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Ethacrynic Acid and Citral Suppressed the All Trans Retinoid-Induced Monocyte Chemoattractant Protein-1 Production in Human Dermal Fibroblasts

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Abstract − Skin irritation caused by retinol and retinoic acid results in mild erythema called as retinoid dermatitis. To develop compounds modulating the retinoid dermatitis, we tried to establish the screening method for retinoid dermatitis. At first we examined the inflammatory cytokine profile in neonatal human dermal fibroblasts which are known to be one of main site of retinoid action. As a result, interleukin-8 (IL-8) and monocytes chemoattractant protein-1 (MCP-1) were significantly produced by all trans retinoic acid (ATRA) and all trans retinol (ATROL) in dermal fibroblasts. Especially the production of MCP-1 was more than that of IL-8. The production of MCP-1 by retinoid was dose-dependently increased, continuing up to 24 hrs. After then using ethacrynic acid (ECA) known to reduce mouse ear edema induced by ATRA, we checked whether ECA suppressed the production of MCP-1. As a result, ECA effectively suppressed the production of MCP-1 in the ATRA- or ATROL-treated-fibroblasts. These results suggested that screening method effectively reflects the *in vivo* anti-inflammatory activity of ECA. It was reported that citral inhibited the enzyme involved in the conversion of ATROL to ATRA. We showed that citral suppressed the production of MCP-1 in ATROL-treated fibroblasts. We expect these finding might be helpful to find useful compounds modulating the side effects of retinoid or retinoid dermatitis.

Keywords: MCP-1, All trans retinoic acid, Dermal fibroblast, All trans retinol, Ethacrynic acid, Citral

INTRODUCTION

Retinoids, as a class of natural or synthetic compounds structurally related to vitamin A, play an essential role in various biologic functions, such as vision, embryogenesis, cell proliferation and differentiation (Orfanos *et al*., 1985; Li *et al*., 2000). Retinoids have been widely used for dermatological applications to acne, psoriasis, keratinization disorders and cutaneous malignancies and for cosmetic formulations for reducing wrinkles and improving the appearance of cellulite (Boehm *et al*., 1995; Schwarz *et al*., 1997; Varani *et al*., 1998; Kligman *et al*., 1999). Parallel with retinoid beneficial effects, however, topical application of retinoids often cause severe irritation resulting in mild erythema and stratum corneum peeling in skin (Kang *et al*., 1995). This erythematous reaction is clinically similar to a mild irritant dermatitis and also called as retinoid dermatitis (Fisher *et al*., 1991; Shalita *et al*., 1996).

Pharmacological mechanisms of retinoids on their biological effects have been well known by nuclear receptor studies. At the cellular level, retinoids exert their effects via two families of nuclear receptors, known as retinoic acid receptors (RAR) and retinoid X receptors (RXR). RARs and RXRs are ligand-dependent transcription factors that belong to the superfamily of steroid/thyroid hormone nuclear receptors (Petkovich *et al*., 1987; Brand *et al*., 1988; Mangelsdorf *et al*., 1992). However, the cellular mechanism of retinoid dermastitis is not fully understood. Some reports suggested that retinoid-induced irritation is most likely mediated through a separated mechanism from reti-

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noid biological effects via retinoid receptor activation (Kang *et al*., 1995). Erythema induced by retinoids was studied in human (Kang *et al*., 1995) and mouse ear edema evoked by retinoic acid was also reported (Wille *et al*., 2000), but study on the irritation and following inflammation from retinoid in celluar level is limited to keratinocytes from epidermis. Inflammatory effect of retinoid on cell from dermis part (for example fibroblasts) is rarely studied in vitro. Especially fibroblasts, major component of dermal part regarded as playing the role of synthesizing the extracellular matrix for integrity of tissue and repair. However several findings suggested that fibroblasts begin inflammation response and recruit leukocyte and have an immunological function as tissue defense for connective tissue (Postlethwaite, 1993; Newby *et al*., 2000). But the role of dermal fibroblasts in retinoid dermatitis is not fully examined. The modulation of retinoid-induced irritation is difficult due to unclear the mechanisms. Especially retinoid induced inflammation in dermal layers is rarely known. Therefore we investigated the inflammatory effects of retinoid on dermal fibroblasts. At first we checked the profiles of cytokines in dermal fibroblasts treated with all trans retinoic acid (ATRA) or all trans retinol (ATROL). We evaluated the usefulness of the screening method measuring monocyte chemoattractant protein-1 (MCP-1) in dermal fibroblasts using ethacrynic acid (ECA) and citral.

