Palladium and Platinum Nanoparticles Activate AHR and NRF2 in Human Keratinocytes—Implications in Vitiligo Therapy

TO THE EDITOR
Metal nanoparticles, including palladium (Pd) and platinum (Pt), reportedly exert catalytic activities, suggesting that these nanoparticles can be used as antioxidants against skin inflammation (Yoshihisa et al., 2010).

PAPLAL (Toyokosei Seiyakusyo, Tokyo, Japan), a Pd and Pt nanoparticle solution approved in Japan, has been utilized for treating vitiligo and aging skin (Shibata et al., 2015; Shibuya et al., 2014) because it induces superoxide dismutase 1. The expression of aryl hydrocarbon receptor (AHR), a ligand-activated transcriptional factor, and nuclear factor erythroid-2-related 2 (NRF2), a master switch of the antioxidant machinery, is related to superoxide dismutase 1 expression (Jin et al., 2014); thus, we hypothesized that PAPLAL activates AHR and NRF2 in keratinocytes.

To demonstrate this, we analyzed PAPLAL-treated cultured normal human epidermal keratinocytes (NHEKs). The details for this experiment are described in Supplementary Materials and Methods online. Transmission electron microscopy analysis revealed that nanoparticles were located in the cytoplasm of NHEKs treated with PAPLAL for 3 hours (Figure 1a and b).

Transmission electron microscopy analysis at different time points is shown in Supplementary Figure S1 online. Energy dispersive X-ray spectrometry identified the nanoparticles as Pt and Pd (PAPLAL) (Figure 1c). Energy dispersive X-ray spectrometry also detected carbon, oxygen, and chlorine contained in Epon resin and silicon of microscopy pedestal. The PAPLAL level in NHEKs was determined using a Palladium API Screening Fluorescent Detection Kit, which measures the Pd level in the cell lysate of PAPLAL-treated NHEKs. The peak Pd level (7.02 ± 3.22 nM) was achieved at 3 hours, after which the level remained relatively stable (2.86 ± 0.09 nM) for 24 hours (Figure 1d).

