PMA-induced down-regulation of the receptor for α2-macroglobulin in human U937 cells

Massimo Conese[a], Ugo Cavallaro[a], Nicolai Sidenius[a], David Olson[b], Marco R. Soria[a], Francesco Blasi[a,b,*]

[a]*Department of Biological and Technological Research, Istituto Scientifico H.S. Raffaele, and Department of Genetics and Microbial Biology, University of Milano, via Olgettina 60, 20132 Milan, Italy*
[b]*Microbiology Institute, University of Copenhagen, Copenhagen, Denmark*

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Abstract Transcription and expression of the urokinase (uPA) receptor (uPAR) are strongly stimulated by PMA. As for uPAR, the expression of α2-MR is regulated by PMA in U937 cells. Ligand blotting experiments with the 39 kDa receptor-associated protein RAP, a ligand for α2-MR, indicated that α2-MR levels first increased and then decreased after PMA treatment. FACscan as well as immunoblotting analysis with α2-MR-specific antibodies showed an identical trend: α2-MR levels increased within the first day of treatment with PMA, decreased at later times, and totally disappeared by three days of treatment. The effect of PMA was not due to transcriptional down-regulation, as the α2-MR mRNA level did not decrease at later times. Sensitivity of U937 cells to uPA-saporin, a toxin conjugate that reguires binding to uPAR for killing activity, was also markedly decreased. These results suggest that uPAR-mediated endocytosis depends on α2-MR expression.

Key words: Phorbol ester; Urokinase receptor; α2-Macroglobulin receptor; Endocytosis

1. Introduction

The urokinase receptor (uPAR) focusses proteolytic activity at the cell surface by specifically binding the plasminogen activator urokinase (uPA) [1]. Receptor-bound uPA is not shielded from the specific inhibitor PAI-1 (plasminogen activator inhibitor type-1), which binds uPAR-bound uPA and regulates cell surface proteolysis [2]. Unlike free uPA, uPA:PAI-1 complexes are internalized and degraded [3–5], a process also requiring the α2-macroglobulin receptor (α2-MR [6,7]. α2-MR, also called low density lipoprotein (LDL) receptor-related protein, is a member of the LDL receptor family [8], and plays a role in binding and endocytosis of many ligands, like α2-macroglobulin:proteinase complexes, β-migrating very low density lipoproteins enriched with apolipoprotein E, and lipoprotein lipase (reviewed in [9]). α2-MR is also involved in the internalization of bacterial and plant toxins, such as *Pseudomonas aeruginosa* exotoxin A and the plant type I ribosome-inactivating protein saporin (SAP) [10,11]. A conjugate between uPA and SAP was shown to be highly cytotoxic towards cells expressing uPAR, whereas cells devoid of uPAR and α2-MR were resistant [12].

During PMA (tetradecanoyl-phorbol acetate)-induced differentiation of U937 cells, transcription of the uPAR gene is strongly stimulated [13,14]. We have therefore studied the effect of PMA on the levels of α2-MR protein and mRNA. We find a time-dependent effect of PMA on α2-MR protein, with an initial increase and subsequent decrease. The α2-MR mRNA levels are increased at earlier times and remain detectable after prolonged stimulation. Down-regulation of α2-MR is accompanied by inhibition of uPA-SAP cytotoxicity.

2. Materials and methods

2.1. Reagents

Anti-uPAR monoclonal antibody R2 [15] was a generous gift of Drs. E. Rønne and G. Høyer-Hansen. The conjugate between active uPA and the toxin saporin, uPA–SAP, was prepared and purified as described [12]. Purified, bacterially expressed 39 kDa receptor-associated protein RAP [6], a ligand for α2-MR, was a generous gift of Drs. A. Nykiær, S. Moestrup and J. Gliemann, as also were the anti-α2-MR polyclonal antibody that recognizes the α chain of α2-MR [16] and the purified α2-MR [17]. PMA was from Sigma.

2.2. Cell culture

U937 cells were grown in RPMI 1640 (Gibco Life Sciences) supplemented with 10% fetal bovine serum. Differentiation was obtained by incubating cells (150–300,000/ml) with 150 nM PMA for different time intervals.

