Vimentin expression as a late event in the in vitro differentiation of human promonocytic cells

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Summary
The administration of either 12-O-tetradecanoyl phorbol-13-acetate (TPA, 3×10⁻⁶M), sodium butyrate (SB, 10⁻³M), N⁶,2'-O-dibutyryl adenosine-3':5'-cyclic monophosphate (dbcAMP, 10⁻³M), cytosine arabinoside (ara-C, 10⁻⁷M), amsacrine (mAMSA, 10⁻⁷M) or retinoic acid (RA, 10⁻⁶M) inhibits the growth activity of human promonocytic U-937 cells, by arresting them at G₁ or at the G₁/S border (SB, RA, ara-C), at G₂ (mAMSA) or at G₁ and G₂ (dbcAMP). All these agents trigger cell differentiation, as proved by the increased expression of the maturation-associated CD11b and CD11c surface antigens, and induce the expression of the vimentin gene at both the protein and the mRNA levels. TPA, SB and dbcAMP behave as “early” inducers, in the sense that vimentin mRNA levels are rapidly increased (hour 6) upon drug administration. In contrast, mAMSA and RA behave as “late” inducers, since they do not increase vimentin mRNA levels until 48 to 72 hours, following the stimulation of surface antigen expression. The action of RA is characterized by an initial inhibition period, in which the basal level of vimentin mRNA is abolished (hour 24). Nevertheless, this RNA is later re-induced, to reach at 72 hours higher levels than in untreated cells. Moreover, RA is capable of delaying the early induction of vimentin expression caused by TPA and SB, without affecting the normal expression of differentiation markers. Taken together, these results strongly suggest that vimentin expression is not required at the initial stages of promonocytic cell differentiation, although it could play a role at an advanced stage.

Key words: vimentin expression, differentiation, promonocytic cells.

Introduction
The intermediate-size filament protein vimentin is expressed in cells of mesenchymal origin, as well as in most cell types in culture. Vimentin behaves as a cell growth-dependent gene, in the sense that its expression is rapidly stimulated when quiescent, G₀-arrested cells are induced to proliferate by serum or specific mitogens (Hirschhorn et al., 1984; Ferrari et al., 1986). Also, vimentin is a differentiation-dependent gene, since its expression is modulated during cell maturation, according to the cell lineage. For instance, in vivo studies using normal hemopoietic precursors showed that vimentin is conserved in mature granulocytes and monocytes, whereas it disappears in megakaryocytes and erythroid cells (Dellagi et al., 1983). In addition, in vitro studies using mammalian leukemia cell lines indicated that vimentin expression is inhibited in murine (MEL) cells differentiating along the erythroid pathway (Ngai et al., 1984), while it is increased when murine (M1) or human (HL-60 and U-937) cells differentiate along the monocyte pathway (Rius and Aller, 1989; Hass et al., 1989; Tsuru et al., 1990). In the latter case, the observations that vimentin is increased by diverse differentiation inducers such as the phorbol ester TPA (Hass et al., 1989), sodium butyrate (Rius et al., 1990) and conditioned medium from rat embryo fibroblasts (Tsuru et al., 1990), and that it reverts to basal levels when cells “retro-differentiate” upon withdrawal of the inducer (Hass et al., 1990), seem to indicate that vimentin expression is functionally linked to the maturation of promonocyctic cells. Moreover, vimentin expression is rapidly stimulated upon treatment with those inducers, suggesting that this protein could play a role at the initial stages of the differentiation process (Bernal and Chen, 1982). Nevertheless, Taimi et al. (1990) have recently reported that 1,25-dihydroxyvitamin D₃ and retinoic acid induce monocyte-like properties in U-937 cells in the absence of vimentin expression. Hence, vimentin stimulation could be a particular effect of some inducers, instead of being associated with differentiation itself.

