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S-phase inhibitors induce vimentin expression in human promonocytic U-937 cells

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The administration of hydroxyurea (3×10^{-4} M) and cytosine arabinoside (10^{-7} M) greatly induces the expression of the vimentin gene in human promonocytic leukemia U-937 cells. The induction takes place at both the mRNA and protein levels, as demonstrated by Northern blot, immunoblot and immunofluorescence assays. On the contrary, the drugs inhibit the expression of c-*myc* and ornithine decarboxylase, and do not modify significantly the expression of β-actin. Since hydroxyurea and cytosine arabinoside trigger the phenotypic differentiation of U-937 cells, as demonstrated by the induction of the differentiation-specific CD11b and CD11c antigens, it is concluded that vimentin expression might be implicated in the maturation of these cells.

Hydroxyurea; Cytosine arabinoside; Differentiation; Gene expression; Vimentin; U-937 cell

1. INTRODUCTION

One of the goals in the studies of cell differentiation consists in the characterization of the genes implicated in this process. It has been suggested that vimentin, the major intermediate-size filament protein in cells from mesenchymal origin, might be involved in the maturation of hemopoietic cells. Much of the evidence supporting this idea is given by the observation that the phorbol ester TPA, a potent maturation inducer of cultured myeloid cells, stimulates at the same time vimentin expression [1,2]. This observation must be considered with caution since TPA, a protein kinase C activator, is capable per se of stimulating many biochemical functions, including the expression of several genes, irrespective of the cell species and of the final consequence for the cell phenotype (see [3] for review). For instance, it has been shown that TPA stimulates vimentin synthesis in chicken embryo fibroblasts, but this is a non-differentiating cell line [4]. Also, TPA stimulates the expression of the ODC gene in human myeloid cells [5], whereas ODC does not appear to be directly implicated in the differentiation of these cells [5,6]. Thus, the establishment of a clear correlation between cell differentiation and the expression of vimentin (and, in general, of any other gene) may require the examination of the effects of other inducers with different action mechanisms at the molecular level.

In this work we study the action of two S-phase inhibitors, HU and ara-C, on U-937 cells, a human promonocytic leukemia cell line. We observe that HU and ara-C efficiently trigger the phenotypic differentiation of these cells. At the same time, the drugs greatly induce the expression of vimentin, both at the mRNA and protein levels.

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Abbreviations: HU, hydroxyurea; ara-C, cytosine arabinoside; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate: ODC, ornithine decarboxylase; TfR, transferrin receptor: SDS-PAGE, polyacrylamide gel electroforesis

2. MATERIALS AND METHODS

2.1. *Cell culture and drug treatment*

U-937 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 5 mM Hepes buffer, 0.2% (w/v) sodium bicarbonate, in humidified 5% Co_2 atmosphere at 37°C. Cells were seeded in 100-mm plastic dishes and maintained in continuous logarithmic growth by passing them every 2-3 days. TPA, HU and ara-C were obtained from Sigma (St. Louis, MO). TPA was dissolved in DMSO at 1.5×10^{-3} M. HU and ara-C were dissolved in RPMI at 3×10^{-2} M and 10^{-5} M, respectively. The drugs were applied at final concentrations of 3×10^{-8} M TPA, 3×10^{-4} M HU and 10^{-7} M ara-C.

2.2. *Immunofluorescence and flow cytometry*

To detect the expression of surface antigens, indirect immunofluorescence determinations were carried out using the MAbs Bear 1 (anti-CD11b) [7], HCl/1 (anti-CD11c) [8], and FG1/6 (specific for the TfR) [9]. The cells were labelled with the antibodies for 30 min at 4°C. After two washes with RPMI medium, FITC-labelled sheep anti-mouse IgG (Amersham, England) was added and the incubation followed for an additional period of 30 min at 4°C. After washing the cells twice with RPMI medium, their fluorescence was estimated by flow cytometry with an EPICS-flow cytometer, with an argon laser operated at 200 mV and excitation wavelength at 488 nm.