2.3. Radio-iodination

The α2-MR ligand 39 kDa RAP (receptor associated protein) was iodinated with Iodogen (Pierce Chemical Co.) as described before [18] to a specific activity of 2.5–12.5 × 10^5 cpm/pmol.

2.4. Cytofluorimetric analysis

Surface expression of uPAR and α2-MR was analysed by cytofluorimetry. Cells were briefly acid-washed to dissociate ligands [19], incubated in 0.1 ml PBS with 50 μg/ml anti-uPAR antibody R2 or with a 1:500 dilution of anti-α2-MR polyclonal antibody, for 30 min at 4°C. The cells were washed twice with PBS and resuspended in PBS containing a 1:50 dilution of fluorescein-conjugated anti-mouse or anti-rabbit IgG antibody (Dakopatts, Copenhagen, Denmark). After 30 min at 4°C, cells were washed twice and analysed by flow cytofluorimetry with a FACScan apparatus (Becton Dickinson, San Jose, CA).

2.5. Ligand blotting analysis

Cells were lysed in 0.1 M Tris-Cl, pH 8.0, 0.15 M NaCl, 1% Triton X-100; lysates (150 μg proteins) were separated by 5–8% gradient SDS-PAGE in the absence of reducing agents and transferred to a nitrocellulose filter. The filter was preincubated in 140 mM NaCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.8, containing low fat (2%) dry milk and 0.05% Tween 20, and subsequently 16 h at 4°C in the same buffer containing 1% bovine serum albumin and 100,000 cpm

*Corresponding author: DIBIT-H.S. Raffaele, via Olgettina 60, 20132 Milan, Italy. Fax: (39) (2) 2643 4844.

Abbreviations: α2-MR, α2-macroglobulin receptor/low density lipoprotein receptor-related protein; PAI-1, plasminogen activator inhibitor type-1; PMA, tetradecanoyl-phorbol acetate; RAP, α2-macroglobulin receptor-associated protein; SAP, saporin; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

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^{125}I-labeled RAP (50 pM). The filter was washed twice and autoradiographed at −80°C using an intensifying screen. As a positive control, purified α2-MR was run and blotted alongside. Competition (10–100 nM) was used to check specificity.

2.6. Enrichment and immunoblotting of α2-MR

Cells were lysed in 0.1 M Tris-HCl, 1% Triton X-100, 1 mM PMSF, pH 8.1, cleared at 14,000 rpm for 10 min at 4°C, and supplemented with 3-[3-cholamidopropyldimethyl-ammonio]-1-propanesulfonic acid (CHAPS) in 10 mM HEPES buffer, pH 7.8, containing 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, to a final concentration of 0.2%. Cleared lysates were incubated overnight at 4°C with RAP-Sepharose (66 μl resin/mg protein) (prepared by standard procedures, 0.3 mg RAP/ml CN-Br-Sepharose), the resin subsequently washed twice in the same buffer, the beads resuspended, boiled in Laemmli buffer without β-mercaptoethanol, and centrifuged. Supernatants were electrophoresed (5% acrylamide) in the absence of reducing agents. The gel was blotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), which was blocked in TBS buffer, 3% bovine serum albumin (BSA), included in a 1:1000 dilution of polyclonal rabbit anti-α2-MR (α chain) serum in TBS, 0.5% BSA for 2 h at room temperature, extensively washed and incubated with ^{125}I labeled protein A (Amersham; 0.2 μCi/ml TBS, 0.5% BSA) for 40 min at room temperature. The filters were then washed and autoradiographed at −80°C.

2.7. Cytotoxicity assays

Cytotoxicity assays with U937 cells were based on [^{3}H]leucine incorporation and have been described before [12]. PMA-containing medium was removed before the cell killing assay and replaced by fresh, PMA-free medium.

2.8. RT-PCR analysis of α2-MR mRNA levels

Total RNA was purified from PMA treated or untreated U937 cells as described [20]. RNA was reverse-transcribed according to a published procedure [21] except that oligo$(dN)_6$ was used instead of oligo$(dT)_{12-18}$ to prime the reaction. First strand cDNA corresponding to 50 ng total RNA was amplified in 50 μl reactions containing 2.5 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.2 mM each dNTP, 1 U *Taq* polymerase and 1 μM of the primers α2-MR2d (5′-GGATGTCCAGGCTTAGCCCAT-3′) and α2-MR2u (5′-ACAATGTGGAGGGTGTGGCC-3′). The primers α2-MR2d and α2-MR2u amplify a 321 bp fragment from human α2-MR cDNA. Amplification was performed by 40 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 1 min, extension at 72°C for 1 min. Negative control reactions without reverse transcriptase and without RNA were run in parallel.

