Retinoic Acid Upregulates the Plasminogen Activator System in Human Epidermal Keratinocytes

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The activation of the proteolytic plasminogen activator system is important for the re-epithelialization of skin wounds. Keratinocytes synthesize and secrete the urokinase-type plasminogen activator, which binds to its specific receptor on keratinocytes. Receptor-bound urokinase-type plasminogen activator efficiently activates cell surface bound plasminogen. This results in pericellular proteolysis, which facilitates keratinocyte migration. Urokinase-type plasminogen activator activity is specifically controlled by plasminogen activator inhibitor-1 and -2. As retinoids have been reported to accelerate epithelialization of skin wounds in animal studies and clinical settings, we investigated the effects of all-*trans* retinoic acid on the plasminogen activator system in human epidermal keratinocytes. As tested in a chromogenic plasminogen activation assay, incubation with 10 μM all-*trans* retinoic acid caused a marked induction of cell-associated plasminogen activity after 24 h, and this induction was blocked by neutralizing anti-urokinase-type plasminogen activator antibodies, but not anti-tissue-type plasminogen activator antibodies. All-*trans* retinoic acid lead to a strong increase in urokinase-type plasminogen activator (enzyme-linked immunosorbent assay) and urokinase-type plasminogen activator receptor cell surface expression (flow cytometry) after 24 h. At this time-point, tissue-type plasminogen activator and plasminogen activator inhibitor-1 and -2 proteins were not or only slightly increased. Northern blot analyses revealed that all-*trans* retinoic acid caused an early and short-lived increase of plasminogen activator inhibitor-1, but a prolonged induction of urokinase-type plasminogen activator and urokinase-type plasminogen activator receptor mRNA levels. Collectively, these data suggest that all-*trans* retinoic acid activates the plasminogen activator system in human epidermal keratinocytes by differentially regulating activating and inhibiting components. The activation of the plasminogen activator system may be one mechanism by which all-*trans* retinoic acid exerts beneficial effects in cutaneous wound healing. ***Key words: epithelialization/keratinocyte/plasminogen activator system/retinoic acid/wound healing. J Invest Dermatol 116:778–784, 2001***

Keratinocytes synthesize and secrete the urokinase-type plasminogen activator (uPA), which binds to its specific receptor (uPAR) on keratinocytes (Kramer *et al*, 1995). Receptor-bound uPA effectively activates cell surface-associated plasminogen, which in turn cleaves various extracellular matrix components providing pericellular proteolysis. Keratinocytes may also produce tissue-type plasminogen activator (tPA), for which no cell surface receptor is known. UPA and tPA activity is controlled by the specific plasminogen activator inhibitor (PAI) proteins PAI-1 and PAI-2. Activation of the PA system plays a central part for extracellular matrix degradation under various physiologic and pathologic conditions, including cancer invasion and cutaneous wound healing (Kramer *et al*, 1995; Schmitt *et al*, 1995; Blasi, 1997).

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Abbreviations: uPA, urokinase-type plasminogen activator uPAR, urokinase type plasminogen activator receptor tPA, tissue-type plasminogen activator; PAI, plasminogen activator inhibitor.

Cutaneous wound healing is a complex and well-ordered process that starts with rapid provisional wound closure by a blood clot and leads to the reconstruction of a coherent dermal and epidermal layer (Clark, 1999). Re-epithelialization is achieved by keratinocytes that migrate from wound edges or hair follicles into the wound and later proliferate and differentiate in order to form regular epidermis. Both migration and proliferation of keratinocytes are fostered by various biologic response modifiers produced at the wound site, including epidermal growth factor, keratinocyte growth factor, or transforming growth factor-β. These factors initially originate from activated platelets of the blood clot and are later provided by infiltrating leukocytes and fibroblasts (Martin, 1997). Keratinocytes migrate on a provisional dermal matrix and underneath the blood clot that will be shed after re-epithelialization. In order to dissect along this migration path, keratinocytes need to express, among other things, adhesion receptors (e.g., integrins) for extracellular matrix components (e.g., collagen I, vitronectin, and fibronectin), but also proteases such as matrix metalloproteinases or components of the PA system (Martin, 1997; Clark, 1999).

Several lines of evidence suggest that the PA system is necessary for re-epithelialization. Whereas in normal human and murine epidermis uPA, uPAR, and tPA are only detectable at low basal

levels, in human and murine skin wounds uPA, uPAR, and, to a lesser extent, tPA are upregulated in keratinocytes of the epithelial outgrowth (Grondahl-Hansen *et al*, 1988; Jensen *et al*, 1988; Baird *et al*, 1990; Romer *et al*, 1994; Jensen and Lavker, 1996). Exogenously applied uPA stimulates re-epithelialization of skin wounds in diabetic and nondiabetic mice (Jimenez *et al*, 1997). In plasminogen-deficient mice, re-epithelialization and wound closure are dramatically retarded (Romer *et al*, 1996). In uPA-deficient mice, although not formally evaluated, extensive nonhealing skin ulcerations were noted at least in a small proportion of animals (Carmeliet *et al*, 1994). Impaired cutaneous wound healing was also observed in a patient with homozygous plasminogen deficiency (Schott *et al*, 1998).

