Effective inhibition of melanosome transfer to keratinocytes by lectins and niacinamide is reversible


Abstract: Skin pigmentation results in part from the transfer of melanized melanosomes synthesized by melanocytes to neighboring keratinocytes. Plasma membrane lectins and their glycoconjugates expressed by these epidermal cells are critical molecules involved in this transfer process. In addition, the derivative of vitamin B<sub>3</sub>, niacinamide, can inhibit melanosome transfer and induce skin lightening. We investigated the effects of these molecules on the viability of melanocytes and keratinocytes and on the reversibility of melanosome-transfer inhibition induced by these agents using an in vitro melanocyte–keratinocyte coculture model system. While lectins and neoglycoproteins could induce apoptosis in a dose-dependent manner to melanocytes or keratinocytes in monoculture, similar dosages of the lectins, as opposed to neoglycoproteins, did not induce apoptosis to either cell type when treated in coculture. The dosages of lectins and niacinamide not affecting cell viability produced an inhibitory effect on melanosome transfer, when used either alone or together in cocultures of melanocytes–keratinocytes. Cocultures treated with lectins or niacinamide resumed normal melanosome transfer in 3 days after removal of the inhibitor, while cocultures treated with a combination of lectins and niacinamide demonstrated a lag in this recovery. Subsequently, we assessed the effect of niacinamide on facial hyperpigmented spots using a vehicle-controlled, split-faced design human clinical trial. Topical application of niacinamide resulted in a dose-dependent and reversible reduction in hyperpigmented lesions. These results suggest that lectins and niacinamide at concentrations that do not affect cell viability are reversible inhibitors of melanosome transfer.

Introduction

Skin coloration is a result of many complex processes. Epidermal melanocytes synthesize pigmented melanosomes that are subsequently transferred to and retained by the surrounding keratinocytes. Normal skin coloration is a result of both efficient melanization of the melanosome by the melanocyte and proper transfer to and receipt of the melanosome in the keratinocyte (1,2). The process of melanin production has been well studied, and the major enzymes involved in the melanization pathway have been identified (3,4). However, little is known about the process of intercellular melanosome transfer. Several theories have been proposed to explain this transfer process. These theories consist of: (a) release of melanosomes into the intercellular space, with subsequent endocytosis by the keratinocytes; (b) cytophagocytosis, i.e. the phagocytosis of dendritic tips of the melanocytes by keratinocytes; (c) direct inoculation of the melanosomes into the keratinocytes; or (d) transfer of melanosomes via a communication conduit between the melanocytes and keratinocytes (5–8). These processes may not be mutually exclusive; transfer of melanosomes may occur through a combination of these mechanisms (6).
The amount and regulation of melanosomal transfer contributes to the ultimate pigmentation of human skin. Alterations in this melanosomal-transfer process may be the basis for several pigmentary/complexion diseases and disorders. This is supported by several pigmentary disorders involving containment of the melanosomal within the melanocyte, such as hair-graying (9) and nevus depigmentosus (10). Cutaneous hyperpigmentation occurs in numerous conditions such as aging (11), pregnancy (12), postinflammation (13), and scarring (14). Inhibition of melanosomal transfer by the protease-activated receptor 2 (PAR-2) (15) or niacinamide (16) results in lightening of cutaneous pigmentation. In addition to skin health issues related to altered pigmentation, a cosmetic desire for light and even skin tone is prevalent in many areas of the world, especially among Asian populations. Approximately 60% of Japanese women and 75% of Chinese women desire lighter skin (16). Therefore, the process of melanosomal transfer presents unique opportunities to therapeutically and cosmetically influence human skin pigmentation.

Plasma membrane lectins and their corresponding glycoconjugates, putatively expressed by melanocytes and keratinocytes, may play a critical role in melanosomal transfer for a variety of reasons. These molecules are responsible for cell–cell recognition in general (17), and phagocytosis by macrophages in specific (18). Previous studies have indicated increased production of these molecules in melanocytes after UVA irradiation (19), suggesting that they facilitate melanosomal transfer.