3. Results

3.1. Expression of α2-MR is first up-regulated and then down-regulated by PMA in U937 cells

Surface exposure of UPAR and α2-MR was tested with specific antibodies and FACscan analysis. PMA treatment of U937 cells caused a large time-dependent increase of uPAR-specific fluorescence (Fig. 1) mostly at 20 h in agreement with previous observations [13]. PMA treatment caused an initial increase of cell surface α2-MR fluorescence at 20 h, but was then followed by a decrease. By 72 h, essentially no specific signal could be observed at the U937 cell surface.

To confirm the decrease of α2-MR, we have used ligand blot analysis to the iodinated α2-MR ligand RAP [22,23]. To this goal, equal amounts of proteins extracted from U937 cells treated for various lengths of time with PMA were separated by 5–8% gradient SDS-PAGE in the absence of reducing agents, blotted on a nitrocellulose filter and probed with [^{125}I]RAP. Purified α2-MR was used as positive control. [^{125}I]RAP recognized an about 600 kDa band in U937 cell extracts which co-migrated with authentic purified α2-MR, but nevertheless was barely visible (compare lanes 1 and 2; Fig. 2A). The amount of [^{125}I]RAP-binding protein in cell extracts was increased at 20 h of PMA treatment, decreased after this time, and had completely disappeared at 72 h (lanes 3–5). Binding was specific and could be out-competed by unlabeled RAP (not shown).

Immunoblotting analysis of the level of α2-MR during PMA-induced differentiation also gave similar results. To detect α2-MR, a prior enrichment from the cell lysates with a RAP-Sepharose resin was necessary (see section 2). In control U937 cell lysates we observed a band co-migrating with purified α2-MR. Its intensity was increased at 20 h of PMA treatment but became hardly visible at 48 and 72 h (Fig. 3).

3.2. PMA treatment reduces sensitivity to the cytotoxic uPA–SAP conjugate

We have previously shown that the uPA–SAP conjugate was highly and specifically cytotoxic towards cells expressing uPAR and α2-MR, including U937 cells [12], indicating that both receptors are necessary for mediating uPA–SAP endocytosis. Increase of uPAR by PMA treatment at 72 h would be expected to increase the cytotoxic activity of uPA–SAP. On the other hand, loss of α2-MR would be expected to have the opposite effect. We have first tested the uPA–SAP binding to purified α2-MR using unlabeled uPA–SAP as a competitor of [^{125}I]RAP binding (Fig. 2B). The control behaved in the expected way, i.e. 10 nM unlabeled RAP (200-fold excess with respect to [^{125}I]RAP totally competed for binding. At 10 nM, the uPA–SAP conjugate had a weaker competing activity than RAP, but completely extinguished the signal at 100 nM. We have therefore tested the effect of PMA treatment on the killing activity of uPA–SAP in U937 cells. As shown in Fig. 4A, a 72 h PMA treatment of U937 cells resulted in a several-order of magnitude decrease in sensitivity to uPA–SAP. We also studied the sensitivity to uPA–SAP of cells which had been exposed to PMA for different times. As shown in Fig. 4B, U937 cells acquired resistance to uPA–SAP in a time-dependent fashion which was similar to the effect of PMA on α2-MR (see Figs. 2A and 3).

3.3. α2-MR down-regulation is not due to decreased transcription

To gain further insight in the mechanism underlying α2-MR down-regulation, we extracted total RNA from control and PMA-treated U937 cells and tested for the presence of α2-MR mRNA by RT-PCR. As a positive control, we used HepG2 cells that express high levels of α2-MR [24]. In untreated cells, α2-MR was barely detectable (Fig. 5, lane 1); however, treatment with PMA resulted in a clear signal both at 6 and 24 h of treatment (lanes 2–3). At 72 h, the level of mRNA was not decreased with respect to the 24 h signal (lane 4).

