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Retinoic Acid Potentiates TNF-α-Induced ICAM-1 Expression in Normal Human Epidermal Keratinocytes

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ICAM-1 protein in keratinocytes is thought to contribute to cutaneous inflammatory reactions. Its induction depends—among others—on cytokines such as TNF-α, IFN-γ, IL-1 or on retinoic acid (RA), a key regulator of epidermal homeostasis. We investigated the effect of treatments with TNF-α, RA or their combination on ICAM-1 expression on proliferative or differentiating keratinocytes over an 8 day culture period. Basal ICAM-1 levels were undetectable at low (30 μM) and standard (88 μM) Ca^{2+} and RA alone did not induce ICAM-1. However, at high Ca^{2+} (1500 μM), ICAM-1 levels were augmented in response to RA-treatment. TNF-α induced a transient ICAM-1 increase in NHK, which reached peak-levels 2-4 days post cytokine stimulus. RA potentiated the TNF-α-induced ICAM-1 response in all Ca^{2+}-concentrations. This potentiating effect of RA was confirmed at the mRNA level. In summary, our results establish retinoic acid as an enhancer of TNF-α-induced ICAM-1 levels in NHK.

Intercellular adhesion molecule-1 (ICAM-1), a cell surface glycoprotein of 80–114 kDa which belongs to the immunoglobulin family (1), plays a prominent role in keratinocyte mediated immune reactions (reviewed in 2, 3). As ligand for lymphocyte-function associated antigen-1 (LFA-1) (4), which is expressed on the plasma membrane of leukocytes, ICAM-1 is critical for the interaction between leukocytes and keratinocytes (5) and thus for the migration of activated T cells from the vasculature to the epidermis during inflammation (2, 6). The ICAM-1/LFA-1 interaction has proven to be essential for the accessory cell function of the keratinocyte in the costimulation of helper T cells (7) and is necessary in $CD8^+$-cell mediated cytotoxicity (8). In normal, uninflamed skin, keratinocytes (NHK) show minimal basal ICAM-1 levels (5). However, in many cutaneous diseases, such as psoriasis (9), allergic contact dermatitis (10), delayed hypersensitivity reaction (11) or graft-vs-host disease (12), ICAM-1 levels are markedly upregulated and associated with an increased epidermal T cell infiltrate (10, 13). A variety of stimuli induce ICAM-1 expression in keratinocytes: pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (10), interferon-gamma (IFN-γ) (10, 14) or interleukin-1 (IL-1) (14) as well as many non-cytokine agents such as UV (15), substance P or histamine (3). In many tumor cell lines, retinoic acid (RA) is described as a potent inducer of ICAM-1 (16–18).

Retinoids exert many functions in skin through binding to and activation of their nuclear receptors (19, 20). The predominant receptors in skin are RAR-γ and RXR-α (21). Because of their inhibitory effects on keratinocyte differentiation (22) and on proliferation (9), retinoids are applied as therapeutic agents in many skin diseases (19, 20). Topical treatment with RA often results in erythema and inflammation (23). It is suggested that this can be partly explained by the induction of ICAM-1 by RA (19). However, literature data about RA induction of ICAM-1 in keratinocytes are conflicting. Aoudjit *et al.* (17) and Kashihara-Sawami and Norris (24) did not observe any effect of RA on basal ICAM-1 expression, in contrast to earlier reports of Fisher *et al.* (25) and Barker *et al.* (6). RA is also able to modulate cytokine-induced ICAM-1-expression. Previous investigations described the synergistic effect of RA on IFN-γ-induced ICAM-1 expression in keratinocytes (24, 26). To our knowledge, no reports have been published about the modulation by RA of TNF-α-induced ICAM-1 in keratinocytes. In tumor cells, such as human thyroid carcinoma cells, tumor necrosis factor increased ICAM-1 surface expression to a greater extent in combination with RA (18). In contrast, in human dermal microvascular endothelial cells, no ef-

Abbreviations used: ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-gamma; NHK, normal human keratinocytes; RA, all-*trans*-retinoic acid; TNF-α, tumor necrosis factor-alpha.

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fect was observed of RA on TNF-α-induced ICAM-1 levels (27).

We examined the effect of RA both on basal and on TNF-α-induced ICAM-1 protein and mRNA level in normal human keratinocytes cultured at three different calcium concentrations, reflecting the undifferentiated and the differentiating state. The observations establish RA as a potentiating agent of TNF-α-induced ICAM-1 expression in epidermal keratinocytes.

