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Secretion of proinflammatory cytokines by normal human melanocytes in response to lipopolysaccharide

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A large body of evidence suggests that epidermal melanocytes are an integral part of the skin immune system and can be considered immunocompetent cells. Recently, it has been reported that human melanocytes constitutively express Toll-like receptors and may be involved in the induction of several inflammatory cytokines. In the study the secretion of IL-1β, IL-6 and TNF-α by cultured normal melanocytes was investigated after stimulation with lipopolysaccharide. LPS increased the secretion of IL-1β in a dose-dependent manner. IL-1β stimulated release of IL-6 and TNF-α by melanocytes, whereas LPS activated production of TNF-α, but not of IL-6. These observations indicate that LPS can participate in the regulation of cytokine activity in normal human melanocytes and suggest that cytokines released by melanocytes could affect melanocytes themselves or/and other cells of the epidermis.

Keywords: melanocytes, proinflammatory cytokines, lipopolysaccharide

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INTRODUCTION

Human melanocytes are unique secretory epidermal cells capable of producing a multifunctional pigment — melanin. Production of melanin is regulated by various factors *via* receptor-dependent and -independent pathways, in a hormonal, auto-, para-, or intracrine manner (Slominski *et al.*, 2004a). The most important regulators of melanogenesis are melanocortin-1 receptor (MC1R) and its ligands α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), that are produced by the enzymatic cleavage of proopiomelanocortin (POMC) in melanocytes, keratinocytes and other skin cell types. Binding of α-MSH or ACTH to MC1R on melanocytes stimulates the expression of the melanogenic cascade and eumelanin synthesis (Slominski *et al.*, 2004b). The pigment, synthesized within melanocytes and then transferred to surrounding keratinocytes, absorbs ultraviolet light and its primary role is to protect skin from solar radiation. Melanin can also act as a scavenger of free radicals, effective metal ions chelator and antioxidant (Herrling *et al.*, 2008; Stępień, 2010). In addition to the photoprotective function, melanocytes also exhibit neuroendocrine activity and produce classical stress neurotransmitters, neuropeptides and hormones (Slominski, 2009).

A large body of evidence suggests that epidermal melanocytes are an integral part of the skin immune system (SIS) and can be considered immunocompetent cells (Tam & Stępień, 2007). These melanin-producing cells express major histocompatibility complex class II molecules and intercellular adhesion molecules (ICAM, VCAM and CD40), and may be capable of antigen processing and presentation (Smit *et* al., 1993; Lu *et al.*, 2002). Melanocytes are able to phagocytose and the melanin pigment itself can act as a physical barrier against microorganisms (Mackintosh, 2001). Moreover, reactive quinone intermediates and hydrogen peroxide generated during melanin synthesis exert strong antimicrobial activity (Plonka *et al.*, 2009). Recently, it has been reported that human melanocytes constitutively express Toll-like receptors 2–5, 7, 9 and 10 at the protein level (Ahn *et al.*, 2008a; Yu *et al.*, 2009; Jin & Kang, 2010). In human, TLRs recognize constituents of microbial cell walls or pathogen-specific nucleic acids, trigger NF-κB (nuclear factor kappa light chain enhancer of activated B cells) and/or MAPK (mitogen-activated protein kinase) signalling pathway, and might be involved in the induction of several inflammatory cytokines and chemokines (Ahn *et al.*, 2008b; Yu *et al.*, 2009; Jin & Kang, 2010). Furthermore, TLR-mediated activation is very similar to that of another essential cytokine receptor, inteleukin-1 receptor (IL-1R). Both TLRs and IL-1R share the MyD88 adaptor molecule and promote the production of proinflammatory cytokines (Verstrepen *et al.*, 2008).

Melanocytes could also act as regulators of the skin's immune response by producing and releasing several immunosuppressive molecules including POMC-derived ACTH and α-MSH (Slominski *et al.*, 2000), cortisol, corticosterone and other steroids (Slominski *et al.*, 1999; 2004b; 2005). In particular, α-MSH has a powerful antiinflammatory potential and affects various pathways implicated in the regulation of inflammation (Brzoska *et al.*, 2008). Furthermore, intermediates of melanogenesis, especially L-DOPA (L-3,4-dihydroxyphenylalanine) and/or products of its oxidation can act as potent immunosuppressors. It has been demonstrated that L-DOPA inhibits lymphocyte proliferation and abolishes production of proinflammatory cytokines by activated lymphocytes (Slominski & Goodman-Snitkoff, 1992; Slominski *et al.*, 2009).

