CD23-Mediated Nitric Oxide Synthase Pathway Induction in Human Keratinocytes Is Inhibited by Retinoic Acid Derivatives

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Retinoids exert various functions including anti-proliferative and anti-inflammatory effects on many cell types including keratinocytes and are widely used in skin diseases, such as psoriasis and acne. We have previously shown that human keratinocytes express low affinity immunoglobulin E receptor (FcεRII/CD23) when stimulated with interleukin-4. Immunoglobulin E ligates CD23 and induces the production of nitrites by low affinity immunoglobulin E receptor induced the production of tumor necrosis factor by these cells by 70%. The level of inducible NO synthase activity in activated human keratinocytes was significantly decreased upon treatment of the cells with RA derivatives (inhibition by 60% of the mean inducible NO synthase activity with 13-cis RA, 2 μM). Treatment for 24 h with RA derivatives almost completely abolished transcription of inducible NO synthase-specific mRNA in activated keratinocytes. Therefore, RA derivatives downregulate tumor necrosis factor-α release and the NO-transduction pathway through the inhibition of inducible NO synthase transcription. Together, our data provide evidence for inhibition of the NO-pathway by 13-cis and all-trans retinoic acid on CD23-activated human keratinocytes. These data may clarify the mechanism of the anti-inflammatory activity of RA derivatives in skin diseases. Key words: CD23/keratinocytes/NO synthase/retinoids J Investig Dermatol 106:1182-1186, 1996

Nitric oxide, a highly reactive free radical produced by many human cell types, including keratinocytes, has emerged as an important mediator of inflammatory responses (Stuehr and Marletta, 1987; Billiar et al., 1992; Hibs et al., 1992; Nüssler et al., 1992; Moncada and Higgs, 1993; Thanhäuser et al., 1993; Mossalayi et al., 1994; Nathan and Qiao-Wen, 1994; Barnes and Liew, 1995). We have previously shown that activation of normal human epidermal keratinocytes leads to the stimulation of the nitric oxide (NO) pathway and to the release of many potent pro-inflammatory mediators, such as tumor necrosis factor (TNF-α) and interleukin (IL)-6 (Bécherel et al., 1994b). Hence, NO produced by activated keratinocytes, in concert with TNF-α and other pro-inflammatory cytokines, such as IL-1 or IL-6, might cause some of the inflammatory lesions in psoriasis or acne (Yoshinada et al., 1995).

We previously observed that the synthesis of inflammatory mediators such as TNF-α by activated keratinocytes was under the direct dependence of the NO pathway, because NO donors such as sodium nitroprussate initiated TNF-α synthesis and because inhibitors of NO synthases (NOS) such as Nω-monomethyl-l-arginine significantly decreased TNF-α synthesis by activated cells (Bécherel et al., 1994b).

13-cis (isotretinoin) and all-trans retinoic acid (tretinoin) are widely used in proliferative and/or inflammatory skin diseases, such as psoriasis, acne, epidermotropic T-cell lymphomas, or epithelial skin cancers (Luceck and Calburn, 1985; Fisher et al., 1991). A major pathway through which RA exerts its antiproliferative and differentiation effects is believed to involve binding to nuclear RARs (retinoic acid receptors α, β, and γ), members of the steroid receptor superfamily (Durand et al., 1992; Chambon, 1994). Cytoplasmic binding proteins, CRABPs (cellular retinoic acid binding proteins), have also been identified, but their functions remain to be determined (Astrom et al., 1991). In a previous work, we identified a new mechanism of action of RA as potent inhibitors of NOS induction in keratinocytes activated through nonspecific stimuli (lipopolysaccharide [LPS]/interferon [IFN]-γ) (Bécherel et al., 1994a). In another report, we demonstrated that these cells expressed the low affinity immunoglobulin (Ig)E receptor (FcεRI/CD23) after stimulation by IL-4 (Bécherel et al., 1994b). Furthermore, IgE binding on this receptor induced the production of

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; INF, interferon; iNOS, inducible NO synthase; HPRT, hypoxanthine phosphoribosyltransferase.