MATERIALS AND METHODS

1. Cells and reagents. Normal human keratinocytes (NHK) were isolated from foreskin and used in experiments at passage 4. All experiments shown are performed with keratinocytes, isolated from the same donor. Cells were cultured in serum-free keratinocyte growth medium (K-SFM, GibcoBRL, Life Technologies, Paisley, UK), supplemented with 0.2 ng/ml EGF (GibcoBRL), 25 μg/ml BPE (GibcoBRL) and 0.5 μg/ml gentamycin (GibcoBRL). To obtain the desired calcium concentrations, calcium-free K-SFM was supplemented with different volumes of a sterile 0.1 M $CaCl_2$ solution.

All-*trans*-retinoic acid was obtained from Sigma (St. Louis, MO). Stock solutions of 10^{-2} M in dimethylsulfoxide were prepared freshly before use and were protected from daylight. Working solutions were prepared in culture medium. All culture manipulations with RA were performed in yellow light conditions. Final DMSO concentrations were 0.01% or less. Tumor necrosis factor-α (TNF-α) was purchased from Genzyme (Cambridge, MA).

2. Cell ELISA. A cell ELISA for ICAM-1 was performed as described (28). Incubated cells were washed with PBS, fixed in 2% paraformaldehyde and blocked with 2% normal goat serum (NGS, DakoPatts, Glostrup, Danmark). 50 ng/ml ICAM-1 monoclonal antibody (Bender-Medsystems, Vienna, Austria) was incubated overnight at 4°C. After incubation, cells were washed and incubated with a horseradish-peroxidase labeled secondary antibody in a 1% NGS/1% BSA solution. The amount of protein expression was measured through use of a colorimetric detection method based on the reduction of ortho-phenylenediamine dihydrochloride (Sigma). The absorbance was measured on an ELISA plate reader at 490–650 nm.

3. MTT assay. Effects of treatment on cell viability and estimations of cell number were assessed with a mitochondrial tetrazolium assay (MTT) (29). The MTT assay was performed as described (28). Briefly, NHK received the same treatments as described for the cell ELISA experiments. After the incubation time, 25 μl/well of an MTT-solution (5 mg/ml MTT (Sigma), in Ca^{2+}/Mg^{2+}-free PBS) was added to the cultures and incubated for 3 h at 37°C. The medium was aspirated and the plates were frozen-thawed twice (−20°C). By adding 200 μl of ethanol/acetone (60/40, v/v, −20°C) and incubation for 30 min at 4°C on the shaker, the monolayers were fixed and the formazan crystals dissolved. Optical densities were measured at 540–650 nm on a Thermomax plate reader (Molecular Devices, CA).

4. RNA isolation and Northern blot. Total RNA was isolated 4 h post cytokine induction, using acid guanidinium thiocyanate-phenol–chloroform (30). RNA was size-fractionated on a 1% agarose gel and transferred to a nylon membrane (Boehringer-Mannheim) by vacuumblotting.

The ICAM-1 cDNA probe was purchased from R&D Systems (Abingdon, UK). The probe was labeled in a PCR-reaction (eLONGase from GibcoBRL, Life Technologies, Paisley, UK) with a 1:3,5 ratio of DIG-dUTP:dTTP nucleotides (Boehringer-Mannheim). The PCR mixture was 1/1000 diluted in DIG Easy Hyb hybridization buffer (Boehringer-Mannheim). The length of the PCR generated probe was approximately 900 bp. The DIG-labeled β-actin RNA probe was obtained from Boehringer-Mannheim and was used at 50 ng/ml. The entire probe solution was filtered through a 0.45 μm filter prior to use. Prehybridizaton and hybridization was performed at 50°C in DIG Easy Hyb for the ICAM-1 cDNA probe and at 68°C for the actin RNA probe. After hybridization, the blot membrane was washed with a final stringency of 0.1× SSC (20× SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.4), 0.1% SDS at 68°C. The hybridized probes were immunodetected with an alkaline phosphatase-linked anti-digoxigenin antibody (Boehringer-Mannheim) and visualized with the chemiluminescent substrate CSPD (Boehringer-Mannheim).