Owing to their multiple effects on cutaneous biology, vitamin A and its derivatives can affect various aspects of wound healing (Kligman *et al*, 1986; Popp *et al*, 1995, and references therein). Among other things retinoids may affect wound repair by improving epithelialization. This is supported by clinical observations: pretreatment with all-*trans* retinoic acid (RA) improved re-epithelialization after superficial skin wounding due to facial dermabrasion, chemical peeling, or electroepilation (Mandy, 1986; Anthony *et al*, 1991; Hevia *et al*, 1991).

One mechanism by which retinoids may improve epithelialization is the activation of the PA system. Treatment of keratinocyte cultures or skin organ cultures with RA increased uPA protein and PA activity in culture supernatants (Varani *et al*, 1994, 1995). In this study, we have systematically analyzed the effects of RA on individual components of the PA system (uPA, uPAR, tPA, PAI-1, and PAI-2) in human epidermal keratinocytes. We show that RA effectively activates the PA system in human epidermal keratinocytes by differentially regulating its activating and inhibiting components. We suggest that the activation of the PA system may be one mechanism by which RA exerts beneficial effects in cutaneous wound healing.

MATERIALS AND METHODS

Cell culture and reagents Normal human epidermal keratinocytes were purchased from Cell Systems (St. Katharinen, Germany) and cultured in the keratinocyte growth medium KGM-2 consisting of the keratinocyte basal medium KBM-2 supplemented with bovine pituitary extract, epidermal growth factor, hydrocortisone, insulin, and transferrin (all from Cell Systems) at 37°C and 5% CO_2. Cell cultures were used for experiments at 60–80% confluency and after incubation for at least 16 h with the basal medium KBM-2 alone. RA (Sigma, Deisenhofen, Germany) was dissolved in dimethyl sulfoxide at a concentration of 10 mM, stored at –20°C, and freshly diluted for each experiment in culture medium. The phorbol ester phorbol-12-myristate-13-acetate (PMA, Sigma) was added to cells as indicated.

Plasminogen activation assay Cell-associated plasminogen activation was assessed using a chromogenic assay. Keratinocyte cultures were grown in 96 well plates and either left untreated, incubated with PMA or RA. Additional cell populations were incubated with RA in combination with one of the following reagents: inhibitory monoclonal antibodies (50 μg per ml) against human uPA or tPA (both from American Diagnostica, Pfungstadt, Germany); the synthetic tPA inhibitor tPAstop (1.5 μM), which according to the manufacturer (American Diagnostica), inhibits tPA 41.5-fold more efficiently than uPA and 78-fold more efficiently than plasmin; or the serine protease inhibitor 2r-L (1 μM) (Stürzebecher *et al*, 1999). Subsequently, cells were washed with phosphate-buffered saline and incubated with 100 μl 0.5 mM plasminogen (Roche Molecular Biochemicals, Mannheim, Germany) at 37°C for 30 min. The chromogenic plasmin specific substrate L1450 (Bachem, Heidelberg, Germany) was added (50 μl of a 1.5-mM solution) and incubation continued for another 20 min, a time-point up to which the accumulation of enzymatically cleaved L1450 was in the linear range. Subsequently, the generation of cleaved substrate was photometrically assessed in an enzyme-linked immunosorbent assay (ELISA) reader by normalizing the optical density (OD) at the L1450 extinction maximum (405 nm) against the extinction at an irrelevant wavelength (614 nm). Normalized ODs are designated $OD_{405–614}$.

Fibrin degradation assay Cell-associated plasminogen activation was also assessed using the fibrin degradation test as described previously (Reuning *et al*, 1995). Briefly, fibrin clots were generated in 24 well plates by incubating 200 μl of 50 mg fibrinogen per ml (Calbiochem, Frankfurt, Germany), 50 μl of 150 mM $CaCl_2$, and 50 μl of 10 U thrombin per ml (Sigma) for 1 h at 37°C. Keratinocyte cultures that either had been left untreated or incubated with RA for 24 h were detached by incubation with Hank's buffered salt solution (Cell Systems) containing 0.5 mM ethylenediamine tetraacetic acid (Sigma), washed, resuspended in KGM-2 containing a fixed concentration (2 mg per ml) of plasminogen, and seeded on fibrin clots. After 4 h of incubation at 37°C supernatants were collected and 25 μl were analyzed for fibrin degradation products (including D-dimer) by ELISA (Dimertest Gold EIA kit, American Diagnostica).