MATERIALS AND METHODS

Chemical reagents

All trans retinoic acid (ATRA), all trans retinol (ATROL), ethacrynic acid (ECA), and citral were purchased from Sigma chemical (St. Louis, MO, USA). ELISA kits for interleukin-8 (IL-8), monocyte chemoattractant protein (MCP-1) and other cytokines were from Pharmingen (San Diego, CA, USA).

Cell culture

Cultures of normal human dermal fibroblasts were established from individual neonatal foreskins according to Tsao's method (Tsao *et al*., 1982). Foreskins were cut in small fragments and treated overnight at 4^{o}C with trypsin-ethylenediamine tetraacetic acid solution (0.05-0.02% in phosphate-buffered saline solution). This allowed dermal epidermal separation by peeling off the epidermis. The dermis was incubated in collagenase solution for 1 hr at 37^{o}C to release dermal fibroblasts from the matrix and then the released fibroblasts were grown in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY). All cultures were maintained in a tissue culture incubator at 37^{o}C in 5% CO_2.

Enzyme linked immunosorbent assay for cytokine determination

Cytokines assays were measured by a commercially

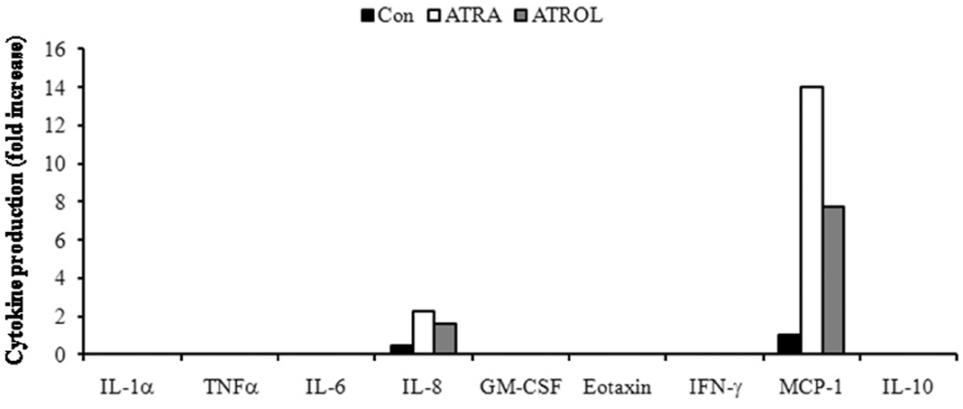

Fig. 1. Induction of MCP-1 and IL-8 protein from dermal fibroblasts by ATRA and ATROL, respectively. Dermal fibroblasts (1×10^5 cells/ml) were cultured with indicated concentrations of ATRA and ATROL for 24 hours. Cytokine protein levels in dermal fibroblasts media were analysed by ELISA. Analysis was performed in conditioned media from fibroblasts under basal conditions (black bar), exposed to ATRA (blank bar), or exposed to ATROL (crossed bar). ELISA determinations were performed in three different samples from fibroblasts. Mean values were compared by Student's t-test for paired samples.

available enzyme linked immunosorbent assay kit.

Briefly, wells of 96 well flat-bottom microtiter plates were coated with 100 μl anti-human cytokine monoclonal antibody (OptEIA™, Pharmingen, USA) diluted in coating buffer (0.1 M carbonate, pH 9.5) at a final concentration of suggested concentration after overnight incubation at 4°C. The wells were blocked with 200 μl of 10% FBS in PBS at room temperature for 1 hr.

Cultured cells were seeded into assay plates and treated with indicated concentrations of ATRA or ATROL. After incubation for indicated times, cell supernatants were collected and the concentration of MCP-1 was measured by ELISA using commercially available kits with manufacturer-recommended protocols. In short, MCP-1 in the culture supernatants was captured by monoclonal anti-human MCP-1 antibody and detected with a biotinylated anti-human monoclonal MCP-1 antibody. Quantification of secreted cytokine was accomplished by the normalization of the ELISA data with a standard MCP-1 dose curve. Measurement of other cytokine release in cultured cells was accomplished as the same method as MCP-1.