In addition, symptoms of the rare metabolic disease, carbohydrate-deficient glycoprotein syndrome type III, which alters carbohydrate moiety synthesis and processing, include pale and unpigmented skin (20). Most importantly, certain lectins and neoglycoproteins have been shown to impede the melanosomal-transfer process when used in a coculture model system that recapitulates melanosomal transfer (21). These various lectins and neoglycoproteins interact with galactosyl, fucosyl, and mannose residues (21) and had been previously implicated in studies of melanin transfer between transformed cells (19). However, it was unknown whether these lectins or neoglycoproteins would have any negative effects on cell viability or whether the transfer inhibition would be permanent. In this study, we investigated the effect of various concentrations of lectins, neoglycoproteins, and niacinamide, a known skin-lightening agent (16), both alone and in combination, on melanocyte and/or keratinocyte viability and on melanosomal transfer between these two epidermal cell types. We also examined the reversibility of melanosomal transfer impeded by these compounds both in a culture model and in a clinical trial. Further insight into these compounds as well as their underlying mechanism of action will indicate potential inhibitors and eventual stimulators to manipulate human skin pigmentation.

Materials and methods

Cell cultures

Melanocytes and keratinocytes were obtained from Cascade Biologicals (Portland, OR, USA). Melanocytes were maintained in M154 basal medium (Cascade Biologicals) and supplemented with 5% fetal bovine serum, 1% antibiotic/antimycotic (Gibco, Grand Island, NY, USA), 1 µg/ml of transferrin, 1 µg/ml of vitamin E, 5 µg/ml of insulin, 0.5 ng/ml of basic fibroblast growth factor, 10⁻⁸ M melanocyte-stimulating hormone, and 10⁻⁹ M endothelin-1 (all from Sigma Chemical Co., St Louis, MO, USA). Keratinocytes were maintained in M154 basal medium supplemented with keratinocyte growth supplements (Cascade Biologicals) and 1% antibiotic/antimycotic (Gibco). Cocultures of keratinocytes/melanocytes were established as previously described (22). In short, established cultures of keratinocytes and melanocytes were trypsinized, washed, and reseeded at a keratinocyte:melanocyte ratio of 10:1 in a media containing equal parts of keratinocytes and melanocytes.

Test compounds

The lectins used in these experiments (and the corresponding carbohydrate moiety they bind to) consist of Narcissus pseudo-narcissus (NP) L5650 (α-d-mannose), Pisum sativum (PS) L5380 (α-mannose), Phosphocarpus tetragonolobus (PT) L2138 (GalNAc, gal), Lycopersicon esculentum (LE) L2886 (GalNAc), and Tetragonolobus purpureas (TP) Lp254 (α-L-fucose). All lectins were purchased from Sigma-Aldrich (St Louis, MO, USA).

The two neoglycoproteins used in these experiments (and the conjugate lectin they interact with) consist of α-L-1,3-galactose–bovine serum albumin (BSA) (N402) (Con A) and galactose α-L-1,3-galactose–BSA (N403) (Erythrina crista-galli). Both neoglycoproteins were purchased from Calbiochem/Novabiochem (La Jolla, CA, USA).

Niacinamide (nicotinic acid amide) was purchased from Sigma Cell Culture.

Apoptosis detection

Cultures of melanocytes or keratinocytes and melanocyte/keratinocyte cocultures were treated with test compound or vehicle [i.e. phosphate-buffered saline (PBS)] alone, once or twice daily for 6 days and assayed for apoptosis/necrosis as described below. Experiments were repeated three times with triplicate samples per experiment. In experiments involving melanocyte–keratinocyte cocultures, we developed a differential tryptsinization procedure whereby melanocytes and keratinocytes were separated at the termination of the experiment and evaluated individually for apoptosis (see Fig. 1a,b). In short, cocultures were treated briefly with 0.25% trypsin (approximately 3 min) to selectively remove the melanocytes. The remaining keratinocytes were obtained further tryptsinization.

Isolated cells were then processed for detection of non-viable cells (i.e. cells in early and late stages of apoptosis and cells undergoing necrosis) by flow cytometry using the Annexin V apoptosis-detection kit (Trevigen, Gathersburg, MD, USA). Counterstaining with propidium iodine (PI) was used to identify
cells in late apoptosis and/or necrosis. Cells were analyzed for Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) reactivity by flow cytometry using an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL, USA). Data were analyzed using COULTER XL software (Coulter).