4. Discussion

The α2-MR protein and mRNA undergo regulation in several instances: they are increased during differentiation of trophoblasts to syncytiotrophoblasts [25] and by colony stimulating factor-1 in murine macrophages [26]. Moreover, tissue macrophages exhibit a higher expression of α2-MR with respect to blood monocytes [27–29]. Therefore, we investigated the regulation of α2-MR in U937 cells during PMA-induced differentiation. Treatment of U937 cells in vitro with PMA

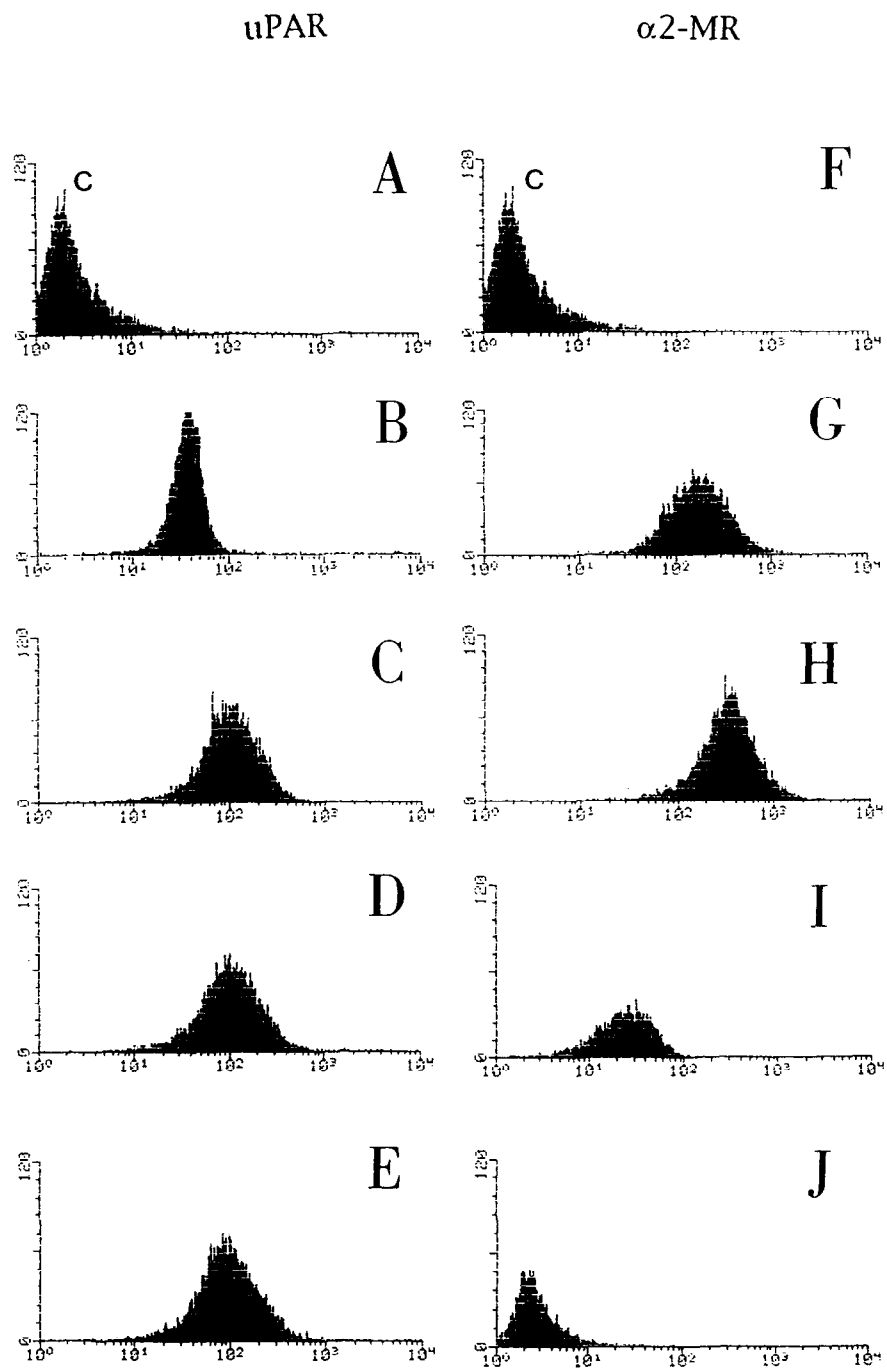


Fig. 1. Effect of PMA on surface expression of uPAR and α2-MR. Acid-washed U937 cells were incubated with 150 nM PMA for 0 (B and G), 20 (C and H), 48 (D and I), and 72 h (E and J). Half of the cells (B, C, D, and E) were challenged with the monoclonal anti-uPAR antibody R2, the other half (F, G, H, I, and J) with α2-MR-2 polyclonal antibody. Controls (A, F) refer to cells treated only with the fluorescein-conjugated anti-antibody.