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To measure the cytoskeleton-associated vimentin, cells were fixed for 5 min at room temperature with 3.7% formaldehyde in a buffer containing 2 mM $MgCl_2$, mM EGTA and 100 mM PIPES, pH 6.8, and then permeabilized for 5 min at room temperature with 0.2% (v/v) Triton X-100, 3.7% (v/v) formaldehyde, in the same buffer. After two washes with RPMI medium, the cells were incubated with a mouse monoclonal anti-vimentin antibody (Amersham), washed twice with RPMI medium and incubated with FITC-labelled sheep anti-mouse IgG for 45 min at 37°C. After two washes with RPMI medium, the cell fluorescence was estimated by flow cytometry, as above.

2.3. *RNA blot assays*

Total cytoplasmic RNA was prepared as described in a previous work [10]. RNA samples (15 μg per lane) were denatured, then electrophoresed in 1.1% (w/v) agarose-formaldehyde gels [11] and blotted onto a nylon membrane. RNA blots were prehybridized, hybridized with excess ^{32}P-labelled probes, washed under highly stringent conditions [12], and finally autoradiographed. The probes used were: the 1.1 kb human vimentin-specific *XhoI* fragment of L_3A_7A plasmid [2]; the 1.5 kb *ClaI-EcoRI* fragment of pMC413rc plasmid, which contains the 3rd exon human c-*myc* [13]; the 1.5 kb human OCD-specific *XhoI* fragment of OB-821 plasmid [5], and the 0.66 kb chicken β-actin-specific *KpnI-BglII* fragment of pAL41 plasmid [14]. The fragments were labelled to $1.0–1.5 \times 10^9$ cpm/μg of DNA with (α-^{32}P) dCTP (3000 Ci/mmol, Amersham, UK) by random hexanucleotide priming [15].

2.4. *Immunoblot assays*

Cells were washed once with PBS and lysed in 62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol. After boiling for 2 min at 98°C, aliquots corresponding to 150 μg of total protein were separated in SDS-polyacrylamide (10%, w/v) slab gels, according to the Laemmli procedure [16]. Electrophoretic blotting onto nitrocellulose and immunological detection of proteins were carried out essentially as described by Towbin et al. [17], using mouse monoclonal anti-vimentin and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Denmark) as the first and the second antibodies, respectively, and developing with α-chloronapthol as substrate.

3. RESULTS

3.1. *Expression of cell surface antigens*

The capability of HU and ara-C to induce the differentiation of U-937 cells was assessed by measuring the surface expression of several antigens, namely CD11b, CD11c and TfR. As a control, we also included TPA, a potent maturation-inducing agent. The concentrations of 3×10^{-4} M HU, 10^{-7} M ara-C and 3×10^{-8} M TPA were adopted on the ground of their earlier use in studies of differentiation in human myeloid cells [5,18,19]. Previous studies had determined that CD11b and CD11c are differentiation-specific antigens [7,8]. On the contrary, TfR is expressed at high levels in rapidly growing myeloid cells, and decays during differentiation [9]. The results obtained are summarized in fig.1. Both HU and ara-C greatly induced the expression of CD11b and CD11c, the percentage of cells expressing these antigens at the 3rd day of treatment being comparable to that obtained with TPA. In addition, the S-phase inhibitors provoked a decrease in the number of cells expressing TfR, although to a lower extent than with the phorbol ester. These results suggest that HU and ara-C induce the phenotypic diferentiation of U937 cells.

3.2. *Induction of vimentin mRNA levels*

To analyze possible changes in the cellular content of vimentin RNA, Northern blot analyses were carried out using total cytoplasmic RNA extracted from either untreated cells or cells treated for increasing periods of time with HU and ara-C. The levels of vimentin RNA were barely detectable in untreated cells, but greatly induced by HU and ara-C (fig.2). This increase was first observed at 24 h and reached a maximum at 48 h.

To exclude a non-specific stimulatory action of HU and ara-C on transcription, we also measured the levels of other RNAs, namely c-*myc*, ODC and β-actin. The drugs reduced the levels of c-*myc* and ODC transcripts, the reduction being more rapid and intense in the case of HU. The levels of β-actin RNA were unaffected except for a slight decrease at the end (72 and 96 h) of the treatment (fig.2). These results suggest that vimentin transcription in U-937 cells is specifically stimulated by S-phase inhibitors.