RESULTS

All trans-retinoids induce increase of MCP-1 and IL-8 production in human dermal fibroblasts

To elucidate the cytokines involving in retinoid dermatitis, we first examined the profiles of cytokines in cultured dermal fibroblasts under basal conditions or exposed to ATRA or ATROL, respectively. In the exposed time, fibroblasts were maintained under no FBS to avoid the influence of growth factors and retinoid present in FBS on cytokine production by added retinoid. Exposure of dermal fibroblasts to ATRA and ATROL at dose of 10 μM induced the increase in the secretion levels of MCP-1 and IL-8, in contrast, IL-6, IL-1α, TNF-α, GM-CSF, eotaxin, IFN-γ, IL-12 and IL-10 in fibroblasts were not detected after all trans retinoids treatment (Fig. 1). Especially MCP-1 production by all trans retinoids was very significant in dermal fibroblasts. Due to high level of MCP-1 induction, we decided the expression level of MCP-1 as a criterion of cell based screening systems for reflecting the retinoid-induced dermatitis.

Effects of concentrations and times of all trans retinoids on the production of MCP-1 in dermal fibroblasts

To screen the compounds modulating the retinoid-induced dermatitis, we tried to establish the screening method measuring the MCP-1 production by retinoid-induced dermal fibroblasts. Induction of MCP-1 by the ATRA and ATROL was examined in human dermal fibroblasts cultured with various concentration or various times of ATRA or ATROL, respectively. Cells treated with ATRA or ATROL (0.01 to 10 μmol/L) for 24 hrs released MCP-1 into the medium in a concentration-dependent manner. In case of ATRA treated, levels of MCP-1 protein reached a 7-fold increase at the highest concentration of ATRA tested. Induction of MCP-1 by same concentration of ATROL was less than that of ATRA (Fig. 2A). MCP-1 induction was rapid and production of MCP-1 was still continued up to 24 hours (Fig. 2B).

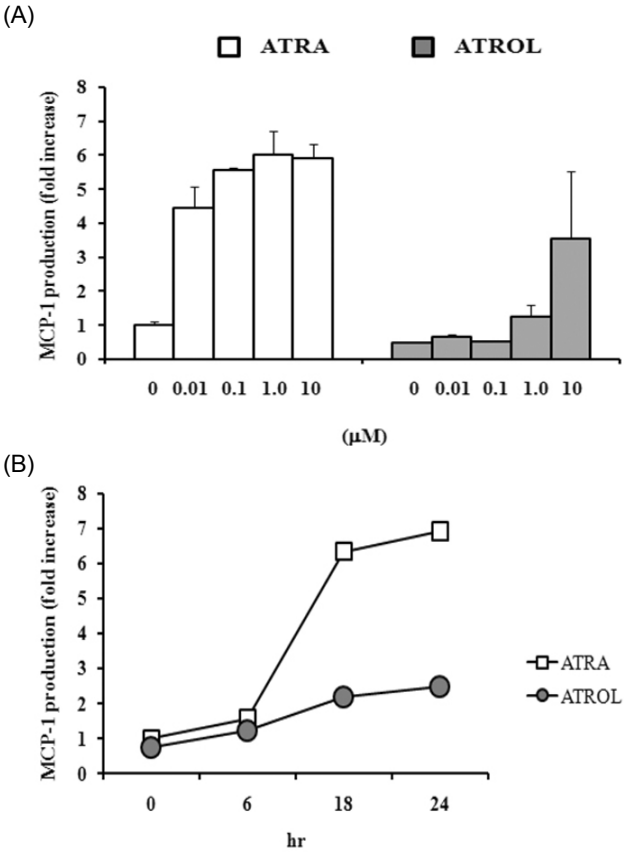

Fig. 2. (A) Effect of various concentration of ATRA and ATROL, respectively on production of MCP-1 from dermal fibroblasts. Dermal fibroblasts (1×10^5 cells/ml) were cultured with indicated concentrations of ATRA and ATROL for 24 hours. (B) Time course of MCP-1 secretion by ATRA and ATROL from dermal fibroblasts. Cell culture medium was harvested from the dermal fibroblasts treated with 10 μM ATRA or ATROL at indicated time points. MCP-1 released into the medium was determined by ELISA.