ELISA Cells were grown in six-well plates and were either left untreated or exposed to 10 μM RA for various time-points. Supernatants were collected and cleared from debris by centrifugation. Cells were washed with Hank's buffered salt solution, extracted with 500 μl Triton/Tris-buffered saline (50 mM Tris pH 7.5; 150 mM NaCl; 1% TritonX-100) for 150 min at room temperature, and harvested with a cell scraper. Preliminary experiments had established that the detectable amounts of uPA, uPAR, tPA, PAI-1, and PAI-2 antigens were not decreased in cell extracts handled at room temperature compared with extracts handled at 4°C, excluding the possibility that proteolysis had a major influence on the detected levels of antigen. Extracts were cleared from cell debris by centrifugation. Extracts and supernatants were stored at –80°C until analysis. Protein concentrations were determined using the BioRad Protein Assay reagent (BioRad, Munich, Germany). uPA, uPAR, tPA, PAI-1, and PAI-2 concentrations in cell extracts and supernatants were determined using the ELISA kits Imubind uPA no. 894, Imubind uPAR no. 893, Imubind tPA no. 860, Imubind PAI-1 no. 821, and Imubind PAI-2 no. 823, respectively (American Diagnostica) as previously described (Schmalfeldt *et al*, 1995).

Flow cytometry uPAR cell surface expression was assessed by an indirect two step staining procedure and subsequent fluorescence-activated cell sorter (FACS) analysis as previously described (Marschall *et al*, 1999). Briefly, cells were detached by incubation in Hank's buffered salt solution containing 0.5 mM ethylenediamine tetraacetic acid at 37°C, 5% CO_2 for 30 min, carefully resuspended in, and washed with, phosphate-buffered saline containing 1% bovine serum albumin and 0.1% NaN_3. Subsequently, cells were incubated at 4°C with either the monoclonal mouse anti-human uPAR antibody HD 13.1 (Todd *et al*, 1997) or with an isotype-matched control antibody (mouse IgG1, DAKO, Glostrup, Denmark). In a second step, cells were incubated with a fluorescein isothiocyanate-coupled goat anti-mouse antibody (Dianova, Hamburg, Germany). Subsequently, cells were analyzed in a FACScanII flow cytometer using the CellQuest software (Becton Dickinson, Heidelberg, Germany).

Assessment of cell viability As a parameter for cell viability, cell membrane permeability was assessed after varying periods of RA exposure by dye exclusion experiments (propidium iodide staining). Propidium iodide staining was assessed by flow cytometry as described previously (Behrends *et al*, 1994). Briefly, 50 ng propidium iodide (Sigma) were added to cells, FACS analysis performed for the distinction of intact (propidium iodide negative) from dead (propidium iodide positive) cells, and the proportions of intact and dead cells were determined.

RNA extraction and northern blot analysis Total cellular RNA from cells grown in 10 cm plates was isolated using the RNA-Clean Kit (AGS, Heidelberg, Germany). RNA (10 μg of each sample) was size fractioned by agarose/formaldehyde electrophoresis, blotted to a positively charged nylon membrane (Roche Molecular Biochemicals), and fixed to the membrane via UV cross-linking. Several digoxigenin (DIG)-labeled hybridization probes were prepared: a 1.1 kb *Xba*I/*Eco*RI fragment of the human uPAR mRNA (Roldan *et al*, 1990) was transcribed *in vitro* using the DIG RNA labeling kit (Roche). The following cDNA probes were used: a 0.9 kb *Pst*I/*BamH*I fragment of the human uPA cDNA (Turkmen *et al*, 1997); a 1.1 kb *Sal*I fragment of the human PAI-1 cDNA (Turkmen *et al*, 1997); or a 1.2 kb *BamH*I/*Hin*dIII cDNA fragment of the human PAI-2 cDNA (Muehlenweg *et al*, 2000). The cDNA fragments were labeled via random hexamer extension using the DIG-High Prime kit (Roche). As a control for loading uniformity all blots were also hybridized with an oligodeoxynucleotide (5′-CGC-CCGCCGCAGCTGGGGCGATCC) complementary to the human 28S ribosomal RNA (Gonzalez *et al*, 1985), which had been DIG end labeled

with the DIG Oligonucleotide 5′-End Labeling Set (Roche). Hybridizations of RNA, cDNA, or oligonucleotide probes were carried out according to the manufacturer. Hybridization signals were quantitated using the DIG-Luminescent Detection Kit for Nucleic Acids (Roche Molecular Biochemicals) and subsequent exposure of Hyperfilm ECL (Amersham, Braunschweig, Germany). Specific signals on films were densitometrically quantitated using the GelDoc system and software (BioRad). Signals generated by the mRNA-specific hybridization probes were normalized to the respective 28S rRNA signals. Different exposure times were used to ensure that the signal strength of the analyzed bands on the autoradiographs was in the linear range.

RESULTS

RA upregulates plasminogen activation in cultured keratinocytes RA may affect the PA system by regulating both its activating and its inhibiting components. In order to assess the overall effect of RA on the activity of the PA system in cultured keratinocytes, a plasminogen activation assay was performed after exposure of cells to RA. RA was used at 10 μM, a concentration that has previously been shown to have profound effects in cutaneous cell types (Varani *et al*, 1989; Gille *et al*, 1997) and in our hands did not affect cell viability for up to 48 h (data not shown). RA increased plasminogen activation compared with untreated controls. A slight increase was noticeable after 4 h of exposure to RA. Induction values were maximal after 16–30 h and declined again thereafter (data not shown). The induction was further characterized at a time-point of maximal induction (24 h, **Fig 1*a***). Untreated cells showed a mean plasmin activity of 0.134 ± 0.029 $OD_{405–614}$. The activity of cells treated with RA, however, was increased to 0.222 ± 0.042 $OD_{405–614}$. A similar induction (0.227 ± 0.083 $OD_{405–614}$) was observed after a 4.5 h incubation with 100 ng per ml PMA, which served as a positive control. The synthetic serine protease inhibitor 2r-L markedly inhibited RA-mediated upregulation (**Fig 1*b***). A uPA-neutralizing antibody blocked cell-associated plasminogen activity by 95% (**Fig 1*b***) implicating that both the basal and the RA-upregulated plasminogen activation are predominantly mediated by uPA. Consistently, a tPA-neutralizing antibody and the tPA-selective synthetic inhibitor tPAstop failed to influence the RA-induced plasminogen activation significantly.