Most of the data on immunocompetence of human melanocytes have emerged from studies on melanoma (Mattei *et al.*, 1994; Lázár-Molnár *et al.*, 2000; Kozłowska

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Abbreviations: ACTH, adrenocorticotropic hormone; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; L-DOPA, L-3,4-dihydroxyphenylalanine; LPS, lipopolysaccharide; MSH, melanocyte-stimulating hormone; POMC, proopiomelanocortin; PCR, polymerase chain reaction; TNF, tumour necrosis factor; TLRs, Toll-like receptors.

et al., 2001; Kholmanskikh *et al.*, 2010; Elias *et al.*, 2010) and little is known about the cytokine-producing potential of normal melanocytes and their role in the network of cytokine interactions. The aim of the present study was to examine the ability of normal human melanocytes to secrete IL-1β, IL-6 and TNF-α, the most important cytokines associated with skin inflammation, in response to bacterial LPS. Additionally, we investigated the potential of IL-1β to induce TNF-α and IL-6 release in melanocyte cultures.

MATERIALS AND METHODS

Materials. Primary human epidermal melanocytes from neonatal foreskin of moderately pigmented donor (HEMn-MP) were purchased from Cascade Biologics/GIBCO Invitrogen. Melanocyte cultures were grown in melanocyte growth medium, consisting of Medium 254, human melanocyte growth supplement (HMGS) and antibiotics (Gentamicin & Amphotericin B solution) (Cascade Biologics/GIBCO Invitrogen). Trypsin neutralizer and 0.025% trypsin/EDTA solutions were from GIBCO Invitrogen. Dulbecco's PBS buffer was obtained from PAA Cell Culture Company. Lipopolysaccharide from *Escherichia coli* (026:B6) was purchased from Sigma-Aldrich, Inc. Recombinant human IL-1β protein and Quantikine Human TNF-α, IL-6 and IL-1β Immunoassays were from R&D Systems (Minneapolis, USA). CytoTox96® Non-Radioactive Cytotoxicity Assay and CellTiter-Glo Luminescent Cell Viability Assay were obtained from Promega Corporation.

Melanocyte culture. Human epidermal melanocytes were cultured according to the supplier's recommendations. Briefly, cryopreserved primary melanocytes were transferred into 25-cm² tissue culture flasks at a density of 5×10^3 cells/cm² and were grown in melanocyte growth medium in a humidified incubator with 5% CO_2/95% air at 37°C. The culture medium was changed every other day. After cells became about 80% confluent the medium was removed and melanocytes were subcultured using conventional trypsinization method with trypsin/EDTA and trypsin neutralizer solutions.

Melanocyte stimulation. For stimulation of cytokine production, melanocytes from the 4th and 5th passages were used. The cells resuspended in supplemented Medium 254 were plated into 96-well microplates at a density of 10^4 cells/ well. After two days of incubation, the media were refreshed and the incubation continued for an additional 24 hours. Then, the media were replaced with fresh media containing various concentrations of LPS (0.01–20 μg/ml) or IL-1β (0.01–10 ng/ml). Melanocytes cultured without the stimulants were used as a control. After a 24-h stimulation culture supernatants were collected, and intracellular adenosine triphosphate (ATP) levels were measured in melanocytes. The supernatant samples were stored at –70°C until assayed for cytokine and lactate dehydrogenase (LDH) activity.

Estimation of cell viability. The parameters used for evaluating melanocyte viability in the stimuli-treated and non-treated cultures were LDH leakage and intracellular ATP level.

LDH release into the culture medium is an indicator of cell death resulting in cell membrane damage. The amount of LDH in the medium was determined using the CytoTox 96® Non-Radioactive Cytotoxicity Kit, according to the manufacturer's instruction. The absorbance of red formazan product was recorded at 490 nm using a TRIAD LT microplate reader (Dynex Technologies, USA). The cytotoxicity of the tested compounds was calculated according to the formula: % cytotoxicity = (stimulated cells LDH release — spontaneous LDH release/maximum LDH release — spontaneous LDH release) × 100. Maximum LDH release was obtained after lysis of the cells with Lysis Solution provided by the producer.