causes terminal differentiation towards a monocyte–macrophage phenotype with the appearance of new functions like adherence to the plastic dish, phagocytosis and the expression of specific surface markers [30,31]. In U937 cells PMA increases transcription and translation of the uPAR gene [13,14] as well as its surface exposure (Fig. 1, and data not shown).

PMA has a dual effect on α2-MR, increasing it at 20 h and decreasing it at later times (48 and 72 h). Surface expression (Fig. 1) and the total content of α2-MR protein (Fig. 2A and 3) indeed correlate with this behaviour.

Several observations indicate that PMA treatment affects the internalization of uPAR-bound ligands, and that this may be due to down-regulation of α2-MR. RAP binding to purified, immobilized α2-MR is displaced by uPA–SAP in a dose-

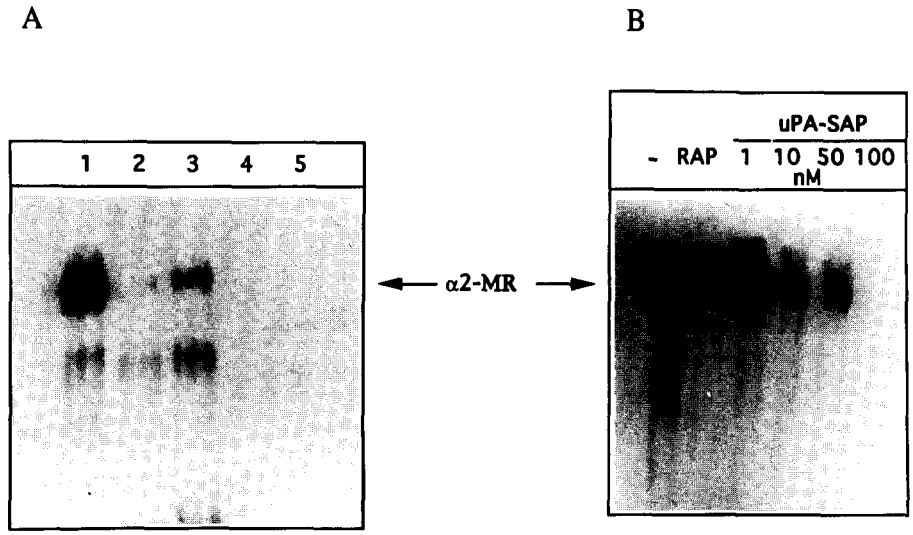


Fig. 2. Effect of PMA treatment on U937 cells α2-MR levels and uPA–SAP competition of [^{125}I]RAP binding to α2-MR. (A) Time-dependence of [^{125}I]RAP binding during PMA-induced differentiation. α2-MR in PMA-treated U937 cell extracts (150 μg total protein) was resolved on 5% SDS-PAGE, blotted on nitrocellulose filters, and incubated with [^{125}I]RAP (5×10^5 cpm/μg) (see section 2). The strips were autoradiographed for 16 h at −80°C. Lane 1 shows the purified α2-MR (1.5 μg). Lanes 2–5, U937 cells incubated with 150 nM PMA for 0, 20, 48, and 72 h, respectively. (B) Competition of [^{125}I]RAP binding to purified α2-MR by uPA–SAP. Purified α2-MR (1.5 μg) was resolved on 5% SDS-PAGE and electroblotted. Individual strips were incubated with different concentration of unlabeled uPA–SAP and then with [^{125}I]RAP (10×10^5 cpm/μg). Autoradiography was carried out for 6 h at −80°C.

dependent manner (Fig. 2B), indicating that a specific interaction between uPA–SAP and α2-MR takes place. Down-regulation of α2-MR expression at the cell surface, after 72 h of PMA stimulation, is accompanied by a 1000-fold reduction in cell sensitivity to uPA–SAP (Fig. 4A). It must be noted, however, that an initial increase in α2-MR levels at 20 h of PMA treatment did not result in an increase in uPA–SAP sensitivity (Fig. 4B). This might be explained by the fact that functions subsequent to internalization (i.e. intracellular routing, specific effects on protein synthesis or other) are required for uPA–SAP cytotoxicity.

