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Kojic Acid, a Potential Inhibitor of NF-κB Activation in Transfectant Human HaCaT and SCC-13 Cells

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The activation of NF-κB induced by kojic acid, an inhibitor of tyrosinase for biosynthesis of melanin in melanocytes, was investigated in human transfectant HaCaT and SCC-13 cells. These two keratinocyte cell lines transfected with pNF-κB-SEAP-NPT plasmid were used to determine the activation of NF-κB. Transfectant cells release the secretory alkaline phosphatase (SEAP) as a transcription reporter in response to the NF-κB activity and contain the neomycin phosphotransferase (NPT) gene for the dominant selective marker of geneticin resistance. NF-κB activation was measured in the SEAP reporter gene assay using a fluorescence detection method. Kojic acid showed the inhibition of cellular NF-κB activity in both human keratinocyte transfectants. It could also downregulate the ultraviolet ray (UVR)-induced activation of NF-κB expression in transfectant HaCaT cells. Moreover, the inhibitory activity of kojic acid in transfectant HaCaT cells was found to be more potent than known antioxidants, *e.g.*, vitamin C and *N*-acetyl-*L*-cysteine. These results indicate that kojic acid is a potential inhibitor of NF-κB activation in human keratinocytes, and suggest the hypothesis that NF-κB activation may be involved in kojic acid induced anti-melanogenic effect.

Key words: Kojic acid, Transfectant HaCaT and SCC-13 cells, NF-κB activation, Keratinocytes, Anti-melanogenic effect

INTRODUCTION

Environmental stimuli and chemicals have been shown to change gene expression in mammalian cells. The molecular mechanisms involved in cell response to stimuli and chemicals reveal that an important control occurs at the transcription level and is coordinated by various transcription factors. Among these transcription factors, NF-κB plays a major role in the regulation of genes responsible for inflammatory and immune responses (Baeuerle and Henkel, 1994; Baeuerle and Baichwal, 1997; Wulczyn *et al.*, 1996). NF-κB, in its latent form, is complexed in the cytoplasm bound to IκB inhibitor proteins. Exposure of cells to various NF-κB activators [e.g., tumor necrosis factor, interleukin-I, lipopolysaccharides, and ultraviolet light] lead to the phosphorylation and degradation of the inhibitory protein IκB. This enables NF-κB to translocate into the nucleus where it can change or alter expression of target genes. The structure, regulation and function of NF-κB have been well studied and reviewed by several investigators (Siebenlist *et al.*, 1994; Legrand-Poels *et al.*, 1998; Huxford *et al.*, 1998; Chen *et al.*, 1998; Grimm and Baeuerle, 1993).

The skin is a primary target for stimuli such as ultraviolet (UV) radiation and various pathogenic synthetic chemicals, frequently leading to inflammation associated with NF-κB activation. Keratinocytes and melanocytes are a functional unit that produces and distributes melanin in human skin. Several studies have shown that keratinocytes regulate both growth and differentiation of melanocytes (Donatien *et al.*, 1993; Gordon *et al.*, 1989; DeLuca *et al.*, 1988). Tyrosinase, the enzyme that controls the synthesis of melanin, is a unique product of melanocytes (Halaban *et*

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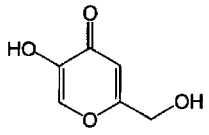

5-Hydroxy-2-hydroxymethyl-γ-pyrone

Fig. 1. Chemical structure of kojic acid

al., 1983; Hearing and Jimenez, 1987). Melanocytes synthesize and transfer melanin to the surrounding keratinocytes. Keratinocytes produce paracrine factors that affect melanocyte proliferation, dendricity, and melanin synthesis (pigmentation) (Gordon *et al.*, 1989). Therefore, the close interactions of keratinocyte-melanocyte, an epidermal-melanin unit were required to regulate melanocyte growth.

Kojic acid (2-hydroxymethyl-5-hydroxy-γ-pyrone) (Fig. 1) isolated from *Aspergillus oryzae* is a well known inhibitor of tyrosinase responsible for melanin biosynthesis in the melanocytes (Virador *et al.*, 1999; Curto *et al.*, 1999; Cabanes *et al.*, 1994), which conferred the cosmetic skin whitening effect of kojic acid. However, the genetic molecular basis regulatory action of kojic acid, i.e., NF-κB activation on melanogenesis in human keratinocytes has yet to be demonstrated.