3.3. *Induction of vimentin protein levels*

Finally, we found it of interest to analyze whether the induction of vimentin at the mRNA level by HU and ara-C is also reflected at the protein level. This was carried out using two different experimental approaches. First, the total cellular vimentin content was measured by immunoblot assays using whole extracts from either untreated cells or cells treated for increasing periods of time with HU and ara-C. The vimentin content was below detectable levels in untreated cells, but it increas-

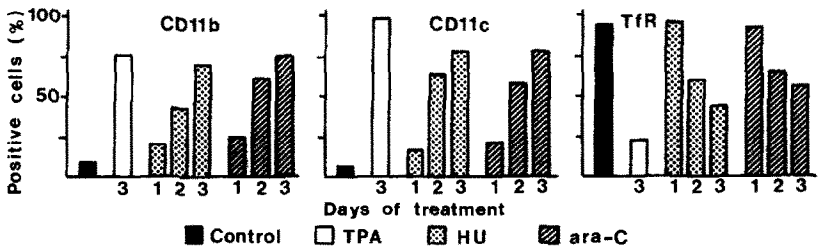

Fig.1. Expression of cell surface antigens in U-937 cells. Untreated (control) cells and cells treated with TPA (3×10^{-8} M), HU (3×10^{-4} M) or ara-C (10^{-7} M) for the indicated times were incubated with the MAbs Bear/1 (anti-CD11b), HC1/1 (anti-CD11c) or FG1/6 (anti-TfR). The percentage of cells expressing the antigens was determined by indirect immunofluorescence combined with flow cytometry. Data represent the average of three experiments.

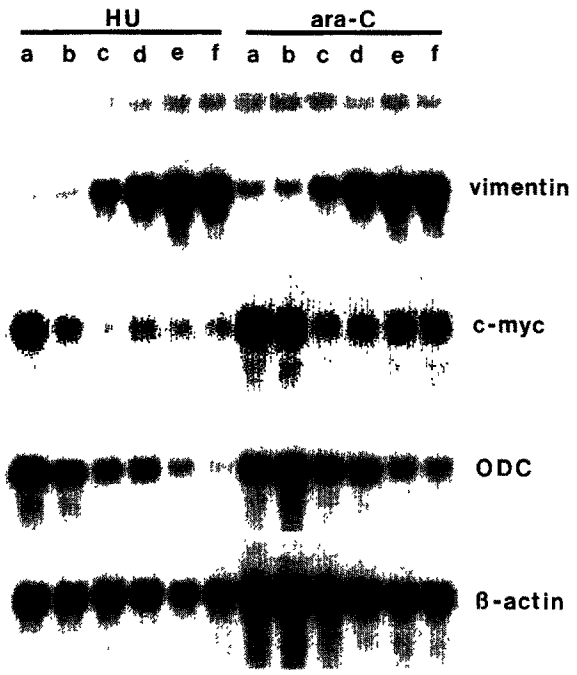

Fig.2. Changes in the levels of specific mRNAs. Total cytoplasmic RNA was extracted from untreated cells (a) and from cells treated for 6 h (b) 24 h (c), 48 h (d), 72 h (e) and 96 h (f) with either HU or ara-C. RNA blots (15 μg per lane) were prepared and hybridized sequentially with the indicated probes. The faint band at the top on vimentin hybridization represents non-specific hybridization of 28S ribosomal RNA. Results of one of two similar experiments.

ed to reach readily measurable levels in drug-treated cells (fig.3). In a second experimental approach, the vimentin content was measured by indirect immunofluorescence combined with flow cytometry. The vimentin was made accessible to specific antibodies by formaldehyde fixation followed by Triton