Melanosome-transfer inhibition

Transfer between melanocytes and keratinocytes in cocultures was evaluated as previously described (21). In short, melanocytes were prelabeled with succinimidyl ester of carboxy fluorescein diacetate (CFDA) (Molecular Probes, Eugene, OR, USA) and used to establish cocultures with keratinocytes. Cocultures were treated with fresh media containing test compound or vehicle, i.e., PBS, every 12 or 24 h during the treatment period. Experiments were repeated twice with triplicate samples per experiment. Following treatment, cells were prefixed in 4% paraformaldehyde, permeabilized with fluorescence-activated cell-sorter-permeabilizing solution (FACS perm, Becton Dickinson, San Jose, CA, USA), and washed. The cells were then treated with a monoclonal mouse anticytokeratin primary antibody (1:300), washed, and incubated with goat antimouse IgG secondary antibody conjugated to phycoerythrin (PE) (Caltag, Burlingame, CA, USA) (1:10). Cells were postfixed in 1% paraformaldehyde and transferred quantitated by evaluating the mean CFDA fluorescence (Mean X) in PE-positive keratinocytes in the presence or absence of inhibitor using an EPICS XL flow cytometer (Coulter Cytometry, Coulter). Data were analyzed using COULTER XL software.

Description of statistics

To determine whether the test compound affected the treated cells as compared with control populations of cells/cocultures in a statistically significant manner, we tested the population mean and variance. We constructed a ×100 99.5% confidence interval for the mean. The relevant test statistic was Z, and this parameter was calculated for each test sample, compared to the control sample. Accordingly, P-values for each Z-statistic value were calculated. All \( P \)-values for the Z-statistic of the one-tailed test from each sample population were assessed for whether they were less than \( z = 0.05 \). All calculations were performed using Microsoft Excel 2000 (Microsoft, Redmond, WA, USA).

Human clinical trial

A double-blinded, randomized, vehicle-controlled, split-face design human clinical study was performed from October 2002 through August 2003 (Kobe, Japan). Seventy-nine Japanese women aged 28–54 years with multiple types of brown hyperpigmentation on both sides of the face were assigned to one of two groups of either 39 or 40 subjects. Most subjects presented with a combination of slight-to-moderate solar lentigines, melasma, or freckles on both sides of the face. All subjects were given basic skin care products (facial cleanser, lotion, and sunscreen, none of which contained skin-whitening ingredients) to use during the first 16 weeks of the study. In a split-face design, Group 1 \( (n = 39) \) utilized 5% niacinamide in a moisturizer to one side of the face vs. the vehicle moisturizer (without niacinamide) to the other side of the face and Group 2 \( (n = 40) \) utilized 2% niacinamide moisturizer vs. the vehicle moisturizer. Subjects applied their assigned test products to the assigned sides of their faces in a dosage-controlled manner (0.3 ml/application/entire half face) twice daily (morning and evening) for 8 weeks (treatment period).

The group that received 5% niacinamide (Group 1) was monitored for an additional 34 weeks after the treatment period to determine reversibility of the lightening effect (recovery period) \( (n = 37) \). To confirm that no side effects occurred, a dermatologist evaluated the subjects’ images captured at baseline and at 8 and 42 weeks (Group 1). All subjects gave written informed consent. The protocol had been approved by the regulatory and safety review committee for human testing of The Procter & Gamble Company (Kobe, Japan). All subjects were given basic skin care products [facial cleanser, lotion, and sunscreen (SPF 15)] none of which contained skin-whitening ingredient(s), to use during the first 16 weeks of the study, while no instruction was provided during the remaining 26 weeks (for Group 1), i.e. they followed their normal skin care habits and practices.

Image analysis of facial hyperpigmented spots

Facial hyperpigmented spot area on each side of the face was objectively measured using a customized image analysis technique similar to the one previously described (16,23). Specifically, images of the right and left sides of each subject’s face were captured at baseline and at 4 and 8 weeks (also at 16, 26, and 42 weeks for Group 1) by a high-resolution digital camera (Fuji SC430 CCD Digital Camera) equipped with a cross-polarized filter (Tiffen glass polarizing filter and Kenko polarizing filter for light source). Facial illumination was provided by National fluorescence light Twin 1 (FPL27EX-N, 6200–6400 K) positioned to the right and left sides of the camera to provide even lighting. Before image capture, subjects were equilibrated in a temperature-controlled room \( (24 \pm 2 ^\circ C) \) for 30 min. At the 4-, 8-, 16-, 26-, and 42-week visits, accurate repositioning of the subjects was facilitated by comparing side by side the live image with the digitally stored image obtained at baseline. The system was calibrated by white balancing the camera each study day. Computer analysis of the digital images allowed quantification of total area of hyperpigmented spots in the selected region of interest (around the cheek to the temple) as a spot area fraction (percentage of total spot area in the area of the selected region of interest).

