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Interactions of promonocytic U937 cells with proteins of the extracellular matrix

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SUMMARY

Monocyte interaction with proteins of the extracellular matrix (ECM) is regulated by expression of specific cell-surface receptors. 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) has been shown to induce the promonocytic cell line U937 to a more differentiated monocyte-like state. In this study we have analysed the attachment of U937 cells to ECM proteins and the effects of treatment with TPA on this process. Non-induced U937 cells attach to fibronectin- and Matrigel-coated surfaces without TPA stimulation, but TPA further increases adherence to these substrates as measured by an enhanced binding and by the lower concentration of proteins needed in the substrate to achieve 50% of maximal cell adhesion. Attachment to type I collagen was seen only with activated U937 cells, whereas no measurable attachment to bovine serum albumin, vitronectin, and type IV collagen was detected. TPA-activated U937 cells showed a two-fold increase in the expression of the RGD-dependent integrin receptors α_3 and α_5, and a reduction in the expression of α_4, another fibronectin-specific receptor, whereas the common β_1 chain was unchanged. Attachment of U937 cells to fibronectin was primarily mediated by the α_3 and α_5 integrins, as revealed by the ability of GRGDS peptides to inhibit attachment, whereas the CS-1 peptide, containing the α_4 binding site, was largely ineffective in blocking attachment.

INTRODUCTION

Cell adhesion to extracellular matrix (ECM) regulates numerous biological processes in which migratory functions are of paramount importance. In the context of cellular migration, white blood cells can exhibit selective targeting to specific locations. In this process they also interact extensively with ECM components of the basement membrane and of the loose connective tissue. These adhesive functions involve several receptors present at the cell membrane. For instance, the interactions between lymphocytes and ECM are mediated to a large extent, although not exclusively, by a family of cell-surface heterodimeric receptors, known as integrins.[1-3]. *In vitro* and *in vivo* studies have confirmed the importance of several physiological factors that regulate the interactions with ECM components, and how the receptors are involved in these interactions and contribute to the state of differentiation and to specific cellular functions. For example, the ability to attach to fibronectin is a property present to a greater extent in immature resident thymocytes than in recirculating peripheral T-mature cells.[4] Similarly, B-cell precursors bind fibronectin through RGD-specific receptors in the early steps of differentiation, then they lose this ability and bind fibronectin via the Hep II domain.[5]

The mononuclear phagocytes can attach to ECM proteins in the bone marrow and during the terminal differentiation to specialized macrophages after their migration into peripheral tissues.[6-9] The interaction of monocytes with ECM proteins can result in cell attachment and spreading and can lead to acquisition or increase of functions typical of activated macrophages. A useful model to study the role of ECM receptors and how these receptors are modulated with maturation is the promonocytic cell line U937.[10-12] In the present study we have analysed several ECM proteins as possible substrates for the attachment of U937 cells and of U937 cells treated with TPA (T-U937). The results indicate that T-U937 adhere to fibronectin, Matrigel and type I collagen to a greater extent compared to untreated U937 and that during the maturation process there is a different modulation of the receptors involved in the recognition of fibronectin.

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MATERIALS AND METHODS

Reagents

12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Sigma Chemical Co., St Louis, MO) was dissolved in dimethyl sulphoxide (Serva, Heidelberg, Germany) at a concentration of 25 mg/ml, divided

into aliquots, and kept at −80°. TPA was added to U937 cells 24 hr before the adhesion assay at a final concentration of 10 ng/ml.

Cell culture

U937 were maintained at a density of $3–10 \times 10^5$ cells/ml in tissue culture dishes (Falcon, Oxnard, CA) containing RPMI-1640 medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 2 mM L-glutamine, 100 μg/ml penicillin G and 100 μg/ml streptomycin.