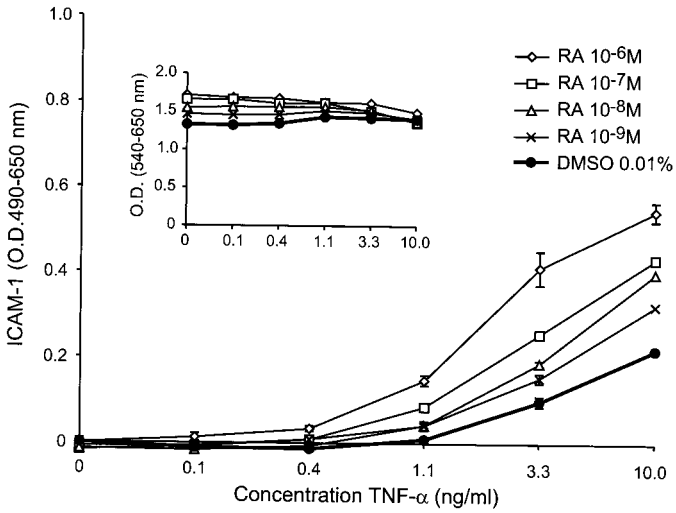

FIG. 1. Modulation of TNF-α-induced ICAM-1 protein expression by retinoic acid. Cell ELISA of ICAM-1 expression 68 h post induction by TNF-α with or without 2 h pretreatment with RA. The ELISA was performed as described in material and methods. Values represent means ± SD with n = 3. Insert graphs show the result of parallel MTT assays, which are performed as described in material and methods. The thick line is the DMSO control. The thin lines are the different RA concentrations with successive lines representing successive doses, the highest dose having the biggest impact on MTT. Values represent means (standard deviations are omitted for clarity).

RESULTS

Retinoic acid enhances TNF-α-induced ICAM-1 protein expression. We investigated by a cell ELISA the effect of different concentrations of all-*trans*-retinoic acid (RA) on TNF-α-induced ICAM-1 protein expression in keratinocytes, cultured in 88 μM Ca^{2+}. NHK were seeded at 4000 c/well in 96-well tissue culture plates and grown to 80-90% confluence. Cells received fresh medium plus RA (10^{-6}–10^{-9} M) 2 h prior to TNF-α -stimulation (1-10 ng/ml). After 68 h incubation ICAM-1 levels were determined and in parallel MTT assays were performed (Fig. 1).

Basal ICAM-1 protein levels proved to be below the detection limit. RA alone was unable to induce ICAM-1, which is in agreement with previously published results (17, 24).

Three and 10 ng/ml TNF-α (Fig. 1) moderately induced ICAM-1 protein. Co-treatment with RA enhanced the TNF-α-induced ICAM-1 signal. The magnitude of the observed potentiation was dependent on the

RA concentration. RA was also able to increase the sensitivity of keratinocytes to lower doses of TNF-α. We did not detect negative effects on cell viability (insert graph in Fig. 1). In contrast, treatment of the cells with RA resulted in a small increase in MTT, which might reflect increased cell metabolic activity or increased cell proliferation. Because this increase was observed in every RA-condition, it can not account for the effect on ICAM-1 protein expression.

Essentially the same observations were made following a 24 h treatment, in foreskin keratinocytes isolated from four different donors and in NHK treated at three different degrees of confluence (data not shown). In all cases, RA enhanced TNF-α-induced ICAM-1 protein expression.

Effects of retinoic acid co-treatment on TNF-α-induced ICAM-1 protein are confirmed at the mRNA level. To assess whether the observed effects of RA on TNF-α-induced ICAM-1 occurred at protein or at mRNA level, we performed Northern blot experiments to measure steady-state mRNA-levels. NHK were seeded at 300,000 c per 6 cm ø petri dish and grown to 80% confluence. 1 h prior to cytokine-stimulation, cells were treated with RA (10^{-6}–10^{-7} M). The optimal concentration and incubation time for TNF-α to induce a strong ICAM-1 mRNA response proved to be 20 ng/ml TNF-α for 4 h.

Figure 2 shows that NHK did not express detectable basal ICAM-1 mRNA, but were induced by TNF-α-treatment to express weak, but clearly detectable ICAM-1 mRNA levels. In agreement with the results obtained at protein level, RA alone (10^{-6} M) did not induce ICAM-1 mRNA, but when given in combination with TNF-α, RA strongly potentiated the TNF-α-induced ICAM-1 mRNA. A densitometric analysis of the ICAM-1 signal, normalized for the β-actin signal showed a 4-fold amplification.

