponding to two major keratins seen in total and high-salt-insoluble extracts (Figure 3e and f). Vimentin could not be detected on immune blots of 1339 extracts (Figure 3i).

1339 clones could be obtained by limit dilution only when mixed with 1246 cells. The accompanying cells could then be washed away after incubation with 1 mM EDTA. Three clones were derived from 1339; they had properties similar to those of the parental line.

In a second experiment two expanding plaques could be subcultured. One of the resulting cell lines (2339) had properties very similar to 1339 but the other (3339) had completely different features. 3339 cells had the morphology of glial cells and contained GFAP (Figure 2k) as well as vimentin (Figure 2l), which is usually the case for cultured astrocytes (Sharp *et al.*, 1982); no other classes of intermediate filaments could be detected. Several GFAP-positive clones could be derived from the 3339 line. One of them was studied for its responsiveness to 5-AzaC. Although rare, TROMA I-positive cells could be detected at high concentrations of 5-AzaC (10^{-5} M). This observation shows that 5-AzaC is able to provoke a second round of phenotypic conversions in a stable 'converted' cell line. The same applies to the myoblastic cell line 4339 (see below).

Treated dishes each contained ~20–30 foci of myotubes. A myoblastic line (4339) was obtained by cloning the cells contained in such a focus. This cell line had a response to 5-AzaC similar to that of the myoblastic line 1168 described above: 5-AzaC provoked the formation of TROMA I-positive cells but myotubes, which eventually formed after differentiation had occurred, were not labelled. No cells of EC morphology or cells labelled with the EC-specific MAb, ECMA7 (Kemler, 1980) could be detected in cultures treated by 5-AzaC.

5-AzaC induces the formation of cytokeratin-positive cells in murine and human fibroblast lines derived from embryos

To exclude the possibility that the induction of TROMA I-positive cells by 5-AzaC was limited to teratocarcinoma cell lines, other mesenchymal cell lines were studied. NIH-3T3 fibroblasts, and two human embryonic dermal fibroblast lines, GM10 and 7000 were induced by 5-AzaC to form many TROMA I-positive cells. Most of them retained a fibroblastic shape but some acquired an epithelial shape. Moreover, some GM10 cells treated by 5-AzaC were labelled with anti-epidermal keratins PAb in a filamentous fashion (Figure 2f).

Discussion

The results described here show that 5-AzaC is able to induce in mesenchymal cells the synthesis of proteins normally specific of other tissues. Moreover, they show that the drug preferentially induces certain proteins. The EndoA cytokeratin recognized by the MAb TROMA I appeared in many cells, while other cytokeratins or other intermediate filament proteins were rarely or not at all observed. Since the analogue must be incorporated at random in the DNA, one might then admit that in a given cell not all genes are equally accessible to DNA-cytosine methyltransferases (Santi *et al.*, 1983). A lower accessibility of certain genes would explain the occurrence of spontaneous conversions and the higher inducibility of cer-

tain proteins. The number of sites that must be demethylated to lead to an activation might also vary from one gene to another. The high frequency at which TROMA I-positive cells are induced could then be explained by a small number of such sites in the corresponding gene. This is compatible with the dose-response curve, showing a limited number of targets.

Most of the TROMA I-positive cells induced by 5-AzaC retained a fibroblastic shape and exhibited either few filaments or a diffuse and weak cytoplasmic staining, suggesting that the appearance of a cytokeratin in a mesenchymal cell is not sufficient to provoke a morphological change. However, some TROMA I-positive cells exhibited an epithelial shape and well-organized filaments. This observation can be related to the results of Steinert *et al.* (1976) showing that the formation of keratin filaments *in vitro* requires the presence of members of the two different classes of cytokeratins, i.e., of products of the two different families of keratin genes (Fuchs *et al.*, 1981). It is thus tempting to postulate that the appearance of a network of filaments in the cells of epithelial shape is due to the induction by 5-AzaC of an additional keratin polypeptide. Double-immunolabelling with a series of specific anti-keratin antibodies should reveal whether this is the case. The low frequency of stable keratinocyte cell lines obtained after the formation of plaques in long-term experiments cannot be used as a measure of the proportion of this cell type since additional events are probably required for invasiveness or immortalization. Markers specific for undifferentiated keratinocytes should help to solve that question.