RA upregulation of plasminogen activation in keratinocytes was also assessed by the fibrin degradation assay. The amount of fibrin degradation products was induced 3.33 ± 1.4-fold (mean ± SEM; data obtained in triplicates in three independent experiments) in cells exposed to RA for 24 h compared with untreated cells (data not shown).

RA induces uPA and uPAR protein expression In order to investigate how RA affects individual components of the PA system in keratinocytes, the concentrations of uPA, uPAR, tPA, PAI-1, and PAI-2 proteins were determined in protein extracts and supernatants after various incubation periods with 10 μM RA. All components of the PA system were present in extracts of untreated keratinocytes (**Table I**), and the amount of PAI-2 was markedly higher than those of the other components confirming previous observations (Jensen *et al*, 1995). Similar amounts of uPA and PAI-1 were detected in supernatants and cell extracts, whereas uPAR, tPA, and PAI-2 were detected in supernatants in much smaller amounts than in cell extracts (**Table I**). RA induced cell-associated uPA and uPAR in a similar time pattern with discernible inductions after 4 h of RA, maximal induction levels between 16 and 32 h of RA incubation and declining, but still elevated levels after 48 h of RA exposure (data not shown). Cell-associated tPA was maximally induced after 8 h of exposure to RA (approximately 2–3-fold, data not shown) and had declined to slightly above prestimulation values after 24 h of exposure to RA. Cell-associated PAI-1 also displayed a short-lived induction (up to 3-fold) in response to RA peaking after 8 h to 16 h of exposure to RA (data not shown). There was a slight increase of cell-associated PAI-2 discernible after 16 h to 24 h of RA incubation, and PAI-2 levels had declined again to prestimulation levels at 48 h (data not shown). The effects of RA on uPA, uPAR, tPA, PAI-1, and PAI-2 protein levels at a time-point at which the upregulation of plasminogen activation by RA was maximal (24 h of exposure to RA), is illustrated in **Fig 2**, **Table I**. Cell-associated uPA and uPAR (**Fig 2*a***) as well as secreted uPAR (**Fig 2*b***) were markedly increased, whereas uPA in supernatants was only slightly elevated. TPA and the inhibitory component PAI-1 were not increased by RA at this time-point either in cell extracts or supernatants (**Fig 2**) reflecting already declining levels of these molecules. There was a slight increase in cell-associated PAI-2 and a marked increase in secreted PAI-2 after 24 h incubation with RA, yet, even the