The relationship between vimentin expression and promonocyte cell differentiation is examined in this work by comparatively analyzing the action of several maturation inducers on U-937 human promonocytic
cells. The inducers used were: the phorbol ester TPA, sodium butyrate (SB), dibutyryl cyclic AMP (dbcAMP), cytosine arabinoside (ara-C), amsacrine (mAMSA) and retinoic acid (RA). The results indicate that although these agents have different action mechanisms at the molecular level and different effects on the growth cycle, all of them stimulate vimentin expression both at the protein and the mRNA levels. Nevertheless, the kinetics of vimentin induction vary very much with the agent used, suggesting that this protein is not required at the early stages of the differentiation process.

**Materials and methods**

**Cell growth and drug treatment**

U-937 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.2% sodium bicarbonate and antibiotics, in a humidified 5% CO₂ atmosphere at 37°C. Cells were seeded in 100 mm plastic dishes at approximately 2x10⁶ cells per milliliter and maintained in continuous logarithmic growth by passing them every 2 or 3 days. TPA, dbcAMP, ara-C and RA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SB was obtained from Merck (Darmstadt, FRG). mAMSA (Lamasine) and α-difluoromethylornithine (DFMO) were generous gifts from Bristol Myers, SAE (Spain) and the Merrel Dow Research Institute (Strasbourg, France), respectively. Stock solutions of TPA (1x10⁻⁵M), mAMSA (1x10⁻⁵M) and RA (1x10⁻⁶M) were prepared in DMSO, RPMI and absolute ethanol, respectively, and kept at −20°C. At the final concentrations used, DMSO and ethanol were without significant effects on cell growth and differentiation. Stock solutions of dbcAMP (1x10⁻⁴M), SB (1x10⁻⁴M), ara-C (1x10⁻⁷M) and DFMO (1x10⁻⁴M) were freshly prepared in RPMI just before their application. Cells were seeded for experiments, in a mixture of conditioned medium and fresh medium (approximately 1/3, v/v). Cells from control cultures were represented in Fig. 1. All treatments inhibited the proliferation activity to a similar extent (Fig. 1a), but they had different effects on cell cycle distribution (Fig. 1b). In fact, while SB-, RA- and DFMO-treated cells were arrested at G₁, mAMSA-treated cells were arrested at G₂, and dbcAMP blocked cells at both G₁ and G₂, ara-C is believed to block cells at the G₁/S border or at very early S-phase (Tobey and Crismann, 1972), but this could not be distinguished from the G₁-blockade by the technique employed here (Fig. 1b).

We afterwards determined the capacity of these growth inhibitor agents to induce cell differentiation. This was carried out by measuring the surface expression of the leukocyte integrins CR3 (CD11b/CD18) and p150,95 (CD11c/CD18) was analyzed by measuring reactivity with the mAbs Bear 1 (anti-CD11b) and HCl/1 (anti-CD11c). These antibodies were obtained by immunization of Balb/c mice with human monocytes in the case of Bear 1 (Keizer et al., 1985), and with TPA-stimulated cells, in the case of HCl/1 (Cabañas et al., 1988). Cells were first incubated for 30min at 4°C with the mAb, then washed with RPMI medium and incubated again under the same conditions with FITC-labelled sheep anti-mouse IgG (Amersham, Buckinghamshire, UK). Some cell samples were incubated only with this second antibody to determine the background of non-specific fluorescence. After washing the cells with RPMI, their fluorescence was estimated by flow cytometry, as above.

To measure the cytoskeleton-associated vimentin, cells were fixed for 5min at room temperature with 3.7% (v/v) formaldehyde, in a buffer containing 2mM MgCl₂, 1mM EGTA and 100mM PIPES, pH 6.8, and then permeabilized for 5min with 0.2% (v/v) Triton X-100, 3.7% (v/v) formaldehyde, in the same buffer. After washing with RPMI medium, the cells were incubated for 45min at 37°C with a mouse anti-vimentin mAb (Amersham), washed again, and incubated under the same conditions with FITC-labelled sheep anti-mouse IgG. The cells were washed with RPMI medium and either examined by flow cytometry, as described above, or mounted on glass slides and examined by fluorescence microscopy.

