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Vanillin protects human keratinocyte stem cells against Ultraviolet B irradiation

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ABSTRACT

Ultraviolet-B (UVB) irradiation is one of major factors which induce cellular damages in the epidermis. We investigated protective effects and mechanisms of vanillin, a main constituent of vanilla beans, against UVB-induced cellular damages in keratinocyte stem cells (KSC). Here, vanillin significantly attenuated UVB irradiation-induced cytotoxicity. The vanillin effects were also demonstrated by the results of the senescence-associated β-galactosidase and alkaline comet assays. In addition, vanillin induced production of pro-inflammatory cytokines. Attempts to elucidate a possible mechanism underlying the vanillin-mediated effects revealed that vanillin significantly reduced UVB-induced phosphorylation of ataxia telangiectasia mutated (ATM), serine threonine kinase checkpoint kinase 2 (Chk2), tumor suppressor protein 53 (p53), p38/mitogen-activated protein kinase (p38), c-Jun N-terminal kinase/stress-activated protein kinase (JNK), S6 ribosomal protein (S6RP), and histone 2A family member X (H2A.X). UVB-induced activation of p53 luciferase reporter was also significantly inhibited by vanillin. In addition, while ATM inhibitor had no effect on the vanillin effects, mouse double minute 2 homolog (MDM2) inhibitor significantly attenuated suppressive effects of vanillin on UVB-induced activation of p53 reporter in KSC. Taken together, these findings suggest that vanillin protects KSC from UVB irradiation and its effects may occur through the suppression of downstream step of MDM2 in UVB irradiation-induced p53 activation.

1. Introduction

Increased ultraviolet (UV) irradiation at the Earth's surface due to the depletion of the stratospheric ozone layer have enhanced interest in the mechanisms of various effects it might have on organisms. Especially, DNA is one of the main targets for UV-induced damage in a variety of organisms ranging from bacteria to humans (Sinha and Häder, 2002; Häder and Sinha, 2005). UV Irradiation induces the formation of several types of mutagenic DNA lesions. Cellular senescence is also an irreversible cell cycle arrest in response to UVB-induced DNA damage (Harley et al., 1990; Serrano et al., 1997; te Poele et al., 2002). UV has three different UV wavelength components, including UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm), which have distinct mutagenic properties.

Among three UV wavelength components, UVB irradiation is a main causer of DNA damage in the epidermis and is the most important environmental mutagen and carcinogen of epidermal cells. Epidermal keratinocytes absorb the bulk of cutaneous UV exposure. As a result of carcinogenic UVB exposure, keratinocytes have acquired extensive protective measures to handle UVB-induced DNA damage (Zhuang et al., 1999). The human epidermis is a stratified epithelium that maintains its integrity through a process of constant regeneration, driven by a population of keratinocyte stem cells (KSC) in the basal layer (Green, 1977). The search to identify human KSC has focused on the principle of adhesion to the basement membrane. *In vitro*, it has been shown that the keratinocytes that rapidly adhere to cell culture plates form tightly packed colonies, termed holoclones, which have the greatest long-term growth potential and so are likely to contain stem cells (Barrandon and Green, 1987; Jones and Watt, 1993). In the epidermis, KSC are promising clinical candidates for the treatment of photodamage, chronic wounds, and ulcers (Kim et al., 2004).

Several natural plant-derived compounds, including vanillin, cinnamaldehyde, coumarin, umbelliferone and tannic acid, have moderate anti-mutagenic properties. They also sensitise cells to the lethal effects of DNA-damaging agents (Ohta, 1993). Among them, vanillin (4-hydroxy-3-methoxybenzaldehyde), an

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anticlastogen, has been demonstrated to inhibit gene mutations in both bacterial and mammalian cells (Watanabe et al., 1990; Keshava et al., 1998; Gustafson et al., 2000; Akagi et al., 1995). However, the mechanisms underlying its effect against UVB radiation-induced cellular damage remain undefined. Therefore, this study was conducted to investigate the effects of vanillin on cellular damages induced by UVB irradiation, and the possible mechanism underlying this protective effect.