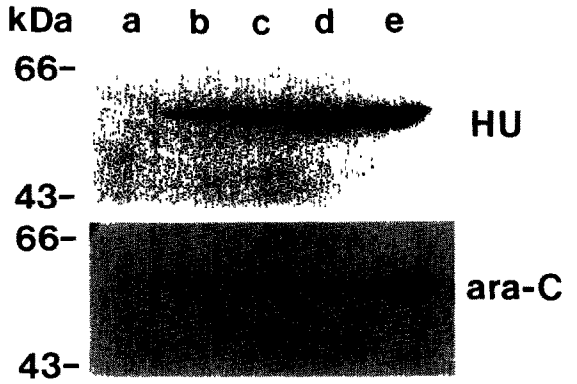

Fig.3. Changes in total vimentin content. Whole lysates were obtained from untreated cells (a) and from cells treated for 24 h (b), 48 (c), 72 h (d) and 96 h (e) with either HU or ara-C. Extracts corresponding to 150 μg of total protein were separated by SDS-PAGE, transferred onto nitrocellulose and assayed for reactivity with an anti-vimentin antibody. The reaction was developed using α-chloronaphtol as substrate. Only the region containing the vimentin band is shown in the figure. Results of one of two similar experiments.

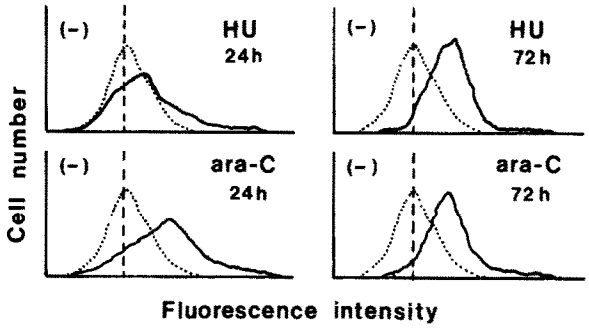

Fig.4. Changes in cytoskeleton-associated vimentin content. Following formaldehyde fixation, Triton X-100 permeabilization and incubation with an anti-vimentin antibody, the vimentin content in the cells was determined by indirect immunofluorescence combined with flow cytometry. Dotted lines: untreated cells. Continuous lines: drug-treated cells. The dotted, vertical lines, delimit the regions corresponding to non-specific fluorescence (-) which were given by the distribution of cells incubated only with the second antibody (FITC-labelled anti-IgG). Results of one of two similar experiments.

permeabilization of the cells. Thus, the fluorescent values reflect the fraction of non-extractable, cytoskeleton-associated vimentin. Treatment with HU and ara-C produced an increase in the fluorescence intensity in relation to untreated cells, demonstrating that the drugs effectively augment the vimentin in the cytoskeleton (fig.4).

4. DISCUSSION

The results presented here show that treatment with S-phase inhibitors triggers the phenotypic differentiation of human promonocytic leukemia U-937 cells. This was evidenced, firstly, by the induction by HU and ara-C of two surface differentiation-specific markers, namely the CD11b and CD11c antigens, with an efficiently similar to that obtained with the potent maturation inducer TPA. Another evidence at the molecular level is provided by the decrease of c-*myc* mRNA levels upon drug treatment, since differentiation induction of myeloid cells seems to be associated with down-regulation of c-*myc* expression (see [20,21] for reviews). In fact, c-*myc* expression decreases rapidly upon treatment with typical maturation inducers, such as TPA, DMSO or dibutyryl cyclic AMP [5,22,23]. On the contrary, HU does not inhibit c-*myc* expression in the absence of cell differentiation [24].

In addition, our results show that treatment with S-phase inhibitors greatly induces the expression of the vimentin gene in U-937 cells, both at the mRNA and protein levels. It must be noted: (i) that this effect is produced by two compounds, HU and ara-C, which inhibit DNA synthesis through different action mechanisms; and (ii) that none of these compounds modified vimentin expression when used as growth inhibitors in non-differentiating cell systems ([25] and unpublished observations). This suggests that vimentin in-

duction in U-937 cells is neither a mere consequence of cell growth inhibition nor a side effect of a particular drug, but instead it is related to the differentiation process. Earlier observations indicated that TPA induces vimentin expression in myeloid cells [1,2,5]. Nevertheless this agent also causes a transient activation of other genes, including ODC (5) and β-actin [26]. In contrast, the action of HU and ara-C seems to be more specific, since only vimentin and not the other genes examined by us were activated.