**RNA blot assays**

Total cytoplasmic RNA was prepared as described previously (Aller and Baserga, 1986). RNA samples (15µg) were denatured, electrophoresed in 1.1% agarose-formaldehyde gels (Lehrach et al., 1977) and blotted onto nylon membranes (Hybond-N, Amersham). RNA blots were prehybridized, hybridized with excess 32p-labeled probes, washed under highly stringent conditions (Hirschhorn et al., 1984) and finally autoradiographed. The probes used were: the 1.1kb human vimentin-specific XhoI fragment of p4Fl plasmid (Ferrari et al., 1986) and the 0.66kb mouse β-actin-specific XhoI/BglII fragment of pAL41 plasmid (Alonso et al., 1986). The fragments were labeled to approximately 10⁶cts/min per µg of DNA with [α-32P]dCTP (3000Ci/mmol, New England Nuclear, Boston, MA, USA) by random hexanucleotide priming (Feinberg and Vogelstein, 1984).

**Results**

**Cell growth and differentiation**

We first determined the action of SB (1x10⁻³M), dbcAMP (1x10⁻³M), ara-C (1x10⁻⁷M), mAMSA (1x10⁻⁷M), RA (1x10⁻⁶M) and DFMO (5x10⁻⁴M) on the growth of U-937 cells. Some of the results obtained are represented in Fig. 1. All treatments inhibited the proliferation activity to a similar extent (Fig. 1a), but they had different effects on cell cycle distribution (Fig. 1b). In fact, while SB-, RA- and DFMO-treated cells were arrested at G₁, mAMSA-treated cells were arrested at G₂, and dbcAMP blocked cells at both G₁ and G₂, ara-C is believed to block cells at the G₁/S border or at very early S-phase (Tobey and Crismann, 1972), but this could not be distinguished from the G₁-blockade by the technique employed here (Fig. 1b).

We afterwards determined the capacity of these growth inhibitor agents to induce cell differentiation. This was carried out by measuring the surface expression of CD11b and CD11c antigens. CD11b and CD11c are the α subunits of the leukocyte adhesion molecules CR3 (CD11b/CD18) and p150,95 (CD11c/CD18), respectively (Arnaout, 1990), the expression of which has been found to be increased during the in vitro differentiation of human myeloid cells (Keizer et al., 1985; Cabañas et al., 1988; Dudley et al., 1989; Rosmarin et al., 1989). We also examined the action of the phorbol ester TPA, a potent maturation inducer of these cells. With the exception of DFMO, all
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agents increased the number of cells expressing CD11b and CD11c (Fig. 1c), suggesting that they trigger the functional maturation of U-937 cells. This conclusion was cytochemically confirmed by observing an increase in the capacity of the treated cells to reduce nitroblue tetrazolium (results not shown). The inability of DFMO to induce the expression of differentiation markers in U-937 cells is consistent with results obtained earlier using the human myelomonocytic HL-60 cell line (Sugiura et al., 1984).

Vimentin expression

The effect of the assayed agents on vimentin expression was first determined at the protein level by using a specific anti-vimentin antibody and examining the cells by fluorescence microscopy. Some of the results obtained are represented in Fig. 2. The fraction of cells with detectable amounts of vimentin, which was below 5% in untreated cells, increased upon treatment with TPA (2 days), SB, dbcAMP, ara-C, mAMSA (3 days) and RA (4 days) to values ranging from 40% (ara-C) to 80% (TPA). DFMO, which did not induce differentiation, also failed to stimulate vimentin expression.

Then, we attempted to determine vimentin expression at the mRNA level. For this purpose, Northern blot assays were carried out using RNA extracted from either untreated cells or cells treated for different periods of time with the indicated agents. The results in Fig. 3 show that all differentiation inducers augmented the vimentin mRNA content. This up-regulation was not due to non-specific stimulation of transcription, since the level of β-actin mRNA was little modified, or even decreased at the latest times of treatment (Fig. 3, and results not shown). Nevertheless, the kinetics of vimentin mRNA increase varied greatly with the agent used. In fact, while a significant stimulation of this mRNA level was already observed upon 6 hours of treatment with TPA, SB or dbcAMP, stimulation was not observed until after 24 or 48 hours of treatment with ara-C and mAMSA, respectively. The action of RA was characterized by an initial inhibition period, lasting for at least 24 hours, in which the basal vimentin mRNA level was completely abolished. Nevertheless, this RNA was later re-induced, to reach after 72 hours higher levels than in untreated cells. This re-induction cannot be attributed to cell de-differentiation, caused by drug exhaustion or inactivation. In fact, over the 72 hours treatment period studied, RA increased progressively the expression of differentiation-associated antigens (see Fig. 4), and decreased progressively the growth activity.