We now report that the inhibitory effect of kojic acid on the activation of NF-κB in two human keratinocytes and suggest the hypothesis that the modulation of NF-κB in keratinocytes may be involved in anti-melanogenic effect induced by kojic acid.

MATERIALS AND METHODS

Great EscAPe Fluorescence detection kit was obtained from Clontech Laboratories, (Palo Alto, CA). Cell culture media and geneticin (antibiotic G-418) were from Gibco BRL (Grand Island, NY). Kojic acid, vitamin C and *N*-acetyl-*L*-cysteine (NAC) were from Sigma Chemical Co. (St. Louis, MO). Other chemicals and solvents were from Aldrich Chemical Co.(Milwaukee, WI). Human HaCaT cells were originally obtained from Dr. Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany) and human squamous cell carcinoma-13 (SCC-13) cells subcultured by this laboratory were used. The preparation of pNF-κB-SEAP-NPT plasmid used in this study and its transfection into human HaCaT and SCC-13 cells were described in detail previously (Moon *et al.*, 2001).

Cell culture

Transfectant human HaCaT and SCC-13 cells were cultured with 500-600 μg/ml of geneticin (100 mg/ml) for selection and maintenance of stable transformants. These cells were maintained at subconfluence in a 95% air, 5% CO_2 humidified atmosphere at 37°C. The medium used for routine subcultivation was Dulbeccos Modified Eagles Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were counted with a hemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

Reporter (SEAP) gene assay

Reporter enzyme (SEAP) was measured essentially as described previously using a fluorescence detection assay method (Moon *et al.*, 2001). Single cell-derived stable transfectant HaCaT (3×10^6) and SCC-13 (1.5×10^6) cells were plated on 5 ml of T-25 flask, respectively and the media was decanted 24 h later. At this time, cultures were washed twice with PBS, and incubations were initiated by addition of new media. The aliquots (25 μl) of medium in both a control and chemical-treated cultures were taken at 0, 3, 20, 24, and 48 h, heated at 65°C for 5 min to eliminate the endogeneous alkaline phosphatase activity, and used immediately or stored at -20°C. Kojic acid, vitamin C and NAC were directly added to the culture medium after 24 h of incubations. Mixtures, containing dilution buffer (25 μl), assay buffer (97 μl), culture media (25 μl), and 4-methylumbelliferyl phosphate (MUP, 1 mM, 3 μl) in 96 well plate were incubated for 60 min in the dark at room temperature. Fluorescence from the product of the SEAP/MUP were measured using a 96 well plate fluorometer (Molecular Devices, F max) by excitation at 360 nm and measuring light emission at 449 nm.

Ultraviolet irradiation of cells

Transfectant human HaCaT (3×10^6) and SCC-13 (1.5×10^6) cells were irradiated to a UVB with a peak emission at a wavelength of 312 nm in the tissue culture dishes (Aberdam *et al.*, 1993; Saliou *et al.*, 1999). The source of ultraviolet radiation was a Spectrolinker XL-1000 UV (Spectronics Co., USA) stimulator equipped with a UVB irradiation source and with microprocessor-controlled energy (mJ/cm^2). In brief, single cell-derived stable transfectant HaCaT (3×10^6) cells were plated on 5 ml of tissue culture dish, and the media was decanted 20 h later. Then, cultures were washed twice with PBS, and added to new media. Kojic acid was directly added to the culture medium at 0 h and incubated for 3 h. UVB was radiated to tissue culture dish directly at 60 mj/cm^2. The aliquots (25 μl) in both control and kojic acid-treated cultures were taken at 0, 3, 6, and 9 h, heated at 65°C for 5 min to eliminate the endogeneous alkaline phosphatase activity, and used immediately or stored at -20°C.

Statistical analysis

Data were summarized as mean ± SE. For analysis of

two groups the unpaired Students t-test was perfomed.

RESULTS AND DISCUSSION

The activation of NF-κB induced by kojic acid was evaluated in two human keratinocyte cell lines, e.g., transfectant HaCaT cells and transfectant SCC-13 cells to see the influence of activation of NF-κB in relation to melanogenesis. It has been reported that antioxidants such as vitamin C, a-lipoic acid and NAC reduce the activation of NF-κB in human HaCaT keratinocytes after exposure to a solar UV stimulator, resulting in the prevention of UVR-induced skin diseases through the modulation of NF-κB activation (Saliou *et al.*, 1999).