Calcium does not significantly alter retinoic acid effects on cytokine-induced ICAM-1. Calcium is a crucial factor in the differentiation of keratinocytes. We investigated the influence of Ca^{2+} on the modulatory role of RA on TNF-α-induced ICAM-1 expression. Cells were seeded at 2750 c/w in standard K-SFM medium (88 μM) and reached 60-70% confluency after 3 days. They received fresh medium with 30, 88 or 1500 μM Ca^{2+} with or without RA and were grown for 2 days (day 0 to day 2) (see Fig. 3). While maintaining the Ca^{2+}- and RA-conditions constant, cells were treated with 3 ng/ml TNF-α and refreshed every other day. At different time points (day 0, 2, 4, 6 and 8) ICAM-1 protein levels were determined by *in situ* ELISA and in parallel, MTT assays were performed.

The effects of RA and TNF-α-treatment, in function of time, on ICAM-1 induction and cell viability are shown in Fig. 3. In Fig. 3 parts d–f, which shows MTT results, it can be seen that the control cultures continue

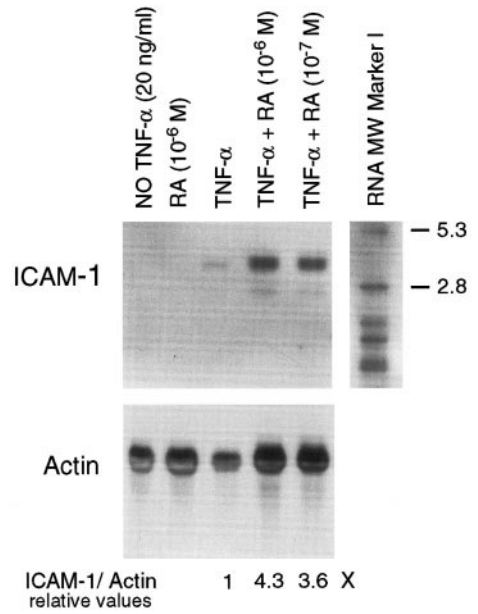

FIG. 2. Effect of retinoic acid co-treatment on cytokine-induced ICAM-1 mRNA. 60–70% confluent NHK were pretreated with 10^{-6} M or 10^{-7} M RA or solvent (DMSO, 0.01%) for 1 h and were then stimulated with TNF-α (20 ng/ml) for 4 h. Northern blot analysis of total RNA (5 μg), using DIG-labeled ICAM-1 and DIG-labeled β-actin as probes.The right lane shows a RNA molecular weight marker I (Boehringer-Mannheim). Fold induction values obtained after densitometric scanning and normalization for the actin signal, are given below the blot.

to proliferate throughout the experiment to the same extent at the different Ca^{2+}-concentrations, as is reflected by increasing MTT values over time. The combined treatments with Ca^{2+}, RA and/or TNF-α all result in differences in the MTT values. As in Fig. 1 (88 μM Ca^{2+}), RA-treatment increased the MTT values over controls and this effect augmented in function of time (Fig. 3e). At 1500 μM Ca^{2+}, similar observations were made, but at 30 μM Ca^{2+} this RA effect was only marginal. TNF-α reduced cell growth, as also reported previously for IFN-γ (31). The combination RA and TNF-α always yielded higher MTT values than TNF-α alone. To compensate for the observed differences in MTT, the ICAM-1 protein signal was corrected for the MTT signal in the corresponding well and ELISA values were expressed as

$$OD_{490\text{–}650\ nm\ (ELISA\ value)} / OD_{540\text{–}650\ nm(MTT\ value\ in\ the\ corresponding\ well)}.$$

No ICAM-1 protein expression could be detected in the untreated cells at either of the three Ca^{2+}-concentrations. At 30 and 88 μM calcium, RA-treatment did not induce ICAM-1. However, an increase of basal ICAM-1 expression by RA alone was observed in NHK grown at 1500 μM Ca^{2+} (Fig. 3c). At