Visual assessment of hyperpigmented spot

Subjective visual grading of the captured images was carried out to compare pretreatment (baseline image) vs. post-treatment at the 8-, 26-, and 42-week time points as previously described (16). Briefly, paired blinded pre- and post-treatment images appeared side by side randomly on the monitor. Eight treatment-blinded judges independently viewed pairs of images for each subject. The judges indicated which of the two images had fewer areas of hyperpigmented spots around the eye and the cheek, then rated the magnitude of the difference between images on a scale of 1–4: 1, I think there is a small difference; 2, I know there is a small difference; 3, I know there is a moderate difference; and 4, I know there is a big difference. There was no ‘no difference’ option to increase the sensitivity of the judges’ evaluations. The magnitude rating was assigned a positive (+) value when the post-treatment
was selected to have less hyperpigmented spots, while it was assigned a negative (–) value when the pretreatment was selected to have less hyperpigmented spots. Thus, positive values indicated efficacy and negative values indicated no efficacy. The mean ratings of the eight judges were used for the statistical analysis.

**Data analysis**

Image analysis data (hyperpigmented spot area fraction) were compared for the change from the baseline by a paired t-test at each subsequent time point. Visual assessment data (the mean rating of the eight judges) were also compared by a paired t-test. Statistical significances were assessed for whether t-values were less than t = 0.05 or not. All calculations were performed using SPSS for Windows, version 11.5.

**Results**

**Effect of lectins and neoglycoproteins on cell viability of cultured melanocytes and keratinocytes**

The viability of pure cultures of melanocytes or keratinocytes exposed to lectins and neoglycoproteins was assessed. Test concentrations included those previously demonstrated to reduce melanosome transfer in cocultures of melanocytes and keratinocytes (21) as well as concentrations that were one-half and twice these reported concentrations. Cells were then evaluated for Annexin V and/or PI reactivity by flow cytometry as an indicator of non-viable cells. Cells negative for both Annexin V–FITC and PI were considered healthy. The data demonstrated that the viability in pure cultures of both melanocytes and keratinocytes was significantly compromised by certain lectins and neoglycoproteins generally in a dose-dependent manner (Fig. 2a,b). Of exception were the lectin PT and the neoglycoprotein alpha-galactose, which exhibited no deleterious effect on melanocytes at the dosages tested.

The negative effects of lectins and neoglycoproteins on the viability of melanocytes or keratinocytes in monocultures were not apparent in previous experiments where melanocytes and keratinocytes were grown in coculture (21). The negative effects on cell viability by several lectins

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**Figure 2.** Effect of lectins or neoglycoproteins on viability of melanocyte or keratinocyte monocultures or cocultures. Pure cultures of (a) melanocytes and (b) keratinocytes were treated twice daily for 6 days with the various concentrations of lectins and neoglycoproteins listed. Cells were then assessed for viability as described in Materials and methods. (a) All lectins and neoglycoproteins except PT and alpha-galactose, respectively, exhibited a negative effect on the viability of isolated cultures of melanocytes in a dose-dependent manner. (b) All compounds exhibited a negative effect on viability of isolated cultures of keratinocytes in a dose-dependent manner. Cocultures were treated twice a day for 6 days with the various concentrations of lectins and neoglycoproteins listed. Subsequently, (c) melanocytes and (d) keratinocytes were isolated by differential trypsinization and assessed for viability as described in Materials and methods. All lectins exhibited no or minimal effect on cell viability of either melanocytes or keratinocytes, especially at the lowest concentration tested, except for PT that exhibited a significant negative effect on keratinocyte viability at the lowest concentration tested. In contrast, both neoglycoproteins affected cell viability of melanocytes and keratinocytes. Error bars represent SD; asterisks represent \( P < 0.05 \). Table presents the highest relative concentration (µg/ml) of lectin allowing normal cell viability in comparison between monoculture and coculture conditions.