Ethacrynic acid suppressed the ATRA and ATROL-induced MCP-1 induction in dermal fibroblasts

We evaluated the usefulness and adequacy of the retinoid-induced MCP-1 production in dermal fibroblast re-

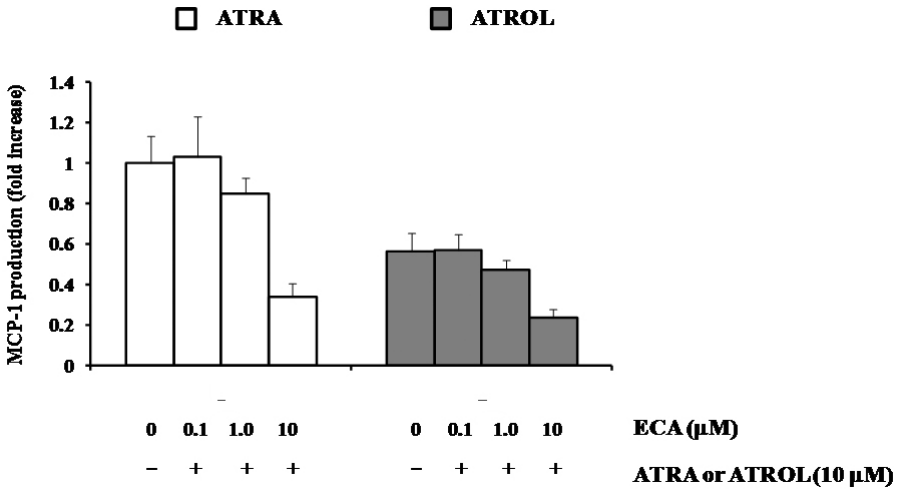

Fig. 3. Ethacrynic acid inhibits effect of ATRA and ATROL on MCP-1 induction. Dermal fibroblasts (1×10^5 cells/ml) were cultured with varied concentration of ECA (0.1 to 10 μM) and ATRA or ATROL respectively. MCP-1 released into medium was determined by ELISA after cells were cultured for 24 hours with indicated agents.

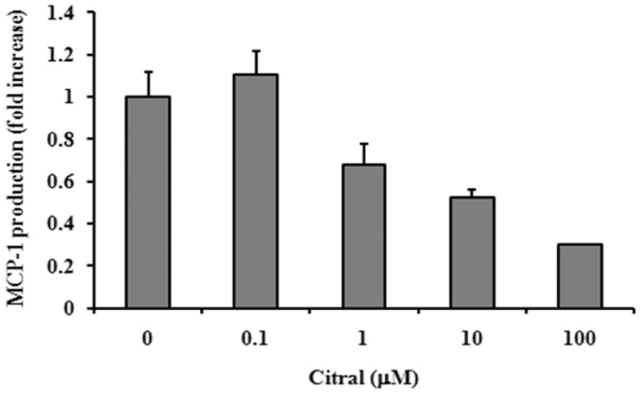

Fig. 4. Citral inhibits MCP-1 production in dermal fibroblasts induced by ATROL. Dermal fibroblasts (1×10^5 cells/ml) were cultured with varied concentration of citral (0.1 to 10 μM) and ATRA or ATROL. MCP-1 released into medium was determined by ELISA after cells were cultured for 24 hours with indicated agents.

flecting the retinoid-induced dermatitis using ECA, which is known to reduce the retinoid-induced ear edema in mice (Wille *et al*., 2000). When dermal fibroblasts were exposed to varied concentration of ECA (0.01 to 10 μM) and ATRA or ATROL respectively, ECA inhibited dose dependently the induction of MCP-1 production from dermal fibroblasts by ATRA or ATROL (Fig. 3).