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nitrates (reflecting the mobilization of the NO pathway) and TNF-α by human keratinocytes.

This study was designed to investigate the effects of retinoic acid (RA) derivatives on the production of NO by human keratinocytes activated with immunoglobulin (IgE/anti-IgE or anti-CD23 mAb). Our results suggest that retinoids, and in particular all-trans and 13-cis RA derivatives, are potent inhibitors of the CD23-mediated activation of the NO pathway and TNF-α secretion by human keratinocytes.

**MATERIALS AND METHODS**

**Materials**

RA derivatives (13-cis retinoic acid, all-trans retinoic acid, retinol, retinol-aldehyde) were purchased from Sigma (St Louis, MO). Stock solutions of retinoids (10⁻⁴ M) were prepared in dimethyl sulfoxide and stored protected from light at −80°C. Immediately before use, the stock solution was diluted to the desired concentration with the keratinocyte culture medium. EGTA, leupeptin, aminomethylbenzenesulfonylfluoride, tosyl, lysylchymotrypsin, pepstatin, bestatin, chymostatin, L-arginine, L-citrulline, NADPH, and 0.66 μM/ml [³H]GTPγS. The reaction was stopped with a resin suspension that binds selectively to L-arginine but not to L-citrulline (Bio-Rad, Richmond, VA), and the supernatant removed into scintillation vials with an aqueous liquid scintillator (ALCα, Amersham). The reaction was made with or without EGTA (2 mM) to determine the Ca²⁺-independent activity of the inducible enzyme, and with N⁰ mono-methyl-L-arginine to determine the background.

**Inducible NOS mRNA Analysis**

Human CD23⁻⁺ keratinocytes were treated with anti-CD23 or IgE/anti-IgE for various periods of time in the presence of 2 μM of 13-cis or all-trans RA to investigate the effect of these compounds on the inducible NO synthesis (iNOS) mRNA transcription. For mRNA isolation, 3⁻¹⁰⁷ cells/assay were treated in RNAzole (Bio-probe, Montereau-Bois, France) for 3 h at 4°C. Total RNAs were extracted with a modified single-step guanidinium isothiocyanate and phenol/chloroform extraction method. Reverse transcription, semi-quantitative mRNA amplification, and visualization of the polymerase chain reaction products were performed exactly as previously described using the following specific primers (Reiling et al., 1994; Becherel et al., 1995): iNOS sense (5' ATGCCAGATGGCAGATCGAG 3'), exon 8, bases 1020–1040, and iNOS antisense (5' ACCTTCTCCAGGATGTGTA 3'), exon 11, bases 1371–1390; hydroxyphosphorylretinoic acid (HPR) mRNA sense (5' TATGGACAGACTGAGGCTTCGC 3') and HPRPT mRNA antisense (5' CACGACACTAGTCAAATCTCTGATA 3'). The expected fragment amplified from iNOS mRNA is 371 bp long, while a 496-bp fragment is expected to be amplified from HPRPT message. The molecular weight marker VI (Bgl II-digested pBR328 DNA + HindIII-digested pBR328 DNA; Boehringer-Mannheim) was used.

**RESULTS**

CD23 expression by keratinocytes requires induction by IL-4. Therefore, all experiments were performed with IL-4-pretreated keratinocytes.