ECM proteins

Fibronectin from human plasma was purified by affinity chromatography on Gelatin Sepharose 4B (Pharmacia, Uppsala, Sweden) according to Vuento and Vaheri.[13] Matrigel (Collaborative Research, Bedford, MA), a reconstituted complex ECM substratum containing basement membrane components including laminin, nidogen, type IV collagen, and heparan sulphate proteoglycan,[14] was from the Engelbreth–Holm–Swarm (EHS) murine sarcoma, and type I collagen was from rat tail (Serva, Feinbiochemica Gmbh and Co., Heidelberg, Germany). Vitronectin was purified from human plasma by heparin affinity chromatography according to Yatohgo *et al.*[15]

Synthetic peptides

The peptides LLHGPEILDVPST (CS-1 peptide), GRGDS and GRGES were synthesized using an automated Milligen 9050 Synthesizer (Waters, Millipore Corporation, Bedford, MA) according to the technical manual, and purity was verified by high-performance liquid chromatography.

Cell attachment assay

The assays were performed in 96-well flat-bottomed microtitre plates (EIA no. 3590, Costar, Cambridge, MA). Briefly, the plates were coated with 100 μl of appropriate dilutions of the different substrate proteins or peptides in phosphate-buffered saline (PBS) for 12 hr at 4°. Plates were rinsed with PBS and incubated at 37° with 100 μl of 1% boiled bovine serum albumin (BSA) for 1 hr. The plates were rinsed again and 100 μl of 10×10^5/ml cell suspension in attachment medium (PBS containing 0·9 mM $CaCl_2$ and 0·5 mM $MgCl_2$) were added to each coated well. After 30 min at 37°, non-adherent cells were removed by two gentle washes with PBS. Attached cells were fixed with 3% paraformaldehyde for 10 min and then for 10 min with 2% methanol, and finally stained with 0·5% crystal violet in 20% methanol. After 10 min the plates were washed and the stain was eluted with 0·1 M sodium citrate, pH 4·2, in 50% ethanol and analysed in a Titertek (Flow) using a 540 nm filter.

Immunofluorescence

1×10^6 U937 or T-U937 cells were preincubated with an excess of unlabelled human IgG (1·5 mg/ml) in order to saturate Fc receptors, and then reacted with the following mouse monoclonal antibodies (mAb): MAB 1981 (anti-β_1) from Chemicon (Temecula, CA); TS2/7 (anti-α_1) and HP2/1 (anti-α_4) from Dr F. Sanchez (Madrid, Spain), 10G11 (anti-α_2) from Dr A. E. G. Kr von dem Borne (Amsterdam, The Netherlands); J143 (anti-α_3) from Dr L. J. Old (New York); P1D6 (anti-α_5) from Telios Pharmaceuticals Inc. (San Diego, CA); and GoH3 (anti-α_6) from Dr A. Sonnenberg (Amsterdam, The Netherlands). As negative controls, non-relevant mouse monoclonal antibodies of the same isotype were used. After 45 min at 4° the cells were washed with 1% BSA in PBS, resuspended in 20 μl of FITC-labelled goat anti-mouse IgG (Serva), and further incubated for 45 min at 4°. The cells were washed and analysed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA).

RESULTS

TPA induces differentiation of U937 and increases the adhesion to fibronectin, Matrigel and type I collagen

It has been already reported that TPA can up-regulate the expression of maturation markers on U937 cells and can induce U937 cells to a more differentiated monocyte-like state.[10–12] To investigate whether TPA also has any effect on the interaction between U937 and proteins of the ECM, untreated U937 and T-U937 were compared for their ability to adhere to microtitre plates coated with fibronectin, vitronectin, Matrigel and types I and IV collagen. In a preliminary experiment the dose of 10 ng/ml of TPA was found to be innocuous for the cells and effective in promoting cell adhesion and in modulating mature cell-surface differentiation antigens. As shown in Fig. 1, non-activated U937 cells exhibited considerable attachment to both fibronectin and Matrigel. However, treatment with TPA increased cell adhesiveness to both substrates with up to 90% of the cells attached. Incubation of U937 and T-U937 cells on wells coated with boiled BSA showed only minimal adhesion. Untreated U937 cells did not adhere to type I collagen but pretreatment with TPA resulted in the acquisition of low adherence (Fig. 1). Both U937 and T-U937 did not show any attachment to type IV collagen and vitronectin (data not shown). That these proteins were absorbed onto the plates was checked by an ELISA assay using specific rabbit polyclonal antisera. The concentration of fibronectin and Matrigel required to achieve 50% of maximal cell adhesion was approximately 0·2 μg/well and 1·0 μg/well for T-U937 and U937, respectively. Thus binding to both fibronectin and Matrigel was about five-fold higher for T-U937 cells than for unstimulated U937 cells. Cell spreading was negligible on Matrigel or type I collagen even after TPA treatment, whereas both U937 and T-U937 extended thin processes and spread on fibronectin.