Internalization and degradation of uPA:PAI-1 complexes depend on an initial binding to uPAR which subsequently presents the ligand to the α2-MR for internalization [6,7,16]. Indeed, undifferentiated U937 cells bind and degrade uPA:PAI-1 complexes [2,3]. uPA:PAI-1 degradation is inhibited by the α2-MR ligand RAP, and PMA-induced differentiation of U937 cells is accompanied by the inhibition of uPA:PAI-1 degradation (M. Conese and F. Blasi, unpublished), indicating an essential role for α2-MR in uPA:PAI-1 internalization and degradation. The failure of PMA-treated U937 cells to internalize the uPA:PAI-1 complexes, despite the presence of high levels of uPAR, correlates also with the decrease in α2-MR.

The mechanism underlying PMA-induced down-regulation of α2-MR during U937 differentiation remains to be clarified. The RNA data, in fact, while confirming the initial increase of α2-MR protein and ligand-binding activity after 24 h of treatment with PMA, do not account for its down-regulation at later times (Fig. 5). PMA-induced down-regulation of α2-MR, therefore, does not depend on decreased transcription. Other possibilities (shedding of receptor molecules from the plasma membrane or augmented degradation) are now under investigation (M. Conese and F. Blasi, in preparation).

The stimulatory effect of α2-MR mRNA and protein by PMA at early times appears to be in agreement with the

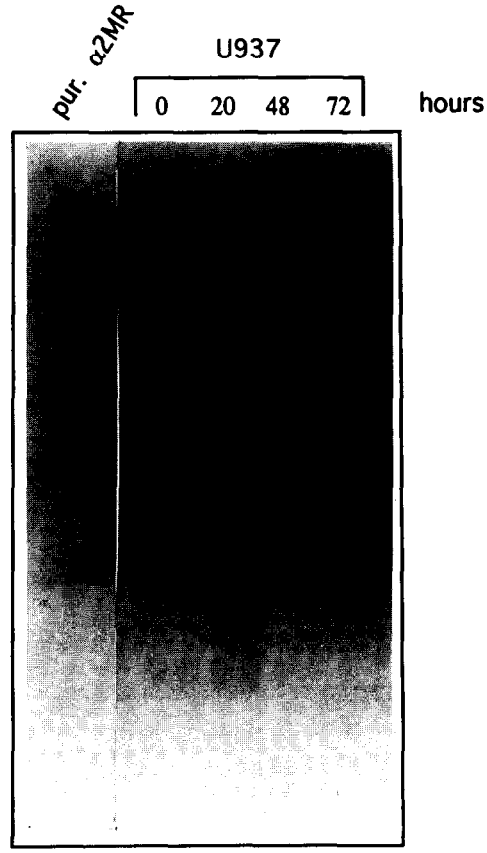
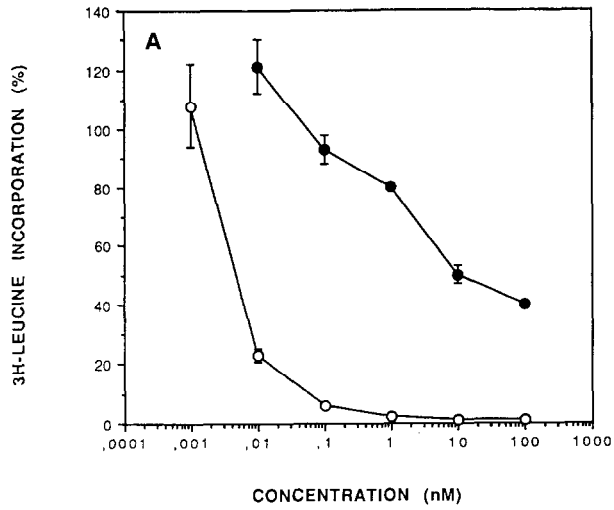