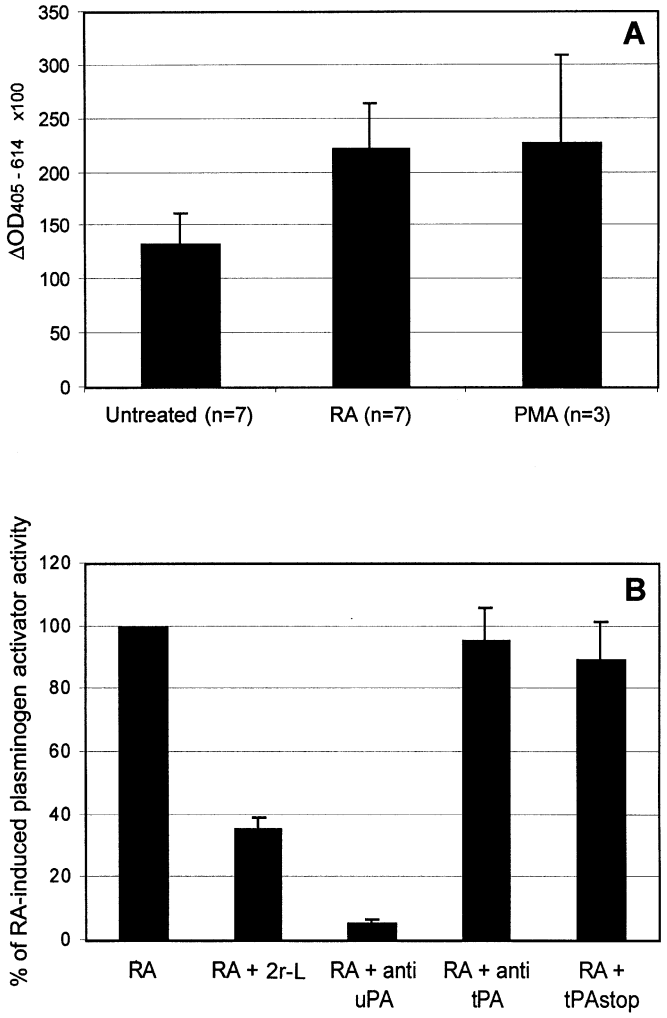

Figure 1. RA upregulates plasminogen activation in cultured keratinocytes. (*a*) Plasminogen activation assay of cultured keratinocytes that were either left untreated or exposed to 10 μM RA for 24 h or 100 ng PMA per ml for 4.5 h. Photometric measurements of the plasmin-cleaved chromogenic substrate L1450 were performed as described in *Materials and Methods*. Activity is depicted as mean ± SEM from seven (untreated controls and RA) or three (PMA) independent experiments with quadruplicate determinations. (*b*) The type of PA responsible for cell-associated RA-mediated plasminogen activation was determined after exposure of keratinocytes for 24 h to 10 μM RA alone or in combination with either the serine protease inhibitor 2r-L, a monoclonal uPA-inhibitory antibody (anti-uPA), a monoclonal tPA-inhibitory antibody (anti-tPA), or a synthetic tPA inhibitor (tPAstop). Total PA-activity of RA-treated keratinocytes (0.210 ± 0.030 $OD_{405–614}$) was regarded as 100%, and the percentage of plasminogen activity remaining after the various treatments was calculated. Values are displayed as mean ± SEM from four independent experiments with quadruplicate determinations.

Table I. RA differentially modulates uPA, uPAR, tPA, PAI-I, and PAI-2 expression in cultured keratinocytes[a]

	uPA	uPAR	tPA	PAI-1	PAI-2
Cell associated[b]					
Untreated	28 ± 4	4 ± 1	15 ± 6	62 ± 10	4424 ± 1592
RA[c]	216 ± 23	17 ± 3	20 ± 5	70 ± 7	7198 ± 2929
Supernatant					
Untreated	72 ± 27	0.04 ± 0.02	6 ± 1	104 ± 28	0.4 ± 0.1
RA[c]	98 ± 19	0.20 ± 0.13	6 ± 1	105 ± 27	2 ± 1

[a]Concentrations of components of the PA system in cell extracts and supernatants were measured by ELISA as described in *Materials* and *Methods*. Values are expressed as ng per mg protein (cell extracts) or ng per ml (supernatants) and represent mean ± SEM (three independent experiments with duplicate determinations).

[b]For a direct comparison of protein amounts in extracts and supernatants, cell-associated values are multiplied by a factor of 2.5 (approximately 0.4 mg extracted protein per well) and values for supernatants by a factor of 1.0 (1 ml supernatant per well).

[c]Measurements after exposure to 10 μM RA for 24 h.

increased amount of secreted PAI-2 was still minute compared with that of the other inhibitor, PAI-1 (**Table I**).

When cell-surface expression of uPAR was assessed by flow cytometry, a marked increase was observed after 24 h incubation with 10 μM RA (**Fig 3**), consistent with the ELISA measurements, and was still elevated after 48 h (data not shown). Taken together, after 24 h RA markedly increased the expression of the activating components, uPA and its receptor uPAR in human epidermal keratinocytes, whereas inhibiting components were not or only slightly increased. This is consistent with the observed increased plasminogen activation at this time-point (**Fig 1**).

RA increases uPA, uPAR, PAI-1, and PAI-2 mRNA levels In order to characterize further the regulatory effects of RA on the uPA, uPAR, PAI-1, and PAI-2 components of the PA system, mRNA analyses were performed (**Fig 4**). In untreated keratinocyte cultures basal expression of uPA, uPAR, PAI-1, and PAI-2 mRNA was observed consistent with the detection of the respective proteins in cell extracts and supernatants from untreated keratinocytes. RA caused a concentration-dependent increase in mRNA levels of all four proteins (**Fig 4*a***). The induction was discernible at 1 μM RA and increased with increasing RA concentrations up to 50 μM RA with the exception of PAI-2, which displayed ever increasing inductions up to 10 μM RA, but a smaller induction after 50 μM RA. Time-course analysis revealed induction of uPA, uPAR, PAI-1, and PAI-2 mRNA after as little as 1 h incubation with RA (data not shown). Different induction kinetics among the components of the PA system were noticed (**Fig 4*b***). PAI-1 induction peaked after 10–16 h exposure to RA and then readily declined to prestimulation levels. This is consistent with the protein measurements demonstrating early and short-lived PAI-1 induction. UPA, uPAR, and PAI-2 mRNA were maximally induced after 24–32 h incubation with RA. At 48 h the uPAR mRNA level remained elevated, whereas uPA and PAI-2 mRNA levels had markedly declined again albeit still being higher than control levels.

Thus, the impact of RA on plasminogen activation appears to be accompanied by distinct modulations of the mRNA levels of individual components of the PA system.