**Inhibitory Effect of RA Derivatives on CD23-Mediated NO (Nitrates) Generation by Keratinocytes**

Unstimulated keratinocytes produced undetectable amounts of nitrates in the presence or absence of RA alone (2 μM, 48 h), whereas stimulation of the cells with IgE/anti-IgE induced high levels of nitrates (Fig 1A). Stimulation experiments conducted in medium devoid of hydrocortisone and/or EGF showed no significant differences in nitrates levels secreted as compared with stimulation in supplemented medium (data not shown). Pretreatment of the cells with 13-cis RA (2 μM), but not vehicle alone, significantly decreased their ability to produce nitrates in response to IgE/anti-IgE (Fig 1A). Similar results were obtained with all-trans RA (data not shown). By contrast, retinol (Fig 1A) and retinal (data not shown) did not inhibit this nitrate generation. Equivalent results were obtained when cells were stimulated with anti-CD23 mAb (10 μg/ml, data not shown). The effect of active RA derivatives on nitrate production by keratinocytes was also concentration- and time-dependent: the inhibitory effect started with 0.25 μM RA derivatives and reached a plateau at 2 μM with a maximum inhibition after 48 h of treatment (Figs 2A and 3A).

**Inducible Effect of RA Derivatives on TNF-α Production by Activated Keratinocytes**

The effect of in vitro RA derivatives treatment on TNF-α release by activated human keratinocytes was next investigated. CD23⁻⁺ cells (10⁷) were activated in 24-well plates with IgE (10 μg/ml)/anti-IgE (50 μg/ml) in the presence or absence of RA derivatives (2 μM). After 48 h, cell supernatants were harvested and assayed for TNF-α activity. Of the retinoids tested, 13-cis RA and all-trans RA were the most potent inhibitors of TNF-α production by activated keratinocytes (Fig 1B). Retinal and retinol did not inhibit TNF-α production (Fig 1B). RA had no effect on TNF-α release by nonactivated keratinocytes and di-methyl sulfoxide (the vehicle used for delivering RA to the cultures) had no effect on IgE/anti-IgE–induced TNF-α production. RA-mediated suppression of nitrites and TNF-α production by activated keratinocytes was time- and concentration-dependent.
Figure 1. **In vitro** effect of RA derivatives (13-cis retinoic acid) treatment on (A) nitrites and (B) TNF-α release by activated normal human epidermal keratinocytes. Human keratinocytes (10⁵) were activated in 24-well plates with IgE (10 μg/ml) for 1 h and then anti-IgE (50 μg/ml) in the presence or absence of 13-cis RA (2 μM, added 24 h before the cell activation). After 48 h, cell-free supernatants were harvested and assayed for nitrites and TNF-α content as described in Materials and Methods. Results are expressed as mean ± SEM of four different experiments.

(Fig 2B and 3B). The maximum inhibition was with 2 μM and after a 48-h treatment. All-trans and 13-cis RA exhibited similar effects, while retinol and retinal did not inhibit TNF-α secretion, even after a 48-h exposure. Similar results were obtained when cells were stimulated with 10 μg/ml anti-CD23 mAb (data not shown).

**Anti-Proliferative Effect and Inhibition of NO Secretion by RA on Activated Keratinocytes Is Reversible** To demonstrate that retinoids were not toxic for keratinocytes, we investigated whether those cells could recover their proliferative capacities and their ability to release nitrites under IgE/anti-IgE stimulation after RA withdrawal. The cells were washed after a continuous 48 h exposure to RA and incubated with the keratinocyte culture medium alone. As shown in Fig 4, the anti-proliferative effect of RA was completely reversed 48 h later, and the cells regained their ability to release NO at 96 h. Therefore, the inhibitory effect of RA is reversible.

**Inhibitory Effect of RA on the Activation of iNOS in Stimulated Keratinocytes** To investigate the mechanism of the inhibitory effect of retinoids on nitrite generation by activated keratinocytes, the effect of cell incubation with retinoids on iNOS enzyme activity through the conversion of [l-¹⁴C]arginine into [l-¹⁴C]citrulline was examined. Results in Fig 5 show significant decrease of iNOS activity with 2 μM 13-cis RA (inhibition by 60%). Like all iNOS so far reported, the iNOS isozyme involved belonged to the Ca²⁺-independent class of NOS because EGTA addition did not modify the enzymatic activity in activated cells (Fig 5). Experiments were carried out to determine at which level 13-cis RA inhibited iNOS activity by activated keratinocytes. RA derivatives had no direct effect on NOS enzyme activity when added in total cytosolic extract (data not shown). Therefore, the effect of 13-cis RA on the induction of iNOS mRNA was next investigated using reverse transcriptase-polymerase chain reaction. As shown in Fig 6, IgE/anti-IgE or anti-CD23 treatment induced the expression of iNOS mRNA expression after 6 h. This expression was maximum at 18 h and disappeared after 24 h. 13-cis RA (2 μM, 24 h pretreatment) strongly inhibited iNOS mRNA expression induced with IgE/anti-IgE or anti-CD23 treatment in keratinocytes. Under the different conditions tested, equivalent amounts of HPRT specific mRNA were amplified (Fig 6).