Fig. 3. Immuno-blotting analysis of α2-MR expression. RAP-Sepharose enriched U937 cell lysates (1 mg proteins) were electrophoresed (5% acrylamide SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was probed with anti-α2-MR polyclonal antibody and developed with [^{125}I]protein A (see section 2). Lane 1, purified α2-MR (0.1 mg); lane 2, untreated cells; lane 3, PMA-treatment for 20 h; lane 4, PMA-treatment for 48 h; lane 5, PMA-treatment for 72 h.

regulatory effect of PAI-1 on cell adhesion. In fact, PMA- and cytokine-induced adhesion of myeloid cells is positively regulated by uPAR occupancy and negatively by PAI-1 [32,33]. Therefore, further studies are required to investigate the role of α2-MR in differentiation-linked phenomena, like cellular adherence. The later decrease in α2-MR suggests that macrophages might require a lower level of uPA-mediated endocytosis. This point needs to be further investigated.



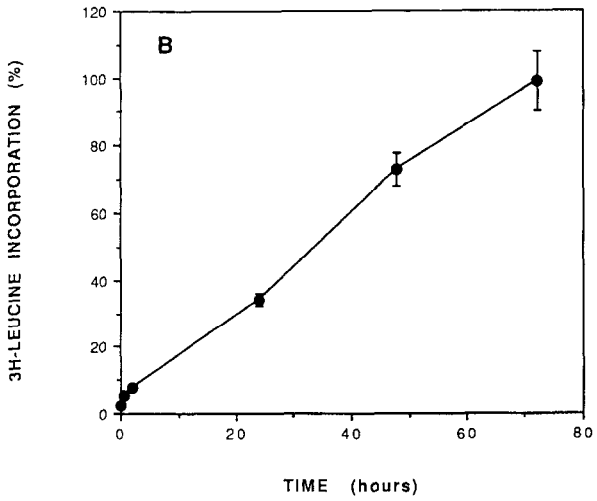


Fig. 4. uPA–SAP cytotoxicity on PMA-treated U937 cells. (A) Loss of uPA–SAP sensitivity upon PMA treatment of U937 cells. Cells cultured for 72 h at 37°C in the absence (○) or in the presence (●) of 150 nM PMA were washed and incubated at 37°C for 48 h in fresh PMA-free medium, in the presence of the indicated concentrations of uPA–SAP. The effect of uPA–SAP on cell viability was measured by [^{3}H]leucine incorporation and is expressed as percent of control cells. The assay was run in quadruplicate. Data represent the means ± S.D. (B) Time–course of uPA–SAP cytotoxicity during PMA-induced differentiation of U937 cells. Cells were incubated at 37°C in the presence of 150 nM PMA for increasing time lengths. After each time point, PMA-containing medium was removed, and cells were incubated at 37°C for 48 h in fresh PMA-free medium, either in the absence or in the presence of 1 nM uPA–SAP. The effect of uPA–SAP on cell viability was measured by [^{3}H]leucine incorporation and is expressed as percent of control cells. Data represent the means ± S.D. of 8 replicates.

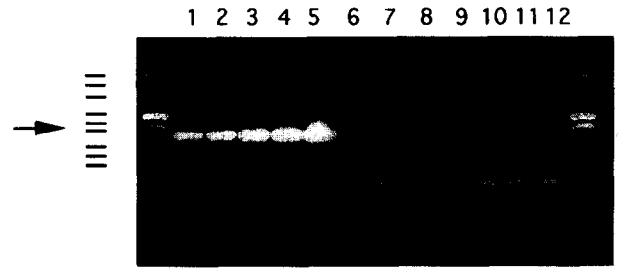


Fig. 5. Effect of PMA on α2-MR mRNA levels in U937 cells. Total RNA was isolated from U937 cells and used to obtain cDNA from which was amplified a sequence of 321 bp (see section 2). Lane 1, untreated cells; lanes 2–4, cells treated for 6, 24 and 72 h, respectively; lane 5, Hep G2 cells; lane 6, as lane 1, RT-PCR run in the absence of RNA; lanes 7–12, as lanes 1–6, RT-PCR run in the absence of reverse transcriptase. Marker VIII (Boehringer-Mannheim) run alongside (on both sides) of the gel; from top to bottom: 1114, 900, 692, 501, 404, 320, 242, 190 and 147 bp.

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