<table>
<thead>
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<th>NP</th>
<th>TP</th>
<th>PS</th>
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<th>LE</th>
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</tr>
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<td>4.4</td>
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<td>&gt;3.5</td>
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<td></td>
<td></td>
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<tr>
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<td>&gt;17.5</td>
<td>&gt;3.5</td>
<td>1.8</td>
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</tbody>
</table>

Highest relative concentration (µg/ml) of lectin allowing normal cell viability

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Materials and Methods

Results

Effect of niacinamide on cell viability of melanocytes and keratinocytes

The viability of pure cultures of melanocytes exposed to niacinamide was assessed. Cells were cultured in the absence (i.e. vehicle alone) or presence of 0.1, 1.0, 10.0, 100.0, and 1000.0 µM niacinamide twice daily for 6 days. The data demonstrated that the health of both cultured melanocytes and keratinocytes was not compromised at niacinamide concentrations tested (Fig. 3). We next investigated the viability of each cell type to treatment with niacinamide when existing together in coculture. All concentrations of niacinamide tested did not compromise viability of either melanocytes or keratinocytes when both cell types also existed in coculture (Fig. 3).

Figure 3. Effect of niacinamide concentrations on viability of melanocyte or keratinocyte monocultures or cocultures. (a) Melanocyte and (b) keratinocyte monocultures were treated twice daily for 6 days with niacinamide and assessed for various stages of apoptosis/necrosis using Annexin V binding and propidium iodine staining as described in Materials and Methods. Concentrations up to 1.0 mM produced no effect on the viability (i.e. apoptosis or necrosis) of melanocyte or keratinocyte monocultures. Cocultures were similarly treated and (a) melanocytes and (b) keratinocytes separated by differential trypsinization were assessed for viability. Concentrations up to 1.0 mM also produced no effect on the viability (i.e. apoptosis or necrosis) of melanocyte or keratinocyte monocultures. Error bars represent SD.

Melanosome-transfer inhibition

To demonstrate melanosome-transfer inhibition, melanocytes were labeled with a succinimidyl ester of CFDA and cocultured with keratinocytes. These cocultures were assessed by confocal microscopy for CFDA transfer in the presence or absence of 10 µM niacinamide after 6 days of treatment (Fig. 4). Control cultures demonstrated brightly fluorescing keratinocytes (arrowheads) within a colony of keratinocytes (star) that also contain CFDA-positive melanocytes (arrows) (Fig. 4a). The corresponding differential contrast image (Fig. 4b) demonstrates that the melanocytes were darkly pigmented (arrows). In contrast, treatment with niacinamide resulted in only weakly fluorescing keratinocytes within a colony (star) that also contained CFDA-positive melanocytes of equal brightness as observed in the control cultures (arrows) (Fig. 4c). The corresponding differential contrast image (Fig. 4d) demonstrated that the melanocytes were darkly pigmented (arrows). These data indicate that a considerable amount of the dye was transferred from melanocytes to keratinocytes in the control cocultures and minimal dye was transferred in the presence of niacinamide.

To quantitate melanosome-transfer inhibition, melanocytes were labeled with a succinimidyl ester of CFDA, cocultured with keratinocytes, and subsequently quantitated for dye transfer in the presence of inhibitors and concurrently assessed for recovery of transfer after removal of inhibitors.
Melanosome transfer to keratinocytes

Figure 4. Cocultures of carboxy fluorescein diacetate (CFDA)-labeled melanocytes and unlabeled keratinocytes were treated twice daily for 6 days with vehicle only (a, b) or 10.0 μM niacinamide (c, d). After 6 days, cocultures were viewed with fluorescence (a, c) or differential interference (DI) contrast (b, d) confocal microscopy simultaneously. (a) Control cocultures containing brightly fluorescing keratinocytes (arrowheads) within a colony (star) that also contained CFDA-positive melanocytes (arrows). (b) Darkly pigmented melanocytes (arrows) were apparent in the corresponding DI contrast image. (c) In contrast, treatment with niacinamide results in only weakly fluorescing keratinocytes within a colony (star) that also contains CFDA-positive melanocytes of equal brightness as observed in the control cultures (arrows). (d) The corresponding DI contrast image demonstrates that the melanocytes remain darkly pigmented. Table presents melanosome-transfer inhibition and recovery using niacinamide (10.0 μM) and/or the lectin cocktail. Cocultures were treated for 3 days with inhibitor and assessed for transfer as described in Materials and methods at the end of the 3-day treatment period and daily for an additional 3 days after termination of treatment with inhibitors.