Next, we investigated whether AHR or NRF2 could be activated by PAPLAL in NHEKs. AHR or NRF2 translocation from the cytoplasm into the nucleus is necessary to activate AHR- and NRF2-dependent signal transduction, including the upregulation of cytochrome P450 family 1 subfamily A member 1 (CYP1A1) (Tsuij et al., 2011) and NAD(P)H quinone dehydrogenase 1 (NQO1), a representative antioxidant enzyme (Tsuij et al., 2012). AHR or NRF2 was stained with a rabbit polyclonal anti-AHR or anti-NRF2 antibody and labeled with a goat-anti-rabbit Alexa Fluor 546 and 488 secondary antibodies. Confocal laser scanning microscopic analysis using a D-Eclipse microscope revealed that AHR and NRF2 were located in the cytoplasm, under unstimulated conditions (Figure 1e and g). In contrast, AHR (Figure 1f) or NRF2 (Figure 1h) was located in the nucleus in NHEKs treated with PAPLAL for 6 hours, indicating that PAPLAL treatment induced AHR and NRF2 nuclear translocation. Five percent PAPLAL treatment did not affect the cell viability of NHEKs in a WST-1 assay (Supplementary Figure 2a online) and did not increase reactive oxygen species production in a 2′,7′-dichlorodihydrofluorescein diacetate (Thermo Fisher Scientific, Waltham, MA) analysis (Supplementary Figure 2b). Consistent with these results, quantitative reverse transcription PCR demonstrated that PAPLAL treatment for 16 hours upregulated CYP1A1, an AHR-specific xenobiotic metabolizing enzyme, NRF2, and NQO1 (Figure 1i, k, and l). In western blotting analyses using a polyclonal rabbit anti-CYP1A1 or anti-NQO1 antibody, the protein levels of CYP1A1 and NQO1 were upregulated in NHEKs treated with PAPLAL for 48 hours (Figure 1j). The PAPLAL-induced upregulation of CYP1A1, but not NRF2, expression was ablated in NHEKs transfected with small interfering (si) RNA for AHR (Figure 1m and n), indicating that PAPLAL-induced CYP1A1 upregulation is dependent on AHR and that PAPLAL-induced NRF2 upregulation is independent of AHR. Furthermore, the PAPLAL-induced
Figure 1. Activation of AHR and NRF2 by PAPLAL in NHEKs. (a), (b) Transmission electron microscopy. NHEKs were stimulated with 5% PAPLAL for 3 hours. (a) Bar = 10 μm, (b) Bar = 2 μm. (c) Energy dispersive X-ray spectrometry analysis of the nanoparticles. The peaks of Pt and Pd were enclosed in black circles. (d) Pd level in the cell lysate of PAPLAL-treated NHEKs (*P < 0.05) (mean ± SEM) (n = 3). (e), (f), (g), (h) Confocal laser scanning microscopy. NHEKs were stimulated with (e, f) distilled water or (g, h) PAPLAL (5%) for 6 hours and then stained with an antibody against (e, f) AHR or (g, h) NRF2, Bar = 100 μm; representative data (n = 3). (i), (j), (k), (l), (m) qRT-PCR. NHEKs treated with distilled water or PAPLAL (5%) for 16 hours. (n) qRT-PCR. Control siRNA-transfected or AHR-siRNA-transfected NHEKs, AHR-siRNA-transfected NHEKs, or NRF2-siRNA-transfected NHEKs were treated with distilled water or PAPLAL (5%) for 16 hours. (p) Western blotting. NQO1 protein from control siRNA-transfected, AHR-siRNA-transfected, or NRF2-siRNA-transfected NHEKs treated with distilled water or PAPLAL (5%) for 24 hours. mRNA expression was measured in triplicate, and mRNA levels normalized for ACTB were expressed as fold induction compared with the control.
Figure 2. CXCL10 production induced by IFN-γ was inhibited by PAPLAL. (a, c, d) qRT-PCR. (a, d) NHEKs; (c) control siRNA-transfected, AHR-siRNA-transfected, or NRF2-siRNA-transfected NHEKs were pretreated with distilled water or PAPLAL (5%) for 24 hours. NHEKs were exposed to IFN-γ (10 ng/ml) for 6 hours. mRNA expression was measured in triplicate, and mRNA levels normalized for ACTB were expressed as fold induction compared with the control group (*P < 0.05) (mean ± SEM) (n = 3). (b) CXCL10 production in the supernatant by ELISA. The supernatant in (a) was collected (*P < 0.05) (mean ± SEM) (n = 3). (e) Western blotting. NQO1 protein from NHEKs pretreated with distilled water or PAPLAL (5%) for 24 hours were exposed to IFN-γ (10 ng/ml) for 6 hours. ACTB was used as a reference gene. Representative data (n = 3). (f) Increase in ear thickness. Mice were sensitized and elicited by TNCB with or without topical application of PAPLAL as indicated. (g) IFN-γ and (h) CXCL10 mRNA expression of ears of the mice treated with TNCB. *P < 0.05 (n = 5). ACTB, actin beta; AHR, aryl hydrocarbon receptor; CXCL, chemokine (C-X-C motif) ligand; NHEK, normal human epidermal keratinocyte; NQO1, NAD(P)H quinone dehydrogenase 1; NRF2, nuclear factor (erythroid-derived 2)-like 2; qRT-PCR, quantitative reverse transcription PCR; SEM, standard error of the mean; siRNA, small interfering RNA; TNCB, 2,4,6-trinitrochlorobenzene.
upregulation of NQO1 expression (Figure 1o) and protein levels of NQO1 (Figure 1p) were ablated by transfection with siRNA for NRF2 but not with siRNA for AHR, indicating that PAPLAL upregulates the NRF2-NQO1 pathway independently of AHR. For knockdown, Hiperfect Transfection Reagent was used, and its efficiency for AHR and NRF2 expression using siRNA transfection has been previously reported (Takesi et al., 2015).