A trivial explanation for the differences in the kinetics of vimentin expression is that they could be
Fig. 2. Changes in vimentin content. Following formaldehyde fixation and Triton X-100 permeabilization, the reactivity of untreated cells (a) and of cells treated for 2 days with TPA (b), for 3 days with dbc AMP (c) and for 4 days with RA (d) with an anti-vimentin mAb was examined by fluorescence microscopy. Bar, 15 μm.

Fig. 3. Time course of changes in vimentin mRNA levels. Samples of total cytoplasmic RNA extracted from untreated cells (Control) and cells treated with the indicated agents were electrophoresed, blotted to membranes, and sequentially hybridized with 32P-labelled vimentin and β-actin probes. The autoradiogram corresponding to vimentin in the RA experiment was overexposed, to show more clearly both the down-regulation and the up-regulation of this RNA. The values in the lower panel were obtained by densitometric readings of RNA blots. Controls were arbitrarily given the value 1.

reflecting differences in the capacity of the agents used to trigger cell maturation. For this reason, we measured the time-course expression of the CD11b differentiation-associated antigen upon treatment with SB, mAMSA and RA. A significant increase in antigen expression was first observed in all cases after approximately 24 hours of treatment. This increase followed the induction of vimentin mRNA by SB, but preceded its induction by mAMSA and RA (Fig. 4). Based on these observations, we can interpret vimentin mRNA induction as an early event in the case of SB, and as a late event in the case of the mAMSA or RA-produced differentiation process.

Since RA transiently decreased vimentin RNA level, we wondered whether this agent could prevent the rapid activation of the vimentin gene caused by other inducers. With this purpose in mind, cells were treated for 12 and 24 hours with TPA or with SB, either in the absence or in the presence of RA. We found that RA suppressed or greatly reduced the stimulation of vimentin expression produced by SB and TPA, as measured at the RNA level by Northern blot assays (Fig. 5a). No treatment significantly altered β-actin mRNA level, which was measured as a control. This excluded possible experimental artifacts such as differences in sample loading in the gel (Fig. 5a). Suppression by RA of most TPA- and SB-produced vimentin induction was also observed at the protein level by immunofluorescence combined with flow cytometry (Fig. 5b). However, RA did not prevent the induction of the CD11b and CD11c antigen expression, observed upon 24 hours treatment with TPA or SB (Fig. 6). Also, RA did not prevent cell to cell adhesion nor cell attachment to the plate surface, which normally occurs upon treatment of myeloid cells with TPA (Fig. 7). After prolonged periods of treatment, RA no longer inhibited, but instead potentiated the stimulation of vimentin expression caused by the other inducers (results not shown).
In vitro maturation of myeloid leukemia cells may be triggered by agents with different primary action mechanisms. Among the agents used here, TPA and cyclic AMP derivatives seem to act through the activation of protein kinases C (Ca²⁺-dependent) and A (cyclic AMP-dependent), respectively (Kikkawa and Nishizuka, 1986; Krebs and Beavo, 1979). SB causes histone hyperacetylation, which may result in the weakening of histone/DNA interactions and in the enhanced expression of some genes, including those critical for differentiation induction (Kruh, 1982). The DNA replication inhibitor ara-C causes early termination of nascent DNA chains (Kufe et al., 1980), while the topoisomerase II inhibitor mAMSA provokes DNA breakage (Nelson et al., 1984). Thus, induction of differentiation by ara-C and mAMSA may represent a cellular response to alterations in the integrity and the normal functioning of the genome (Bloch, 1989). In addition to the diversity in their primary action mechanisms, these agents display different effects on the cell cycle, since the cells are arrested at G₁ by TPA and SB, at G₂ by mAMSA, at G₁ and G₂ by dbcAMP, and probably at the G₁/S border by ara-C (Tobey and Crismann, 1972; Yen et al., 1987; and results in this work). The observation that, in spite of all these differences, the assayed inducers stimulate without exception the expression of the vimentin gene, strongly indicates that vimentin expression is somewhat linked to differentiation itself. Moreover, vimentin is not increased by DFMO, which inhibits cell growth without provoking differentiation, indicating that its induction is not a mere consequence of proliferation arrest.