In summary, it appears that S-phase inhibitors trigger the phenotypic differentiation of U-937 cells, and that vimentin induction is probably implicated in this process. Whether it is an absolute requirement or not, as well as the exact role of vimentin expression in the maturation process, remains to be determined.

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REFERENCES

[1] Bernal, J.D. and Chen, L.B. (1982) Cancer Res. 42, 5106–5116.
[2] Ferrari, S., Battini, R., Kaczmarek, L., Rittling, S., Calabretta, B., de Riel, J.K., Philiponis, U., Wei, J.F. and Baserga, R. (1986) Mol. Cell Biol. 6, 3614–3620.
[3] Kikkawa, K. and Nishizuka, Y. (1986) Annu. Rev. Cell Biol. 2, 149–178.
[4] Laszlo, A. and Bissell, M.J. (1983) Exp. Cell Res. 148, 221–234.
[5] Rius, C. and Aller, P. Cell Differ., in press.
[6] Luk, G.D., Civin, C.I., Weismann, R.M. and Bayleu, S.B. (1982) Science 216, 75–77.
[7] Keizer, D.D., Birst, J., Figdor, C.G., Spits, H., Miedema, F., Terhorst, G. and De Vries, C.G. (1985) J. Immunol. 15, 1142–1148.
[8] Cabañas, C., Sanchez-Madrid, F., Acevedo, F., Bellon, T., Fernandez, J.M., Larraga, V. and Bernabeu, C. (1988) Hybridoma 7, 167–176.
[9] Bernabeu, C., Morago, G., De Landazuri, M.O., Carreira, J. and Sanchez-Madrid, F. (1986) Immunologia 5, 83–90.
[10] Aller, P. and Baserga, R. (1986) J. Cell Physiol. 128, 362–366.
[11] Lehrach, H., Diamond, D., Wozney, M. and Boedtke, H. (1977) Biochemistry 16, 4743–4749.
[12] Hirschhorn, R.R., Aller, P., Yuan, Z.A., Gibron, C.W. and Baserga, R. (1984) Proc. Natl. Acad. Sci. USA 81, 6004–6008.
[13] Dalla Favera, R., Gelmann, E.P., Martinotti, S., Franchini, G., Papas, T.S., Gallo, R.C. and Wong-Staal, F. (1982) Proc. Natl. Acad. Sci. USA 79, 6497–6501.
[14] Alonso, S., Minty, A., Borlet, Y. and Buckingham, M. (1986) J. Mol. Evol. 23, 1–22.
[15] Feinberg, B.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267.
[16] Laemmli, U.K. (1970) Nature 227, 680–683.
[17] Towbin, H., Stachlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Griffin, J., Munroc, D., Major, P. and Kufe, D. (1982) Exp. Hematol. 10, 774–781.
[19] Yen, A., Freeman, L. and Fishbangh, J. (1988) Exp. Cell Res. 174, 98–106.
[20] Muller, R. (1986) Trends Biochem. Sci. 11, 129–132.
[21] Birnie, G.D. (1988) Br. J. Cancer 58, suppl. IX, 41–45.
[22] Watanabe, T., Sariban, E., Mitchel, J. and Kufe, D. (1985) Biochem. Biophys. Res. Commun. 126, 999–1005.
[23] Trepel, J.B., Calamonici, O.R., Kelly, K., Schwab, G., Watt, R.A., Sansville, E.A., Jaffe, E.S. and Trepel, L. (1987) Mol. Cell Biol. 7, 2644–2648.
[24] Grosso, L. and Pitot, H.C. (1984) Biochem. Biophys. Res. Commun., 119, 473–480.
[25] Aller, P., Hirschhorn, R.R. and Rius, C. (1989) Biochem. Int. 18, 119–127.
[26] Sieber, P.D. and Fukuda, M. (1985) J. Biol. Chem. 260, 3868–3874.