Table 1. Assessment of once vs. twice-daily treatment of inhibitors on melanosome transfer

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Lectin × 1 day</td>
<td>10</td>
</tr>
<tr>
<td>Lectin × 2 day</td>
<td>33</td>
</tr>
<tr>
<td>0.1 μM niacinamide × 1 day</td>
<td>0</td>
</tr>
<tr>
<td>0.1 μM niacinamide × 2 day</td>
<td>20</td>
</tr>
<tr>
<td>1.0 μM niacinamide × 1 day</td>
<td>16</td>
</tr>
<tr>
<td>1.0 μM niacinamide × 2 day</td>
<td>28</td>
</tr>
<tr>
<td>10 μM niacinamide × 1 day</td>
<td>21</td>
</tr>
<tr>
<td>10 μM niacinamide × 2 day</td>
<td>32</td>
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fraction were assessed after 4 and 8 weeks of treatment with either 2 or 5% niacinamide (Fig. 5a,b). After both 4 and 8 weeks of treatment, the side of the face receiving niacinamide demonstrated a higher hyperpigmented spot-reduction efficacy vs. vehicle for both concentrations; however, only 5% niacinamide exhibited significant differences at both time points. Visual grading performed on test subjects at the 8-week time point also demonstrated that 5% niacinamide treatment had significantly higher hyperpigmented spot-reduction efficacy vs. vehicle control (Fig. 5c,d), while 2% niacinamide treatment did not (Fig. 5e). These data confirm a dose–response effect in hyperpigmented spot reduction by niacinamide. In addition, visual assessment of captured images by a dermatologist confirmed that there was no side effect due to niacinamide such as hypopigmentation or erythema after 8 weeks of treatment for all subjects.

Image capture was continued in the group that received 5% niacinamide (Group 1) after treatment was stopped at week 8. Specifically, image analysis results of the percentage change from baseline in spot area fraction were assessed at weeks 16, 26, and 42 (i.e. 8, 18, and 34 weeks post-treatment, respectively) (Fig. 5e). After the 18-week regression period, the spot-reduction efficacy of 5% niacinamide was reduced from Δ4.7% (at 8 weeks, significant difference vs. vehicle) to Δ2.8% (at 26 weeks, no significant difference vs. vehicle), and had leveled off through 42 weeks (no significant difference vs. vehicle). Visual grading performed on test subjects also demonstrated that regression from the 5% niacinamide treatment resulted in no significant difference vs. vehicle at both the 26- and 42-week time points (Fig. 5f). After 18 weeks of regression, the spot-reduction effect of 5% niacinamide had regressed from Δ0.33 (at 8 weeks, significant difference vs. vehicle) to Δ0.24 (at 26 weeks, no significant difference vs. vehicle), and continued to regress to Δ0.12 at the 42-week time point (no significant difference vs. vehicle). In addition, visual assess-

**Figure 5.** Dose response of niacinamide spot-reduction efficacy as assessed by image analysis and visual grading plus reversibility of 5% niacinamide hyperpigmented spot-reduction efficacy. Percentage change of hyperpigmented spot area fractions from baseline for (a) 5% and (b) 2% niacinamide and vehicle-treated sides of the face during treatment period of 8 weeks as assessed by image analysis. Asterisks indicate significant difference vs. vehicle at each subsequent time point (*P < 0.05, **P < 0.01). 5% niacinamide reduced spot area fraction vs. vehicle by 4.4% (P < 0.01), while 2% niacinamide did by 2.6% at 8-week time point, indicating a dose dependency of niacinamide effect. (c) Average of visual grading for the area of hyperpigmented spots from baseline for 5 and 2% niacinamide and vehicle-treated sides of the face at the end of the 8-week treatment period. Asterisks indicate significant difference vs. vehicle (*P < 0.05). (d) Representative facial images of 5% niacinamide treatment of the face at week 0, 4, and 8. (e) Percentage change of hyperpigmented spot area fractions from baseline for 5% niacinamide and vehicle-treated sides of the face during treatment period of 8 weeks and the regression period of 34 weeks. Asterisks indicate significant difference (*P < 0.05, **P < 0.01). (f) Average of visual grading for the area of hyperpigmented spots from baseline for 5% niacinamide and vehicle-treated sides of the face at the end of the 8-week treatment period plus the 34-week regression period (i.e. 42 weeks). Asterisks indicate significant difference vs. vehicle (*P < 0.05).
ment by a dermatologist confirmed that there was no side effect due to niacinamide such as hypopigmentation of erythema after 34 weeks of the recovery period for Group 1.