Differentiation is a multistep process, in which each stage is associated with the expression of specific genes (Davis et al., 1987). The observation that TPA rapidly stimulated vimentin expression (Bernal and Chen, 1982), a fact later corroborated with other inducers (Rius et al., 1990; Tsuru et al., 1990), led to the proposition that vimentin could play a role at the early stages of myeloid cell differentiation (Bernal and Chen, 1982). Although “early” and “late” are rather imprecise terms, since they depend much on the criteria used to examine differentiation, the results in the present work seem to argue against this proposition. In fact, vimentin induction was not detected until after 48 to 72 hours of treatment with mAMSA and RA, following the activation of differentiation-specific antigens. Moreover, the activation of vimentin expression by the early inducers TPA and SB was delayed when RA was added together with these agents, without affecting the timing of expression of differentiation markers. Hence, it appears that whatever the time of induction,
vimentin expression is not required at the initial stages of promonocytic cell maturation.

RA was the only assayed inducer capable of decreasing vimentin mRNA levels in U-937 cells. Down-regulation of this RNA by RA was earlier reported by Ferrari et al. (1986) and by us (Rius and Aller, 1989) in human myelomonocytic HL-60 cells. RA seems to exert its biological action through interaction with specific receptors which, upon internalization, behave as nuclear transcription factors (Collins et al., 1990). Since transcription of the vimentin gene is under the control of both positive and negative “cis-acting” regulatory DNA sequences (Rittling and Baserga, 1987), inhibition of vimentin expression could be the result of specific binding of internalized RA receptors to the negative regulatory sequences. Nevertheless, following the initial decrease, vimentin mRNA was reinduced in RA-treated U-937 cells, reaching higher levels at 72 hours than in untreated cells. This contrasts with the result in RA-treated HL-60 cells, where this transcript remained permanently inhibited (Ferrari et al., 1986; Rius and Aller, 1989). RA induces monocytelike maturation of U-937 cells (Taimi et al., 1990), whereas it causes granulocyte-like maturation of the bipotent HL-60 cells (Breitman et al., 1980). Hence, the different behaviour of vimentin expression in these cell lines might indicate that this protein is somewhat involved in cell maturation along the monocyte pathway but not along the granulocyte one. Nevertheless, Taimi et al. (1990) reported that RA confers monocytelike properties to U-937 cells in the absence of vimentin expression, measured at the protein level. This observation contrasts with the results obtained by us (Fig. 2d). We do not know the reasons for such a discrepancy - whether it is due to differences in the applied methodology or in properties inherent to the cell clones used - but it imposes obvious caution when proposing a role for vimentin on cell differentiation.

In summary, the results in this work indicate that vimentin expression is not required to initiate the differentiation of human promonocytic cells, although this protein might have a function at a later stage of the maturation process. Although the physiological significance of the intermediate filaments is not well understood, and different functions have been attributed to these proteins (for reviews see Traub, 1985; Klymkowski et al., 1989), one of their major roles seems to be to participate in the mechanical integration of the intracellular space. The acquisition of the differentiated phenotype often involves changes in cell morphology, such as cell attachment and spreading (TPA), cell to cell adhesion (TPA and, to some extent, RA), growth in size (ara-C, mAMSA) and adoption of elongated or other irregular shapes (SB, dbcAMP, ara-C, mAMSA). Increased amounts of vimentin could then be needed, as a part of the cytoskeleton reorganization, in order to sustain these changes.

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