Modulation of integrin receptors during U937 cell differentiation

Cell interactions with proteins of the ECM are mediated, although not exclusively, by different receptors of the β_1 integrin family.[1–3] We therefore investigated whether the promotion in cell attachment to the various substrates observed with T-U937 compared to U937 cells was dependent upon the levels of the cell-surface expression of β_1 integrins. Upon examination of the cells by flow cytometry using mAb specific for the different β_1 integrins, we noticed that the expression of α_5 increased nearly two-fold after 24 hr of TPA treatment. Similarly, T-U937 cells up-modulated α_3, whose expression showed a 2·8 fold increase compared to untreated cells (Table 1). Conversely, on the same cells the expression of α_4 was reduced 1·25-fold by TPA, whereas α_1, α_2, and α_6 were negative both in the presence and in the absence of TPA. Finally, the level of the common β_1 chain remained constant after 24 hr treatment with TPA. These results

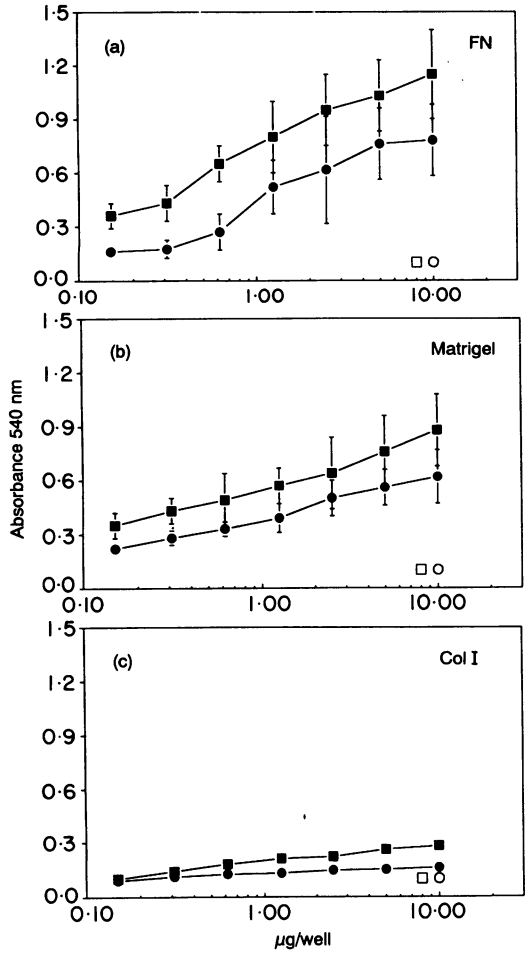

Figure 1. Cell attachment to (a) fibronectin; (b) Matrigel; and (c) type I collagen. Microtitre wells were coated with the proteins at the indicated concentrations. U937 (●) or T-U937 (■) cells were added and the plates incubated at 37° for 30 min. Attached cells (±standard deviation of four experiments) were measured as described in the Materials and Methods. Attachment to heat-denatured BSA (○, □) is also shown.

Table 1. Expression of β_1 integrin subunits on U937 and T-U937 cells

	Mean fluorescence intensity	
Antibody	U937	T-U937
Control	9·0 (7–11)	16·6 (13·8–19·4)
β_1	130·0 (110–150)	152·0 (138–166)
α_1	8·9 (7·9–9·9)	13·3 (10–16·6)
α_2	8·8 (8·1–9·5)	13·6 (9–18·2)
α_3	26·5 (21·3–31·7)	63·3 (56·1–80·3)
α_4	144·4 (129·4–159·4)	118·8 (98–131·6)
α_5	48·5 (42–55)	92·9 (80·9–104·9)
α_6	7·4 (6·9–7·9)	15·7 (13·1–18·3)

The range of two different experiments is included in parentheses.