2. Materials and methods

2.1. Materials

Vanillin was purchased from ChromaDex (ChromaDex, Irvine, CA, USA). Mouse double minute 2 homolog (MDM2) and ataxia telangiectasia mutated (ATM) inhibitors were obtained from EMD millipore (EMD millipore, Billerica, MA, USA). Antibodies in this study were used according to the manufacturers' instructions and purchased as follows: CD29, CD34, CD49f, and CD71-antibodies from BD Pharmingen (BD Biosciences, San Jose, CA, USA), β-catenin-antibody from eBioscience (eBioscience, San Diego, CA, USA), p63-antibody from AbD serotec (AbD serotec, Raleigh, NC, USA), keratin 19 (K19) and ATM-antibodies from abcam (abcam, Cambridge, MA, USA), serine threonine kinase checkpoint kinase 2 (Chk2), tumor suppressor protein 53 (p53), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38/mitogen-activated protein kinase (p38/MAPK), ribosomal protein S6 (S6RP), histone 2A family member X (H2A.X)-anibodies, and Hoechst 33342 from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA).

2.2. Cell isolation and culture

Cells were isolated from human child's foreskin. Skin specimens were processed according to the method of Rheinwald and Green (Rheinwald and Green, 1975), as modified in our laboratory using method of Kim (Kim et al., 2004). In brief, the primary epidermal cells from foreskin were plated onto the collagen-coated dishes which had been prepared by incubating 100 mm dishes with type IV collagen (20 μg/mL) at 4 °C overnight. In this study, we used collagen "type IV" which is the ligand of integrin-β1 (CD29). It was reported that human interfollicular epidermal stem cells express high levels of CD29. Thus, collagen type IV is considered a potential candidate for the selection of epidermal stem cells (Jones and Watt, 1993). Then, a portion of these cells plated was selected according to their ability to adhere to the dishes within 1 h-incubation at 37 °C, and non-adhering cells was discarded. Only rapidly adhering (RA) cells were cultured in EpiLife™ keratinocyte medium (Invitrogen, Carlsbad, CA, USA) at 5% CO_2 and 37 °C under sterile conditions. The medium was changed every 3 days and the cells at 80% confluence were passaged. Before each experiment, the RA cells were trypsinized, counted, washed twice with phosphate-buffered saline (PBS) and resuspended in PBS (Invitrogen). The harvested cells highly expressed KSC-specific markers such as CD29 and integrin-α6 (CD49f), but did not express CD71.

2.3. Characterization of KSC

To immunophenotypically characterize KSC, the cells were stained using monoclonal antibodies against the KSC specific markers (CD29, CD34, CD49f, CD71, β-catenin, p63 and keratin19) according to the manufacturer's instructions. The cells were then analyzed on BD FACSCalibur™ flow cytometer (BD, Franklin Lakes, NJ, USA) with BD FACS software.

2.4. UVB radiation

To understand the biological consequences in skin by UVB irradiation, an *in vitro* model system was used to probe the response of KSC to UVB exposure at the intensity of 30 mJ/cm^2 (Luzchem Research Inc., Ottawa, Canada). For UVB exposure, when the cells were 70% confluent, the medium was removed, and the cells were washed with PBS and gently overlaid with PBS. The cells were then irradiated for 20 s at 30 mJ/cm^2. At the indicated times after UVB irradiation, the cells were harvested and assayed for senescence-related specific experiments.

2.5. MTT assay

Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were exposed to MTT (0.1 mg/mL) for 3 h at 37 °C under 5% CO_2 incubator. The medium was then removed, and the cells were solubilized with dimethyl sulfoxide (1 mL). After complete solubilization, the presence of blue formazan was evaluated spectrophotometrically by measuring the absorbance at a wavelength of 570 nm. Intact cells were also employed and used as a positive control.