Kojic acid has been shown to whiten skin, i.e., depigmentation by the inhibition of tyrosinase activity for melanin biosynthesis in human melanocytes. Kojic acid is also known as an antioxidant through the radical scavenging activity (Niwa and Akamatsu, 1991; Mitani *et al.*, 2001). However, its biological activity at the molecular level linked to NF-κB activation on antimelanogenesis in human keratinocytes has not been suggested.

To examine the effect on the activation of NF-κB by kojic acid in keratinocytes, human HaCaT cells and SCC-13 cells transfected with pNF-κB-SEAP-NPT plasmid were used (Fig. 2). The HaCaT cell line is a human skin equivalent, spontaneously transformed and immortalized human keratinocytes and HaCaT cells have been shown to undergo normal differentiation similar to normal keratinocyte cell line (Boukamp *et al.*, 1988; Boelsma *et al.*, 1999). The SCC-13 cells, the malignant epidermal keratinocyte line derived from a human squamous cell carcinoma can be cultured on a fairly routine basis and serve as suitable model for aberrant terminal differentiation (Rheinwald and Beckett, 1980; 1981). Therefore, these two cell lines were selected to examine the influence of NF-κB activation in keratinocytes by kojic acid. Transfectant HaCaT and SCC-13 cells release the secretory alkaline phosphatase (SEAP) as a transcription reporter in response to the NF-κB activation and also contain the neomycin phosphotransferase (NPT) gene for selection marker. The released SEAP is measured using a fluorescence detection assay method (See Materials and Methods). Kojic acid (10 mM) was added to the culture medium after 24 h of initial incubations and SEAP activities were measured 24 h after exposure. Kojic acid showed the inhibition of NF-κB activation up to 60% and 30% compared to a control in transfectant HaCaT and SCC-13 cells, respectively (Fig. 3A and 3B). Then, we studied the effect of upregulation of cellular NF-κB activity on exposure to UVR in transfectant HaCaT cells. The activation of NF-κB expression in cells was observed compared to the control (Fig. 4). Treatment of the transfectant HaCaT cells with kojic acid (10 mM) inhibited the NF-κB activity by 95% and the degree of inhibition increased in a concentration-dependent manner (Fig. 4). These results indicated that kojic acid is a potent inhibitor of NF-κB activation in human keratinocytes. At

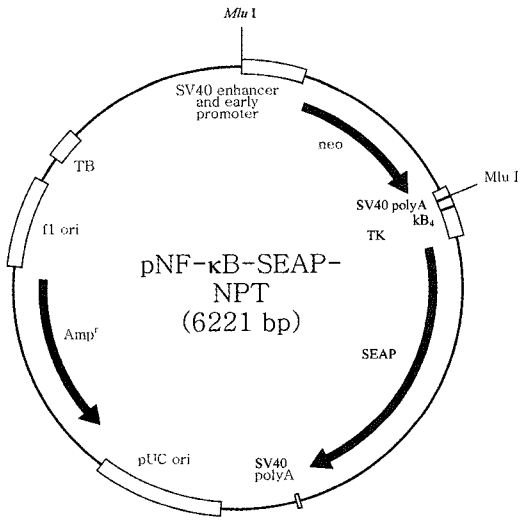

Fig. 2. Diagram of pNF-kB-SEAP-NPT plasmid.

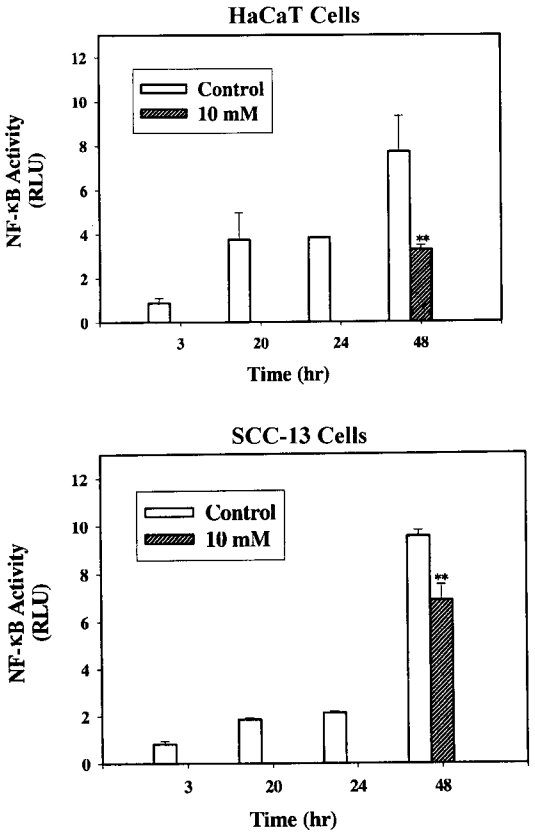

Fig. 3. Downregulation of cellular NF-kB activation by kojic acid in transfectant human HaCaT and SCC-13 cells. Each value represents the mean ± SE of three determinations. A significant difference between test and control group is indicated by *p<0.05 or **p<0.01. RLU stands for relative light units.