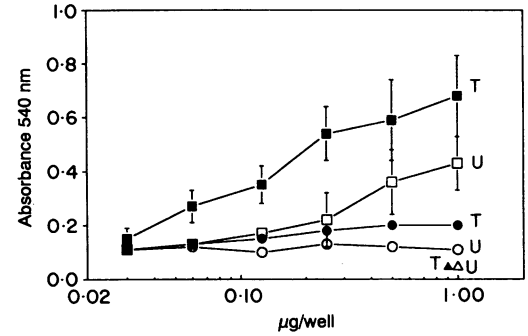

Figure 2. Cell attachment to synthetic peptides. Microtitre wells were coated with CS-1 (○, ●), GRGDS (□, ■) and GRGES (△, ▲) at the indicated concentrations and U937 (U) (○, □, △) and T-U937 (T) (●, ■, ▲) cells were added and the plates incubated at 37° for 30 min. Attached cells (±standard deviations of three experiments) were measured as described in the Materials and Methods.

suggested that the overall expression of β_1 integrins on the surface of U937 cells was not induced by TPA; instead it appeared that what changed was the relative ratio between the different heterodimers.

Increased adhesion of T-U937 cells to fibronectin is mediated by RGD but not CS-1 peptide sequence

To further confirm that the RGD-dependent integrin receptors of the β_1 family were the primary adhesion sites up-regulated in T-U937 cells, we examined first the adhesion of cells to wells coated with the synthetic peptides GRGDS and CS-1. Both U937 and T-U937 cells bound very inefficiently to the CS-1 peptide and to the control GRGES peptide-coated wells. On the contrary, adhesion to GRGDS was already high for U937 cells and increased further for T-U937 cells (Fig. 2).

The peptides were then used to compete for attachment of U937 and T-U937 cells to fibronectin. CS-1 inhibited only about 20–25% of the binding of U937 and 5–15% of the binding of T-U937 (Fig. 3). On the other hand GRGDS, but not GRGES, was a very strong inhibitor of cell attachment, particularly with T-U937, where up to 75% inhibition was observed at the lower fibronectin concentration. The difference in competition between CS-1 and GRGDS was highly significant both for U937 and T-U937 at 1·25 μg of fibronectin but only for T-U937 at 10 μg of fibronectin. Consistent with the above results the addition of both CS-1 and RGD peptides showed an additive inhibitory effect. These results strongly suggest that the enhanced adhesion of T-U937 cells to fibronectin was mainly exerted via the RGD-dependent receptors α_3 and α_5.

DISCUSSION

In this report we have investigated the effect of a treatment with TPA on adhesion of U937 cells to ECM components as an *in vitro* model system to investigate the biology of phagocytic cell functions and the process of cellular activation of monocytes/macrophages. One of the advantages of using U937 cells instead of macrophages to study cell adhesiveness to ECM stems from the fact that macrophages display a broader spectrum of reactivity for ECM components compared to U937 cells when induced by different stimuli, including TPA.[9] Although TPA is all but a physiological stimulus, nevertheless it activates at least