2.6. β-Gal staining

Cells were washed twice with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde at room temperature (RT) for 10 min. After two additional washes with PBS, 2 mL of staining solution (150 mM sodium chloride, 25.2 mM sodium phosphate dibasic, 7.36 mM citric acid, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 ng/ml 5-bromo-4-chloro-3-indolyl-β-d-galactoside, pH 6.0) were added to the cells, and they were incubated at 37 °C overnight (Dimri et al., 1995). After the incubation, the cells were washed with PBS and examined microscopically at 20× magnification (Nikon Eclipse Ti, Nikon, Tokyo, Japan).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Supernatants of the KSC cultured in different conditions were analyzed for various cytokines using commercially available ELISA kits (R&D systems, Minneapolis, Minn, USA) according to the manufacturer's protocols. The supernatants were assayed for epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), transforming growth factor-beta1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and interleukin-6 (IL-6). The standard curve was linearized and subjected to regression analysis. The EGF, FGF-2, TGF-β1, TNF-α, IL-1β, and IL-6 concentration of the unknown samples was calculated using the standard curve. The intact KSC were also employed as a positive control. All samples and standards were measured in duplicate.

2.8. Immunoblotting

Cells were harvested, lysed with lysis buffer and a protease inhibitor cocktail kit (Roche, Mannheim, Germany), and centrifuged at 12,000 rpm for 10 min to obtain the supernatants. Proteins in the supernatants were then separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Uppsala, Sweden) at 250 mA in transfer buffer (20 mM Tris base, 150 mM glycine, 20% MeOH in 1 L distilled water). The membranes were then washed and incubated with primary phospho-p53, phospho-Chk2, phospho-JNK/SAPK, phospho-p38/MAPK, phospho-S6 ribosomal protein (S6RP), phospho-H2A.X, and β-actin antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C, washed and incubated with horseradish peroxidase-conjugated IgG secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Immediately after washing, the immunoreactive proteins were detected by chemiluminescence (ECL kit, GE Healthcare, Uppsala, Sweden). The detected proteins were normalized to β-actin as appropriate.

2.9. Luciferase reporter gene assay

Cells were transiently transfected with 2 μg of the firefly luciferase reporter gene under the control of p53 responsible elements (Stratagene, La Jolla, CA, USA) and 0.2 μg of *Renilla* luciferase expression vector driven by thymidine kinase promoter (Promega, Madison, WI, USA) by superfect reagent (Invitrogen, Carlsbad, CA, USA). Cells (2×10^5 cells/mL) prepared in a 12 well culture plate were transfected transiently with p53-Luc. After 24 h, the cells were irradiated by UVB (30 mJ/cm^2). The non-irradiated intact cells were used as a control. After 12 h, the cells were harvested and a luciferase assay was performed using the dual-luciferase reporter assay system (Promega, Madison, WI, USA), as described previously (Tian et al., 2011). Firefly and *Renilla* luciferase activities were measured using a LB953 luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

2.10. Comet assay

To detect cellular DNA damage by single-strand breaks, alkaline micro-gel electrophoresis was performed essentially as described by Singh et al (Singh et al., 1988). The pelleted cells (2.5×10^4) were washed and resuspended in 85 μL PBS. The cell suspension was then mixed with 85 μL of 1% low gelling temperature agarose dissolved in PBS. Next, 75 μL of the cell-agarose mixture was transferred onto a frosted microscope slide that had been pre-coated with 85 μL of 1% normal gelling temperature agarose. A third layer of 0.5% low gelling temperature agarose (75 μL) was applied over the second layer containing the cells. In establishing each of the three layers of agarose, glass coverslips were applied on top of the liquid agarose to spread the agarose across the surface of the slides. Each layer of agarose was congealed by placing the slides on a metal tray which was positioned in crushed ice. After removing coverslips, the slides were immersed in ice-cold alkaline cell lysis solution and allowed to incubate for 1 h. Slides were then placed carefully in a horizontal electrophoresis unit and left undisturbed at for 20 min. Next, electrophoresis was performed at 300 mA for 20 min. Afterwards slides were gently immersed in neutralization buffer for 5 min, and this step repeated. After applying 60 μL ethidium bromide solution (20 μg/mL) on the top of the agarose and covering with coverslips, the slides were viewed with a fluorescence microscope equipped with camera (Nikon Eclipse Ti, Nikon). The percentage of the total fluorescence in the tail and the tail length of the 50 cells per slide were recorded.