DISCUSSION

This study documents that RA upregulates the PA system in human epidermal keratinocytes. Importantly, it demonstrates that this is achieved by differentially modulating activating and inhibiting components. In other tissues, retinoids have also been found to preferentially induce the activating components of the PA system

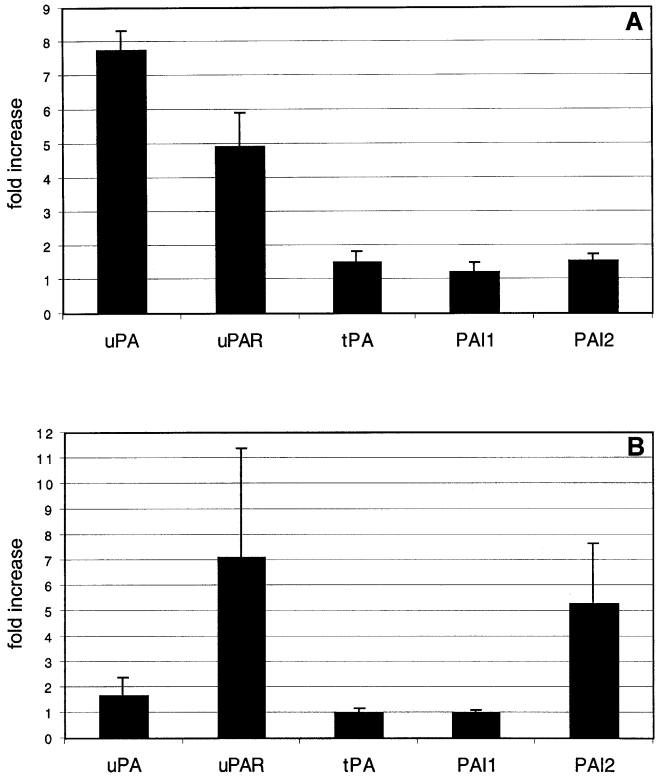

Figure 2. RA induces uPA and uPAR protein expression in cultured human epidermal keratinocytes. Concentrations of components of the PA system in cell extracts and supernatants were measured by ELISA as described in *Materials and Methods*. Induction of protein expression in cell extracts (*a*) and supernatants (*b*) of cells exposed to 10 μM RA for 24 h compared with untreated controls is depicted as mean ± SEM from three independent experiments with duplicate determinations.

while leaving inhibitory factors unchanged, e.g., in human umbilical endothelial cells (Thompson *et al*, 1991) or hepatic stellate cells (Leyland *et al*, 1996).

Whereas it was previously noted that RA incubation of keratinocytes augments plasminogen activity in culture supernatants (Varani *et al*, 1995), this study demonstrates the upregulation of cell-associated plasminogen activation by RA, which is important with regard to the pericellular fibrinolysis required during wound healing. In cultured keratinocytes we (**Fig 1*a***) and others (Varani *et al*, 1995) observed a basal plasminogen activation. In the keratinocyte cell line HaCaT, this basal plasminogen activation is mainly due to uPA, but not tPA, activity (Reinartz *et al*, 1996). In accordance with this, the cell-associated RA-upregulated plasminogen activation observed in this study was due to uPA rather than tPA activity (**Fig 1*b***). Although an early induction of tPA protein expression by RA was observed, at a time-point of maximal RA upregulation of plasminogen activation (after 24 h incubation), RA caused a marked induction of only uPA, but not tPA, protein expression in human cultured keratinocytes (**Fig 2**). This confirms a previous observation at a later time-point (after 48 h incubation with RA), when only increased amounts of uPA, but not tPA, were detected in supernatants (Varani *et al*, 1995). In murine keratinocytes, however, induction of tPA by RA has been observed (Brown *et al*, 1992). The relevance of our observations in keratinocyte monocultures for wound healing is supported by immunohistologic data both in murine and human wounds, which suggest that uPA is the predominant PA in the epithelial outgrowth, whereas very little (mouse) or no (human) immunoreactive tPA is detected (Grondahl-Hansen *et al*, 1988).