Discussion

The molecular mechanisms involved in the intercellular transfer process of melanosomes from melanocytes to neighboring keratinocytes may be harnessed for the amelioration of skin pigmentation disorders and to develop novel and effective means to lighten skin coloration. Initial events in melanosome transfer most certainly involve recognition between melanocytes and keratinocytes. The exact mechanisms facilitating this interaction are currently unknown. Recently, it has been well demonstrated that the PAR-2 on keratinocytes regulates phagocytic activity of the keratinocyte (15,25). In addition, PAR-2 expression on keratinocytes can be regulated by ultraviolet light to increase melanosome transfer (26).

Less-defined endogenous lectin receptors and their glycoconjugate ligands also appear to be involved in melanosome transfer (27). Initial studies demonstrated that melanosome transfer between human melanoma cells and squamous cell carcinoma-derived keratinocytes could be mediated by certain lectins and neoglycoproteins (19). Recent studies have demonstrated that the addition of these agents to cocultures of normal, untransformed human melanocytes and keratinocytes could also downregulate melanosome transfer (21). In addition, it has been demonstrated that exposure of melanoma cells to UV irradiation results in the upregulation of these membrane lectins and neoglycoproteins (27). Therefore, lectins and their glycoconjugate ligands are excellent candidate molecules involved in melanosome transfer for a variety of reasons. These molecules have been well studied for their role in numerous cellular processes, including endocytosis, intracellular trafficking, and cell-to-cell recognition (17).

Niacinamide (28), also called nicotinamide or 3-pyridine carboxamide, is the physiologically active amide of niacin, vitamin B₃. It has multiple medicinal effects on the skin, including anti-inflammatory, antioxidation, prevention of photoimmunosuppression, and increasing intercellular lipid synthesis (25). The lightening of skin color has also been attributed to niacinamide. Recently, we have demonstrated that niacinamide effectively inhibits melanosome transfer by up to 68% in an in vitro coculture model system (16). Niacinamide is a biologically active form of niacin (vitamin B₃) that functions as part of coenzyme I (nicotinamide–adenine dinucleotide, NAD) and coenzyme II (reduced form of NADP) (29,30) and is involved in over 200 enzymatic reactions (31). In the skin, niacin can act as an anti-inflammatory agent in acne (32), as an antioxidant preventing photoimmunosuppression and photocarcinogenesis (33), and a stimulator for intracellular lipid synthesis (34). However, the molecular mechanism of the inhibitory effect of niacinamide or melanosome transfer is unknown and requires further exploration.

Indeed lectins, neoglycoproteins, and niacinamide present interesting molecules that may modulate skin pigmentation. However, it was unconfirmed in earlier studies whether the skin-lightening effect induced by these agents was a result of apoptotic events occurring in the keratinocytes and/or melanocyte, as opposed to actual inhibition of melanosome transfer. Our initial results demonstrated that concentrations previously demonstrated to inhibit melanosome transfer could induce dose-dependent apoptosis in pure cultures of melanocytes and keratinocytes (Figs 1c,d and 2a,b). However, we had not observed any microscopic evidence of these apoptotic effects using these concentrations of inhibitors in our coculture model system (personal observations). Therefore, we tested the apoptotic effect of these agents on both keratinocytes and melanocytes when existing together in coculture (Fig. 2c,d). The apoptotic effects observed in cultures of isolated melanocytes or keratinocytes were, in most cases, greatly reduced, or completely eliminated, when the same concentrations of niacinamide or lectins were tested on melanocytes or keratinocytes existing in coculture (Fig. 2c,d). This suggests that cell-survival factors, produced by one cell type and utilized by the other, may make both cell types more tolerant to stressful stimuli. Many cytokines, produced by epidermal cells such as endothelin-1 (35), insulin (36), transferrin (37), and basic fibroblast growth factor (38) may help give cells added resistance. These compounds are present in conditioned media, often added to slowly proliferating cell monocultures in our laboratory. Alternately, cell–cell contact between melanocytes and recipient keratinocytes might simply occupy receptors or molecules recognized by the test inhibitors and/or alter gene expression rendering cells more resistant to the apoptotic effects of the test compounds. These results also suggest that the lectins and their glycoconjugates responsible for cell-to-cell interactions and that play a vital role in the melanosome-transfer process may also aid in the cell tolerance to the inhibitory compounds. In addition, there was no
negative effect on cell viability induced by combining the lectins in a cocktail. In contrast, all concentrations of neoglycoproteins resulted in compromised viability to melanocytes and keratinocytes whether cultured in isolation or in cocultures (Fig. 2c,d).