Effect of citral on retinoic acid induced MCP-1 release in dermal fibroblasts

The mechanism involved in the increased production of MCP-1 by ATROL-induced fibroblasts is not clear. One possible mechanism is that ATROL itself showed induction of MCP-1. Another mechanism is that conversion of ATROL to ATRA is important for MCP-1 induction by ATROL. To confirm the latter cases, we blocked the enzyme (type-C alcohol dehydrogenase) involving the process of ATROL to ATRA using citral, which is known to inhibit this enzyme (Connor and Smit, 1987). We examined the MCP-1 production from fibroblasts co-treated with ATROL and citral. Production of MCP-1 by ATROL was decreased significantly by citral from dermal fibroblasts (Fig. 4).

DISCUSSION

In this paper, we reported that MCP-1 is induced in ATRA or ATROL-treated human dermal fibroblasts and ECA suppressed the MCP-1 production in ATRA or ATROL-treated fibroblasts. We also proved that type-C alcohol dehydrogenase is involved in the ATROL-induced MCP-1 production using citral (inhibitor of type-C alcohol dehydrogenase).

In Fig. 1, IL-8 induction in dermal fibroblasts by ATRA or ATROL is similar to induction of IL-8 in keratinocytes. However MCP-1 induction is unique in dermal fibroblasts (Dai *et al*., 2004). IL-8 is a member of the C-X-C subfamily of chemokines (Zlotnik and Yoshie, 2000). As a key factor in the pathogenesis of inflammatory diseases, IL-8 has diverse biological properties, including chemotaxis of neutrophils and T lymphocytes (Baggiolini and Clark-Lewis, 1992), regulation of cell adhesion (Djeu *et al*., 1990), activation of neutrophils (Mukaida *et al*., 1992), and modulation of histamine release (Kuna *et al*., 1991). The monocyte chemoattractant protein-1 (MCP-1/ CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes. CCL2 has been demonstrated to recruit monocytes into foci of active inflammation (Ajuebor *et al*., 1998). Especially MCP-1is known to be involved in the

pathogenesis of inflammatory bowel disease, allergic asthma, and rheumatoid arthritis (Ip *et al*., 2006; Spoettl *et al*., 2006; Rantapaa-Dahlqvist *et al*., 2007). Therefore MCP-1 might be a key inflammatory mediators in retinoid-induced dermatitis. Recently MCP-1 is reported to be involved in the acute respiratory distress syndrome induced by retinoic acid therapy of acute promyelocytic leukemia (Tsai *et al*., 2008). Therefore, we speculated that the induction of MCP-1in dermal fibroblasts reflects the inflammatory process of retinoid induced dermatitis and established the screening method for modulating the retinoid dermatitis.

ECA is known to reduce the retinoid-induced ear edema in mice (Wille *et al*., 2000). So, we tested whether the anti-inflammatory effect of ECA affects the induction of MCP-1 in dermal fibroblasts. ECA showed reduction of MCP-1 production from dermal fibroblasts by ATRA or ATROL. These results suggested that dermal fibroblasts-based screening assay for compounds modulating the retinoid dermatitis might be valid to reflect the anti-inflammatory effects of ECA and this assay method might be helpful to find the compounds for modulating the side effect of retinoic acid. However ELISA method is labor-extensive and time consuming for the detection of MCP-1 in ATRA-treated fibroblasts. Therefore further studies are required to apply our findings to establishing high-throughput screening for modulating retinoid dermatitis.

The induction mechanism of MCP-1 by ATROL is still not clear but our results shown in Fig. 4 suggested that ATROL might convert to ATRA and then induced the production of MCP-1. ATROL is converted to ATRA mediated by type-C alcohol dehydrogenase (Connor and Smit, 1987). Citral is known to inhibit this enzyme. Citral with ATROL-treated group showed the reduction of MCP-1 production compared with ATROL only-treated group. These results suggested that type-C alcohol dehydrogenase is involved in ATROL-induced MCP-1 production and conversion of ATROL to ATRA might be required to the MCP-1 induction of ATROL-treated dermal fibroblasts.

Conclusively, in this paper, we found MCP-1 production was significantly increased in ATRA or ATROL-treated dermal fibroblasts and the responsiveness of MCP-1 levels to ECA reflects the retinoid dermatitis. Using citral, we also found that induction of MCP-1 production by ATROL requires conversion to ATRA. We expect these finding might be helpful to find useful compounds modulating the side effects of retinoid or retinoid dermatitis.

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