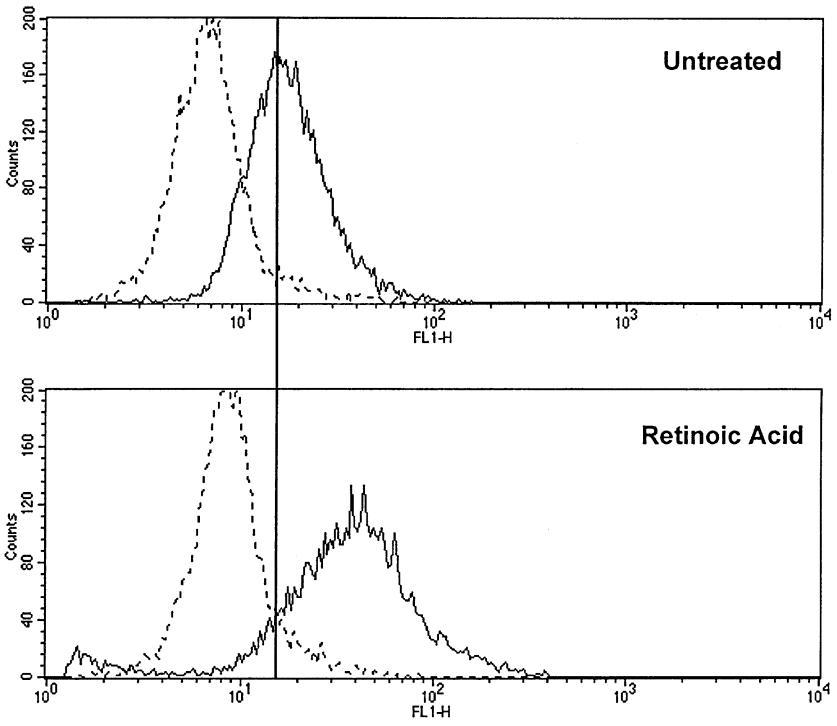

Figure 3. RA induces uPAR cell surface expression in cultured human epidermal keratinocytes. FACS analysis of untreated controls or cells exposed to 10 μM RA for 24 h. Staining was performed with either an anti-uPAR monoclonal antibody (solid lines) or an isotype control monoclonal antibody (dotted lines). One representative of three independent experiments is displayed.

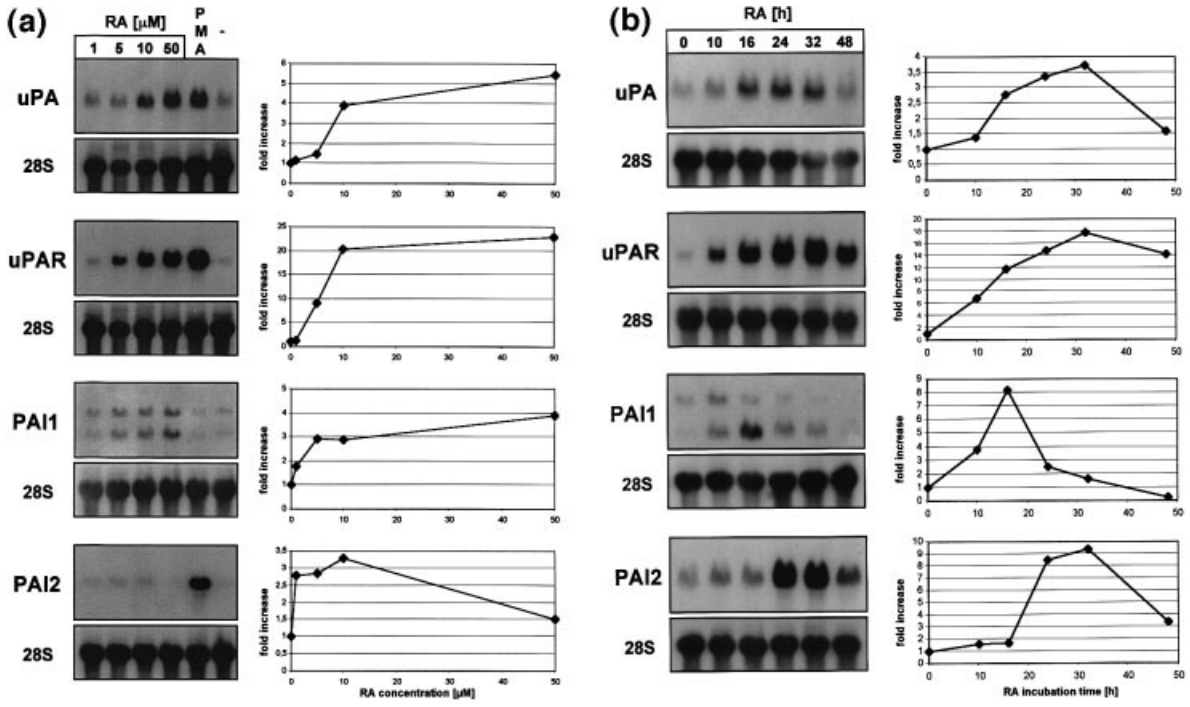

Figure 4. RA increases uPA, uPAR, PAI-1, and PAI-2 mRNA levels in cultured human epidermal keratinocytes. Northern blot analysis was performed as described in *Materials and Methods* with total cellular RNA isolated from cultured keratinocytes that were either left untreated or stimulated with RA or PMA. Blots (left panels) and densitometric quantitations of specific signals (right panels) are displayed. 28S, hybridization with a 28S rRNA oligodeoxynucleotide as a control for loading uniformity. (*a*) Effect of different concentrations of RA (1–50 μM, 10 h incubation) or 100 ng per ml PMA (4 h incubation). The data are from one of three independent experiments with similar results. (*b*) Time course of RA stimulation (10 μM RA). The data are representative of three (10 h and 24 h time-points) or two (16 h, 36 h, and 48 h time-points) experiments with similar results.

Furthermore, disturbances of wound healing were observed at least in a small proportion of transgenic mice deficient in uPA (Carmeliet *et al*, 1994), but not in transgenic mice deficient in tPA (Bugge *et al*, 1996). Taken together, our data further support the view that RA-upregulated plasminogen activation is primarily due to modulation of uPA, but not tPA, expression. It seems, however, possible that, if RA is applied to skin around the time of wounding, RA-induced tPA from other sources, e.g., from dermal fibroblasts (Varani *et al*, 1995) may contribute to plasminogen activation at the epithelial outgrowth.