Although the pathomechanisms of vitiligo are not fully understood, IFN-γ-producing CD8+ T cells have been suggested to have a crucial role in the development of vitiligo (Ezzedine et al., 2015; Rashighi et al., 2014). The production of chemokine (C–C motif) ligand (CXCL) 10, a chemokine of CXC chemokine receptor (CXCR) 3-expressing CD8+ T cells, was triggered via the IFN-γ-signal transducer and activator of transcription 1 axis in keratinocytes, leading to the accumulation of CXCR3+ CD8+ T cells in the epidermis (Villarroel et al., 2014). Therefore, we evaluated whether PAPLAL inhibited IFN-γ-induced CXCL10 expression. As determined by quantitative reverse transcription PCR and ELISA analyses using the Quantikine ELISA Human CXCL10/10-10 Immunoasay, IFN-γ (10 ng/ml) upregulated CXCL10 expression (Figure 2a) and the protein levels of CXCL10 (Figure 2b), which were attenuated in NHEKs pretreated with PAPLAL for 24 hours. Notably, the IFN-γ-induced upregulation of CXCL10 expression was enhanced in AHR-knockdown NHEKs compared with control siRNA-transfected NHEKs (Figure 2c). Moreover, the inhibitory effect on the IFN-γ-induced CXCL10 expression by PAPLAL was partially ablated in AHR, but not in NRF2-knockdown NHEKs (Figure 2c), which is consistent with the results of a study showing that AHR activation negatively regulates signal transducer and activator of transcription 1 signaling (Kimura et al., 2009). Furthermore, we found that IFN-γ treatment (10 ng/ml) for 6 hours significantly reduced NQO1 expression, whereas expression was restored by pretreatment with PAPLAL for 24 hours (Figure 2d). This result was also confirmed at the protein level by western blotting analyses (Figure 2e).

Considering the antioxidant role of NQO1 in keratinocytes (Kleszczyński et al., 2013), PAPLAL may protect keratinocytes from IFN-γ-mediated cell damage via NQO1 upregulation. To further examine whether PAPLAL can attenuate IFN-γ-CXCL10 axis activation in vivo, we utilized a 2,4,6-trinitrochlorobenzene-induced contact hypersensitivity model, which is mainly mediated by the IFN-γ-CXCL10 axis (Dufour et al., 2002; Tsuji et al., 2015). The experiment was approved by the Kyushu University Institutional Animal Care and Use Committee. Topical application of PAPLAL decreased ear swelling by 2,4,6-trinitrochlorobenzene (Figure 2f) with reduced expression of IFN-γ and CXCL10 mRNA in the epidermis of the ears (Figure 2g and h).

Accordingly, PAPLAL exhibited dual effects on the AHR and NRF2 pathways, contributing to inhibition of the IFN-γ-CXCL10 axis; this finding indicates the therapeutic potential of PAPLAL for the treatment of vitiligo. Because Pd could cause metal allergy (Kobayashi et al., 2013), skin conditions should be examined carefully.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was partly supported by grants from the Ministry of Health, Labor and Welfare, Research on the Development of New Drugs program from the Sun Care Institute and the Japan Agency for Medical Research and Development, and Leading Advanced Projects for Medical Innovation.

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Alterations of CXCL12 in Serum of Patients with Vitiligo


TO THE EDITOR

Recently, Rezk et al. (2017) reported a misbalanced CXCL12 and CCL5 chemotactic signaling in vitiligo melanocytes and vitiligo skin samples. Their results on CXCL12, a ligand of CXCR4, suggest that besides chemokine receptor type 3 (CXCR3), CXCR4 signaling could also be involved in vitiligo (Rashighi et al., 2014). The aim of this study was to investigate whether CXCL12 and CCL5 were also elevated in the serum of patients with vitiligo. Biomarker research can progress the understanding of the pathogenesis of vitiligo and reveal potential new targets for treatment. Furthermore, an activity marker may aid the clinical management by identifying patients who will develop disease progression in the following months. This is valuable as clinical disease activity signs are only present in a subset of patients (Sosa et al., 2015).