Although muscular foci are easily detected in 1246 treated cultures and found at roughly the same frequency as epithelial ones, a detailed study of muscular conversion cannot be easily undertaken since no markers are available to distinguish a myoblast from a fibroblast or a preadipocyte. The same comment can be made about the formation of astrocytes; at early steps of differentiation glial cells contain no GFAP and have a fibroblastic shape (Raff *et al.*, 1983); even at a mature stage morphological differences with fibroblasts are subtle (Figure 2k) and, without the fortuitous isolation of a mature and invasive astrocyte line no indication would have been obtained that such transitions can be induced.

The observed effects of 5-AzaC can be interpreted as being the result of a direct activation of structural genes (i.e., the Endo A gene). However, they could instead be a consequence of an activation of other DNA targets. Moreover, one should bear in mind that cell-cell interactions might play a role in the phenotypic changes observed. Complementary events induced in different cells by 5-AzaC might be required for the appearance of a new cell type.

Materials and methods

Cell lines

The phenotypes and culture conditions of 984 (Cl7Sl-DT984) and its subclones 1246 and 1168 have been described previously (Darmon *et al.* 1981; McBurney, 1976). The lineage of these cell lines is summarized in Figure 1. NIH-3T3 cells were a gift from Dr. F.Kelly. GM10 (Institute for Medical Research, Camden, NJ) and 7000 (Flow Lab. Puteaux, France) are lines of normal, human, embryonic, dermal fibroblasts. Cloning and treatment with 5-AzaC were performed as described (Darmon *et al.*, 1981).

Antibodies

TROMA I, TROMA III, LE61, and KL1 anti-cytokeratin MAbs have been described previously (Brûlet *et al.*, 1980; Kemler *et al.*, 1981); Lane, 1982; Viac *et al.*, 1983). Polyclonal antibodies (PAb) against human epidermal keratins were a gift from Dr. Y.Jacques. Anti-EndoA PAb were a gift from Dr. B.Oshima. Affinity-purified anti-GFAP PAb (Manoury *et al.*, 1977) was a gift from Dr. B.Eddé. Affinity-purified anti-70 K neurofilament PAb (Prochiantz *et al.*, 1982) anti-200 K neurofilament MAb (Anderton *et al.*, 1982) and human MAb recognizing vimentin (Dellagi *et al.*, 1982) were a gift from Dr. D.Paulin. ECMA7 MAb was described previously (Kemler, 1981). Affinity-purified PAb recognizing villin (Reggio *et al.*, 1982) or microvilli proteins of dog kidney were gifts from Dr. D.Louvard.

Immunofluorescence and cell counting

Cells were plated on 35 mm dishes at densities of $1-5 \times 10^3/cm^2$. Cultures were either trypsinized and the cells counted as described (Darmon *et al.*, 1981) or stained as follows. A central area of 15 x 15 mm was drawn on the bottom of the dish and the cells outside this area removed with a rubber policeman. The remaining patch of cells was fixed and stained as described (Darmon *et al.*, 1982). Fluorescent cells were counted either in the whole 2.25 cm^2 square, or in six rectangles of 15 mm length and having the diameter of x 16, x 25, or x 40 objective fields or else in ten x 16, x 25 or x 40 objective fields. Diameters of the fields were measured with a micrometer and the corresponding surfaces calculated. When the frequency of positive cells was high enough it was also determined directly by counting both positive and negative cells. Control cell lines known to express the various antigens studied were always included in the experiments.

Gel electrophoresis and immune blots

Total protein extracts and high-salt-insoluble proteins were prepared according to Winter *et al.* (1980). 10% SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Immune blots were performed according to Towbin *et al.* (1979) with the immunostaining procedure of Burnette (1981).

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