After effective and safe concentrations of niacinamide and lectins were determined, we then assessed the reversibility of these agents on the inhibition of melanosome transfer. As previously stated, we had shown that both niacinamide and our lectin cocktail were effective in inhibiting the transfer of melanosomes from melanocytes to keratinocytes (16,21). In addition, dosing the cocultures twice a day vs. once a day was more effective in inhibiting the transfer of melanosomes from the melanocytes to the keratinocytes in coculture.

It was unknown whether inhibition by niacinamide or lectins was permanent and possibly resulting in a toxic effect or reversible and able to resume melanosome transfer after removal of the inhibitory agents. To address this, we investigated the residual effect of lectins and niacinamide after their removal from the melanocyte–keratinocyte cocultures. Cocultures treated with niacinamide, our lectin cocktail, or both were then assayed by flow cytometry for inhibition of melanosome transfer as compared with the untreated control. Cocultures treated with each inhibitor or both were assayed after 3 days of treatment and compared to the untreated control. Subsequently, samples were treated with inhibitors for 3 days and then maintained in the absence of the compound for 1, 2, or 3 days to assess the reversibility of inhibition. Results demonstrated the following: (a) the effect of niacinamide and the lectin cocktail were significant, and the inhibitory effects were reversible after 3 days, although the reversibility appears to happen more rapidly in the lectin-treated cocultures; (b) the effects of treatment with both lectins and niacinamide are not completely reversible in 3 days; and (c) the effects caused by the combined treatment appeared to persist even after removal of the test compounds. It is interesting to note the increased inhibition in the coculture treated with both the lectin cocktail and the niacinamide for 3 days and assayed after 1 day with no treatment. Additionally, the transfer recovery after 3 days was only 21%.

The skin-lightening effect of niacinamide on hyperpigmented facial spots was demonstrated with 2% and 5% niacinamide in an abbreviated dose–response manner, with 5% being statistically significant from the vehicle control. In addition, the skin-lightening effect of niacinamide was temporary since halting application of niacinamide resulted in a normalization of hyperpigmented facial spots toward the vehicle control. These clinical results are consistent with the coculture results and suggest that the inhibition of melanosome transfer by niacinamide is reversible.

In this clinical trial, reduction of hyperpigmented spots on the vehicle-treated sides of the subjects' faces showed a somewhat different pattern between Groups 1 and 2. This fluctuation speaks to the variation between groups of subjects when the base size is relatively small. Actually, statistical test (Student's t-test) between the two vehicle groups revealed no significant differences ($P > 0.1$) at both 4- and 8-week time points, while significant correlations were observed on the reduction efficacy between paired treatments (niacinamide vs. Vehicle) in both groups.

In the clinical study, we had anticipated that the hyperpigmented spot reduction observed in the 8-week treatment phase would have regressed back to the baseline level by the week 16 time point (8 weeks of niacinamide treatment followed by 8 weeks without niacinamide). But at the 16-week assessment, regression had not occurred. This was likely due to the continued usage of sunscreen product during the 8–16-week time period. To ensure that the affected melanocytes were still responsive (i.e. ensure that a persistent or permanent hypopigmentary effect had not been induced), the clinical study was extended (without provided sunscreen) to follow these subjects for an extended time period. Thus, after week 16, subjects were not instructed to use sunscreen but rather were allowed to follow their normal skin care habits and practices. We observed that with their normal sun exposure habits during the ensuing 6-month (26-week) spring-summer season, the reduction in hyperpigmented spots had regressed back to baseline. Therefore, the reduction in spots was a reversible effect and not due to the induction of a hypopigmentary effect.

Vehicle-treated sides of the faces also demonstrated a reduction of hyperpigmented spots from baseline (October) to week 26 (May). This spot reduction is at least partially attributed to the instruction to strictly use the provided sunscreen during 0–16 weeks (fall-winter). As noted above, no sunscreen product was provided later in the study (16–42 weeks), and thus the spot reduction associated with vehicle + sunscreen use also regressed to the baseline by week 42, likely as a result of normal sun-exposure habits and practices of the subjects during the spring-summer season.

In conclusion, lectins and plasma membrane glycoproteins of melanocytes and keratinocytes are required for communication between these cells, and to facilitate melanosome transfer.
Lectins and niacinamide may prove useful in manipulating skin pigmentation. Further understanding of the underlying mechanisms of action of these compounds may have important implications for understanding the complexities of skin coloration.

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References