**DISCUSSION**

Activation of NO-dependent pathway is an important event in the release of pro-inflammatory cytokines (IL-6, TNF-α) by stimulated keratinocytes during innate immunity (LPS, IFN-γ). NO metabolites, such as peroxynitrites produced during this activation, might account for the sustaining of skin inflammatory diseases. In a previous work, we reported the potent inhibitory effect of RA derivatives on NO-pathway induction by LPS/IFN-γ in keratinocytes (Bécherel et al, 1994a). In addition to nonspecific stimulation, the induction of iNOS transcription in human keratinocytes has also been suspected following other stimuli. We have previously demonstrated that these cells expressed CD23 when stimulated with IL-4 or IFN-γ (Bécherel et al, 1994b) and that this CD23 expression was functional because IgE/anti-IgE or anti-CD23 mAb directly mediated the activation of the NO-dependent pathway (Bécherel et al, 1994b). CD23 is the low affinity receptor for IgE (FcεRII) (Delespesse et al, 1991; Mosalayi et al, 1992) and also binds other ligands such as CD11b, CD11c, and CD21 (Aubry et al, 1992). These various ligands may allow keratinocytes to interact with macrophages, and B and T lymphocytes in inflammatory reactions.
Here, we clearly show the induction of a Ca\(^{2+}\)-independent NOS through measurement of nitrate synthesis, enzymatic activity, and mRNA transcription following CD23\(^{+}\) keratinocytes stimulation either with IgE/anti-IgE or with anti-CD23 mAb. To investigate whether RA derivatives could also inhibit CD23-dependent NO-pathway induction, we assayed the effects of the two potent therapeutic RA derivatives (all-trans and 13-cis isomers) on the induction of nitric oxide synthase, NOS, enzymatic activity, and iNOS mRNA transcription in IgE/anti-IgE- or anti-CD23-stimulated human normal keratinocytes. The \textit{in vitro} activity of these two compounds was compared with that of retinol and retinaldehyde, much less potent molecules. Our results demonstrated a concentration- and time-dependent inhibitory effect of RA derivatives on the generation of nitrites and TNF-\(\alpha\) synthesis by IgE/anti-IgE- or anti-CD23-activated keratinocytes. 13-cis and all-trans retinoic acid reduced the production of nitrites and TNF-\(\alpha\) by activated cells by 80% and 70%, respectively. Moreover, our results concerning the effect of these compounds on the iNOS pathway activation confirmed their potent inhibitory action, as evidenced through [\(^{14}\)C]arginine conversion assay and iNOS mRNA expression investigated using reverse transcriptase-polymerase chain reaction with specific primers. Furthermore, we confirmed the anti-prolifer-
Figure 6. iNOS mRNA induction is inhibited by RA derivatives in activated keratinocytes. iNOS mRNA was analyzed by reverse transcriptase-polymerase chain reaction as described in Materials and Methods section. Lanes A, IgE/anti-IgE-stimulated cells; lanes B, anti-CD3 23 mAb-stimulated cells; and lanes C, stimulated cells pretreated with 13-cis-RA (2 μM). The iNOS mRNA-amplified fragment is represented by 371-bp bands, whereas HPRT controls are represented by 496-bp bands.

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