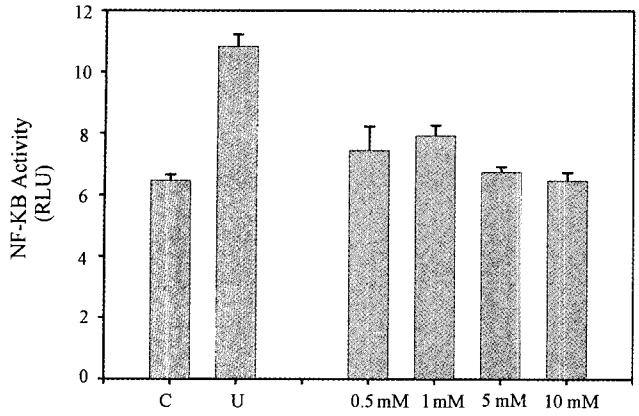

Fig. 4. Inhibitory effect of kojic acid on cellular NF-kB activity in UVR-treated transfectant HaCaT cells. Each value represents the mean ± SE of three determinations. RLU stands for relative light units. C and U represent the control and the values on exposure to UVR, respectively. A significant difference between test and control group is indicated by *p<0.05 or **p<0.01.

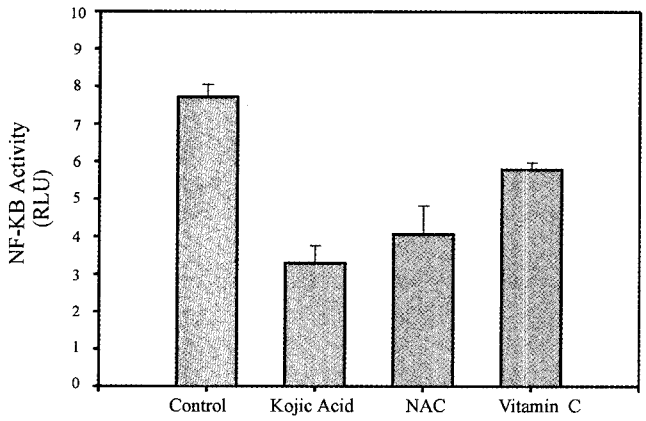

Fig. 5. Inhibitory effects of kojic acid, *N*-acetyl-*L*-cysteine (NAC) and vitamin C on the activation of NF-κB in transfectant human HaCaT cells. Chemicals (10 mM) were added to the culture medium on 24 h of incubations and SEAP enzyme activities were measured 24 h after exposure of chemicals. The SEAP enzyme of control was measured on 48 h of incubation. Each value represents the mean ± SE of three determinations. A Significant difference between test and control group is indicated by *p<0.05 or **p<0.01.

the same concentration (10 mM) of other inhibitors, which are known as antioxidants as well as inhibitors of NF-κB activation, e.g., *N*-acetyl-*L*-cysteins (NAC) (Aruoma *et al.*, 1989; Van den Broeke and Beijersbergen Van Henegouwen, 1995; Verhasselt *et al.*, 1999) and vitamin C (Bowie and O'Neill, 1997; Munoz *et al.*, 1997), the inhibitory action of kojic acid on NF-κB activation was greater than vitamin C and NAC in transfectant HaCaT cells (Fig. 5). Such results indicate that the inhibitory activity of kojic acid may regulate melanocytes behavior through the modulation of NF-κB activation in keratinocytes. Therefore, we put forward a hypothesis that the modulation of NF-κB activation in keratinocytes may be an important mediator associated with the control of melanogenesis. Further molecular mechanisms for the involvement of depigmentation mediated by kojic acid on activation of NF-κB in keratinocytes are necessary, and studies to explore the clinical usefulness of kojic acid are required whether this kojic acid can be used as an anti-inflammatory agent with skin whitening effect.

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