PAI-2 was detected at much higher levels than the other components of the PA system, but only a very small proportion appeared to be secreted. This is in accordance with a previous observation (Jensen *et al*, 1995). Although extracellular PAI-2 has been demonstrated to form complexes with secreted uPA in keratinocytes (Reinartz *et al*, 1996), the vast majority of PAI-2 is retained within the cell (Jensen *et al*, 1995; Risse *et al*, 2000) and has no inhibitory effect on extracellular plasminogen activation. Intracellular PAI-2 is induced in keratinocytes by an increase of the calcium concentration in the culture medium (Jensen *et al*, 1995), and PAI-2 mRNA and antigen is preferentially detected in suprabasal epidermal layers (Lyons-Giordano *et al*, 1994), suggesting an intracellular function in keratinocyte terminal differentiation. The induction of PAI-2 by RA may, therefore, be interpreted as an RA-related differentiation signal.

It has been consistently observed both in animal models (Hung *et al*, 1989) and clinical settings (Mandy, 1986; Anthony *et al*, 1991; Hevia *et al*, 1991) that treatment of the skin with retinoids prior to wounding improves re-epithelialization. In the light of our data, one possible explanation for this phenomenon is that, due to RA pretreatment, plasminogen activation may already be upregulated at the time of wounding and thus accelerate the initiation of keratinocyte migration, whereas in the physiologic course of wound healing, upregulation of plasminogen activation occurs only after a lag phase during which platelets, leukocytes, or fibroblasts at the wound site begin to deliver growth factors and cytokines that upregulate plasminogen activation in keratinocytes (Martin, 1997).

Whereas treatment of the skin with retinoids prior to wounding accelerates epithelialization, continued topical or systemic application of retinoids after wounding has been found to retard wound closure in animal models (Arboleda and Cruz, 1989; Hung *et al*, 1989; Watcher and Wheeland, 1989). Furthermore, impaired wound healing has been observed following laser treatment or dermabrasion in patients taking systemic isotretinoin (Rubenstein *et al*, 1985; Zachariae, 1988). The reasons for such divergent effects are unclear at present. Possibly, in the course of wound healing the enhancement of plasminogen activation by retinoids may become less important due to the presence and strong action of several physiologic enhancers of plasminogen activation, e.g., keratinocyte growth factor (Tsuboi *et al*, 1993). It is also important to appreciate that, apart from plasminogen activation, several other aspects of epithelialization are susceptible to retinoid regulation and may thus affect epithelialization. First, transcriptional repression of matrix metalloproteinases, e.g., interstitial collagenase (matrix metalloproteinase-1) by RA has been documented (Schroen and Brinckerhoff, 1996). Secondly, RA has profound effects on keratinocyte integrin expression (Hakkinen *et al*, 1998) and may, therefore, modulate the integrin pattern during wound healing (Hertle *et al*, 1992; Larjava *et al*, 1993). Thirdly, RA may affect keratinocyte proliferation. In keratinocytes that are growth-suppressed due to culture in growth factor deficient medium, RA initiates proliferation suggesting that RA may *in vivo* stimulate quiescent epidermal keratinocytes (Varani *et al*, 1989) and thus accelerate keratinocyte proliferation at around the time of wounding. On the other hand, keratinocyte proliferation stimulated by keratinocyte growth factor, which is important in later stages of wound healing, is inhibited by concomitant RA treatment (Varani *et al*, 1989), an observation that may help explain the inhibition of wound healing observed when RA treatment is continued during wound healing.

Retinoids are thought to exert their cellular effects by interaction with intracellular RA receptors (RAR). The RAR form heterodimers with retinoic X receptors (RXR); there are several different RAR (α, β, and γ) and RXR (α, β, and γ) (Kang and Voorhees, 1999). Upon binding of RA, the RAR/RXR complexes act as transcription factors either directly by binding to RA response elements (RARE) or indirectly via interaction with other transcription factors, e.g., AP-1 (transcriptional cross-talk) (Pfahl, 1993). The details of the transcriptional effects of retinoids within the PA system have been partially revealed. The human uPA promoter lacks a canonical RARE, but can be activated via transcriptional cross-talk with SP-1 by complexes consisting of RA and RAR/RXR receptors (Suzuki *et al*, 1999). Thus, it may be assumed that transcriptional cross-talk with SP-1 contributes to the observed RA induction of uPA in cultured keratinocytes. In the mouse uPA promoter, transcriptional cross-talk with a different DNA binding factor, AP1, has been reported (Mira-Y-Lopez *et al*, 1998). The tPA and PAI-2 genes both possess RARE that are functionally active within certain cell types (Bachmann, 1995; Bulens *et al*, 1995), and these elements may be involved in the observed tPA and PAI-2 inductions in cultured keratinocytes. The molecular mechanisms by which retinoids modulate PAI-1 and uPAR expression remain to be elucidated.

In conclusion, the data suggest that RA activates the PA system in human epidermal keratinocytes by differentially regulating activating and inhibiting components. The activation of the PA system may be one mechanism by which RA exerts beneficial effects in cutaneous wound healing.

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