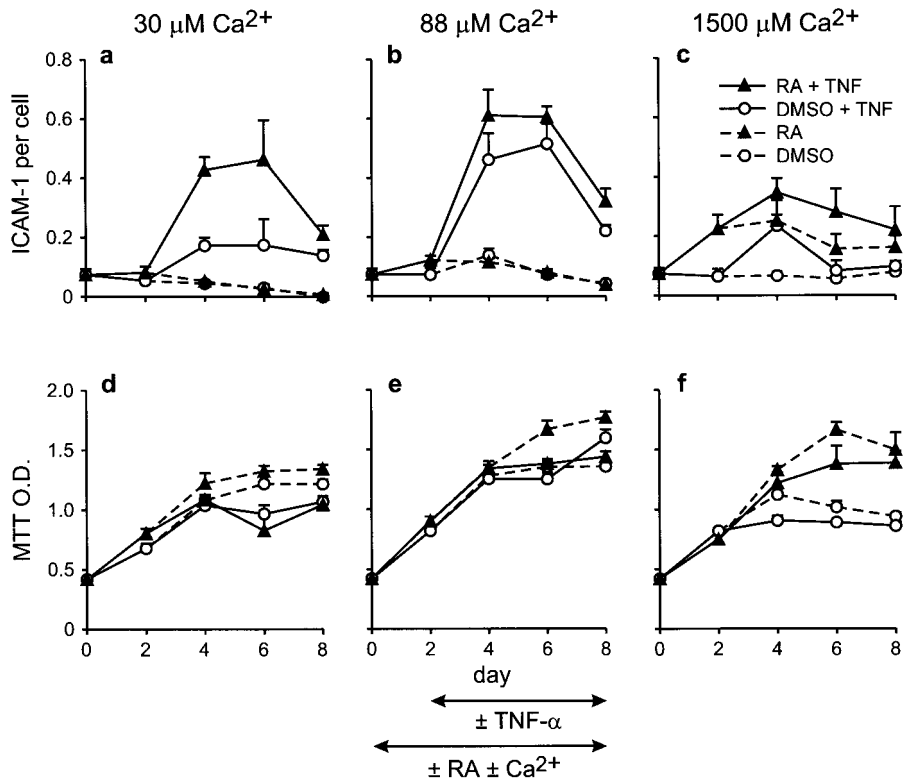

FIG. 3. Influence of different Ca^{2+}-concentrations on retinoic acid effects on TNF-α-induced ICAM-1 protein. Cell ELISA of ICAM-1 expression and parallel MTT assay after combined treatment of NHK with 10^{-6} M RA and 3 ng/ml TNF-α at different time points. The three different columns represent three different calcium conditions. Dashed lines (- - -) represent conditions without cytokine, straight lines (—) conditions with cytokine. Open circular symbols (○) are minus RA conditions, filled triangular symbols (%) are plus RA conditions. Values are means ± SD (with n = 5). The experiment was performed as described in Fig. 1 and under Material and Methods.

all calcium-concentrations, TNF-α caused an increase in ICAM-1 cell surface expression in a time-dependent fashion, which was the strongest at 88 μM Ca^{2+} and the weakest at 1500 μM Ca^{2+}. This confirms earlier observations (24) for IFN-γ-induced ICAM-1 expression in different Ca^{2+}-conditions. The ICAM-1 induction by TNF-α had a transient time course with peak values at 2 and 4 days post cytokine induction.

In line with the data in Fig. 1, TNF-α-induced ICAM-1 expression was upregulated by RA, at all three Ca^{2+}-conditions studied. At the different time points, differences were observed in the extent of the RA effect but throughout time the trends remained the same. If one considers the correction of the ICAM-1 signals for mitochondrial activity, the potentiating effect of RA on TNF-α-induced ICAM-1 is underestimated (compare Figs. 3a–3c with 3d–3f).

In parallel, the expression of a keratinocyte differentiation marker, transglutaminase I was measured (data not shown). Transglutaminase I was induced by confluency and at 1500 μM Ca^{2+} and was completely abolished by RA, indicating that the cells responded normally to RA-treatment.

DISCUSSION

Retinoic acid is a morphogen, thought to contribute to the stratification of the epidermis, possibly by being present in a basal to suprabasal gradient (22). RA also interferes with the epidermal immune response, both positively and negatively (e.g., 23, 32). We further investigated the modulation of epidermal immune responses by RA and focused on the effect of all-*trans*-RA on basal and TNF-α-induced ICAM-1 expression in normal human keratinocytes.