Eighty patients with vitiligo (non-segmental) and 23 healthy controls were enrolled in this study between 2012 and 2015 at the Department of Dermatology of Ghent University Hospital. Disease activity was assessed by patients using our own constructed questionnaire (Table 1). Additionally, disease activity was scored by a physician based on follow-up pictures. The affected body surface area was evaluated using the Vitiligo Extent Score on digital photographs of the patients (van Geel et al., 2016). Further disease evolution was assessed at a follow-up consultation (after 3–6 months). Only topical cream treatment was allowed. The clinical characteristics are summarized in Table 1. The study was approved by the ethical committee of Ghent University Hospital. All patients signed written informed consent. CXCL12 and CCL5 were measured in serum using ELISA (R&D Systems, Abingdon, UK).

Statistical analysis was carried out using SPSS 23.0 (SPSS Science, Chicago, IL). Nonparametric variables were compared using the Mann-Whitney U test or the Kruskall-Wallis test. Data are expressed as mean (median [interquartile range]). To compare CXCL12 values with CCL5 concentrations, Pearson’s correlation was used. All statistical analysis was performed using SPSS 23.0 (SPSS). Statistical significance was considered as P < 0.05.

Serum CXCL12 was increased in patients with vitiligo compared with healthy controls (3.26 ng/ml [3.15 (2.20–3.85)] vs. 2.66 ng/ml [2.56 (2.19–2.89)], P = 0.041) (Figure 1a). Regarding patient-reported disease activity, significantly increased CXCL12 values were found in patients with disease activity in the last 3 months compared with all other patients with vitiligo (3.61 ng/ml [3.78 ng/ml (3.27–4.03)] vs. 2.90 ng/ml [3.09 ng/ml (2.01–3.69)], P = 0.012) (Figure 1b). Similarly, CXCL12 values were different according to the physician-scored disease activity (very active: 3.87 ng/ml [3.87 ng/ml (3.23–4.15)] vs. the other activity categories: 2.94 ng/ml [3.13 ng/ml (2.03–3.70)], P = 0.020). Disease evolution after blood sampling could be assessed in 58 patients. Overall, CXCL12 values were higher in patients with future disease progression although statistical significance was not reached (3.37 ng/ml [3.45 ng/ml (2.90–4.40)] vs. 2.89 ng/ml [3.06 ng/ml (2.11–3.59)], P = 0.056) (Figure 1c). However, in patients who had stable/repigmenting vitiligo in the period before blood sampling (based on follow-up pictures), significantly higher CXCL12 concentrations were found in the group that developed disease progression in the months following blood analysis compared with patients who remained stable or repigmented (3.74 ng/ml [3.50 ng/ml (3.19–4.27)] vs. 2.92 ng/ml [3.02 ng/ml (2.25–3.45)], P = 0.030) (Figure 1d). Receiver operating characteristic analysis in this subgroup showed an area under the curve of 0.771 (95% confidence interval: 0.60–0.984). A cutoff set at 3.27 ng/ml of CXCL12 had a sensitivity of 71.4% and specificity of 68.0% to predict future disease progression in patients with stable/repigmenting vitiligo (P = 0.030). No correlation was found between CXCL12 values and the affected body surface area. CXCL12 values were not influenced by the use of topical treatment. No significant association between RANTES and vitiligo activity was found although CXCL12 and RANTES concentrations were correlated (P = 0.001, r = 0.408, Figure 1c).

These results indicate that serum CXCL12 is indeed elevated in patients with vitiligo compared with controls. Moreover, increased values were found in patients with active vitiligo and CXCL12 carried a predictive capacity on future disease progression. Receiver operating characteristic analyses indicated that CXCL12 had a relative good sensitivity and specificity to predict future disease progression instable patients. CXCL12 is a key chemokine that regulates tissue homeostasis and inflammatory responses. Its tissue expression directs lymphocytes to the site of inflammation (Karin and Wildbaum, 2011; 62:42–9).

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