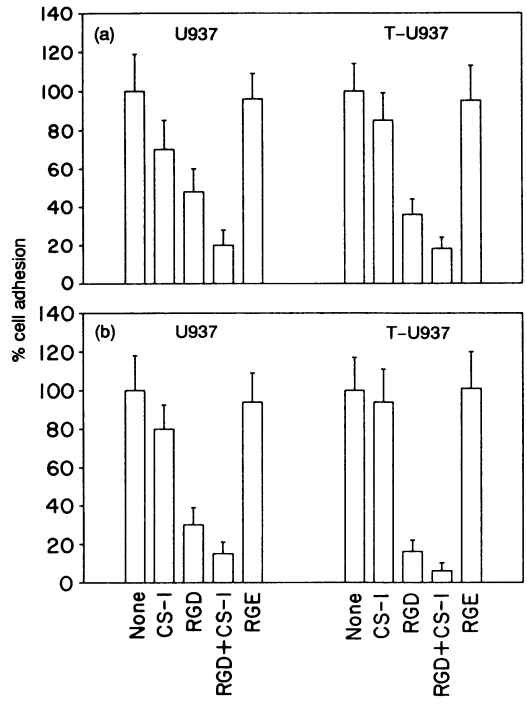

Figure 3. Inhibition of cell attachment to fibronectin by synthetic peptides. Microtitre wells were coated with 10·0 (a) or 1·25 μg (b) of fibronectin. Cells were added to the coated plates together with the competing peptides. Incubation proceeded for 30 min at 37° and attached cells were measured as described in the Materials and Methods. 100% adhesion (±standard deviation of three experiments) corresponds to the absorbance (540 nm) detected on 10·0 or 1·25 μg of fibronectin that was 0·7 and 0·4, respectively, for U937 and 1·0 and 0·7, respectively, for T-U937 cells. Statistical significance of CS-1 versus RGD competition was $P < 0.004$ and $P < 0.001$ at 1·25 μg of fibronectin and $P < 0.1$ and $P < 0.06$ at 10 μg of fibronectin for U937 and T-U937, respectively. The significance of RGD competition in U937 versus T-U937 cells was $P = 0.02$ at 1·25 μg and not significant at 10 μg of fibronectin.

certain cellular pathways that are common to hormonal and cytochine natural ligands.

Non-induced U937 cells displayed a measurable attachment to fibronectin and Matrigel. By contrast, neither U937 nor T-U937 cells exhibited any attachment to vitronectin or type IV collagen. TPA enhanced adhesion of U937 cells to both fibronectin and Matrigel and induced a slight adhesion to collagen type I. It appears that the increased attachment to fibronectin was at least in part due to the up-regulation of α_3 and α_5 integrin receptors. Using two different criteria, immunofluorescence analysis to measure the levels of integrin expression, and inhibition of cell attachment with synthetic peptides, we found an increased expression of both α_3 and α_5 in T-U937 compared to U937 cells and a parallel increased inhibitory effect of the GRGDS peptide on cell attachment to fibronectin.

It is likely that the increased expression of the promiscuous α_3 receptor in T-U937 was also responsible for the enhanced adhesive activity observed on type I collagen and on type IV collagen- and laminin-containing Matrigel. Alternatively, the induction of adhesion of T-U937 cells to type I collagen might depend on other non-integrin collagen receptors that have also been described.[16,17]

The lack of attachment to vitronectin even after TPA stimulation was at variance with other studies,[18] in which T-U937 cells did not display any apparent spreading on vitronectin substrates and the binding was inhibited by RGD-containing peptides.[18] Since different vitronectin receptors were reported on variants of U937 cell lines,[18,19] and it was also suggested that the culture conditions might affect expression of vitronectin receptors,[18] it is likely that the present discrepancy with the previous data depends on the cell subline and/or on the growing conditions used by us.

The increase of α_3 and α_5 receptors observed in T-U937 cells was accompanied by a decrease of the α_4. This was shown both by the reduced expression of this receptor and by a diminished inhibitory effect of the α_4-specific CS-1 peptide on the attachment of T-U937 to fibronectin compared to U937 cells. On the other hand, attachment of T-U937 was inhibited to a greater extent by RGD compared with U937 cells. A direct association between enhanced expression of α_5 in T-U937 cells and increased adhesion to fibronectin was reported recently.[20,21] However, in those studies the expression of the α_3 receptor was not investigated. This molecule at variance with α_5 and α_6, does not recycle at the cell surface[22] and appears to play a primary role in the early recognition events between cell membranes and the substrate whereas it is probably not involved in locomotion. This tenet is supported by the broader ligand specificity, the lower apparent avidity, and the inability to localize into focal adhesive contacts.[22-24] Although α_3 and α_5 were up-regulated and α_4 was down-regulated in U937 cells activated by a non-physiological stimulus, nevertheless these data suggest that integrins might play different roles during monocyte trafficking through activated endothelium, migration into tissues, and terminal differentiation. One way to respond to local stimuli is to up-regulate the number of ECM receptors as in the present example; alternatively, receptor affinity might increase without any changes in cell-surface density, as was reported for CHO cells stimulated with phorbol ester[25] or for U937 treated with certain anti-β_1 antibodies;[26] finally, the specificity of the receptor might change following cell differentiation. In this regard, promiscuous receptors such as α_3 are particularly helpful since they might recognize different substrata with different affinity depending on the local physiological stimuli or cation concentrations.[27]

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