Basal ICAM-1 expression levels were not detected in resting keratinocytes, neither at protein nor at mRNA level as shown previously (5,33). RA alone was unable to induce ICAM-1 expression in NHK, grown at low calcium concentrations (88 μM) confirming earlier observations (17, 24). However, Fisher *et al.* (25) and Barker *et al.* (6) did measure an RA-induced increase in ICAM-1 mRNA levels. This might be due, for the results of Barker *et al.* (6), to the RA-treatment which was in combination with the calcium ionophore ionomycin. In tumor cells, RA has been established as a potent inducing agent for ICAM-1 expression (16), due

to RA activation of the RAR-β receptor and subsequent binding of the RAR-β/RXR-α heterodimer to the RARE element in the 5′ part of the ICAM-1 promotor (34), which is sufficient to activate ICAM-1 gene transcription. Wang *et al.* (16) further showed that in a RA-insensitive tumor cell line (MeWo cells), which did not show enhanced ICAM-1 expression in response to RA-treatment, the RAR-β receptor was not expressed nor induced after RA-stimulation. In skin, RAR-γ is the predominant RA-receptor and RAR-α is present in substantially lower amounts (21). Normal human keratinocytes do not express the RAR-β receptor (43). Therefore, the observed lack of effect of RA-treatment on ICAM-1 might be due to the absence of RAR-β nuclear receptor in NHK. However, we obtained different results when culturing keratinocytes in 1500 μM calcium. RA markedly increased basal ICAM-1 levels. It has been well documented that extracellular calcium is a critical factor in the expression of many differentiation marker genes in keratinocytes, such as transglutaminase-1, involucrin, etc. (35–36). However, preliminary observations (RT-PCR) indicate that RARβ mRNA is not expressed in high calcium conditions (our own observations and (43)), suggesting that RARβ does not account for the potentiation of basal ICAM-1 by RA.

Wang *et al.* (16) also showed that RA was able to enhance cytokine-induced ICAM-1 both in tumor cells which did express RAR-β as in cells lacking RAR-β. This suggests that these effects rely on RAR-α and RAR-γ expression and hence might also be operational in normal human keratinocytes. Synergistic effects of RA on IFN-γ-induced ICAM-1 expression in keratinocytes have been described earlier (24, 26), although others were unable to detect any modulatory effect of RA (2). We focused on the modulation of TNF-α-induced ICAM-1 by RA. RA clearly and consistently amplified TNF-α-induced ICAM-1 expression in keratinocytes in all Ca^{2+}-conditions studied. These results are, to our knowledge, novel and establish RA as a potentiating agent of TNF-α-induced ICAM-1 in normal human keratinocytes. This observation is in line with previously obtained results in tumor cell lines, such as human thyroid carcinoma cell lines (18) or human melanoma cell lines (16), where RA also potentiates TNF-α-induced ICAM-1 expression. However, in dermal microvascular endothelial cells, Gille *et al.* (27) were unable to detect any effect of RA on TNF-α-induced ICAM-1, in contrast to an inhibitory effect of RA on TNF-α-induced VCAM-1. As endothelial cells and keratinocytes are of different lineage (mesodermal versus ectodermal origin), the ICAM-1 gene might be regulated in a different way.

ICAM-1 is not the first TNF-α-induced gene whose expression is potentiated in combination with RA. Harant *et al.* (37) reported that simultaneous treatment of the human melanoma cell line G-361 with all-*trans*-RA and TNF-α resulted in a synergistic induction of IL-8 mRNA and of IL-8 promotor activity. The synergistic induction required an intact NF-κB site. RA was thought to change the composition of NF-κB complexes bound to the NF-κB responsive element in the IL-8 promotor and to remove inhibitory proteins, which resulted in an enhanced p65/p50 binding. The induction of ICAM-1 by TNF-α also relies on the NF-κB pathway. TNF-α induces ICAM-1 promotor activity through binding of predominantly p65/p65 homodimers or p65/c-Rel heterodimers at the NF-κB binding site (1), located between -176 and -206 bp in the ICAM-1 promotor (38). The hypothesis from Harant *et al.* (37) has not been confirmed for keratinocytes. It should be noted that in G-361 cells RA on itself induced IL-8 promotor activity, while we did not see any effect of RA alone on ICAM-1 expression. This might suggest that the mechanism is not the same for the two genes and/or the two cell types.

RA has also been shown to increase the cell surface expression of both TNF receptors two-fold in neuroblastoma SKNBE cells (39) and to augment in HL60-cells the expression of at least the p75 TNF receptor (40,41). Human epidermal keratinocytes express the 55 kDa receptor, but not the 75 kDa receptor (42). A similar mechanism in keratinocytes could explain the enhancing effect of RA on TNF-α.

In summary, we conclude that RA potentiates TNF-α-induced ICAM-1 expression, both at protein and at mRNA levels in human. This was confirmed for different keratinocyte strains, at different time points post cytokine induction and in keratinocytes grown in different calcium conditions.

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