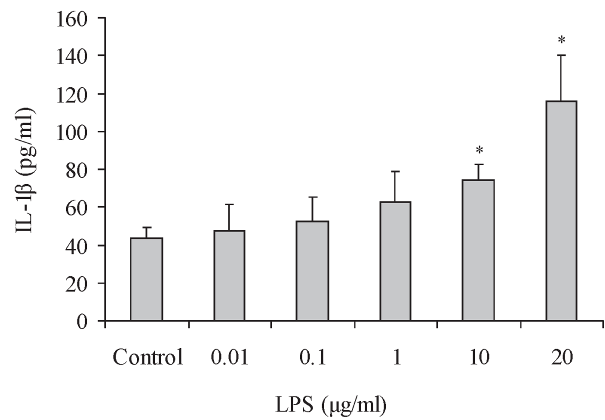

Figure 1. Effect of LPS on IL-1β secretion by normal human epidermal melanocytes.
Culture supernatants were collected 24 hours after LPS stimulation and IL-1β concentrations were determined by ELISA. The values indicate the mean ± S.D. (n = 6). *$P < 0.05$ *vs* control.

The presence of metabolically active melanocytes in the cultures was estimated by measurement of the cellular ATP level. A bioluminescence assay based on the detection of ATP *via* luciferase — catalyzed reaction was performed in accordance with the manufacturer's protocol (CellTiter-Glo Luminescent Cell Viability Assay). The luminescent signal, proportional to the amount of ATP, was recorded on a TRIAD LT microplate reader.

Cytokine measurements. The cytokine contents in cell-free culture supernatants were determined with commercial enzyme-linked immunosorbent assay (ELISA) kits, according the manufacturer's directions (Quantikine). The limits of detection were: 4.4 pg/ml for TNF-α, 0.7 pg/ml for IL-6 and 1 pg/ml for IL-1β. Absorbance was read at 450 nm using a TRIAD LT microplate reader. The quantification was made on the basis of calibration curves prepared for the cytokines analyzed.

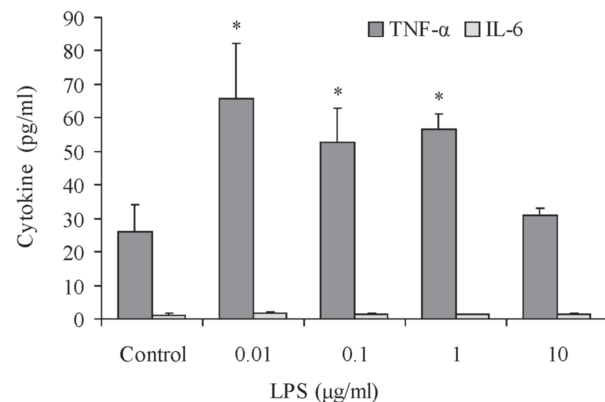

Figure 2. Effect of LPS on IL-6 and TNF-α secretion by normal human epidermal melanocytes.
Culture supernatants were collected 24 hours after LPS stimulation and IL-6 and TNF-α concentrations were determined by ELISA. The values indicate the mean ± S.D. (n = 6). *$P < 0.05$ *vs* control.

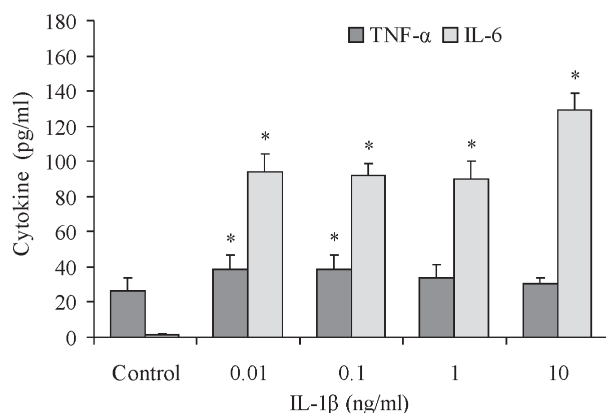

Figure 3. Effect of interleukin 1β on IL-6 and TNF-α secretion by normal human epidermal melanocytes.
Culture supernatants were collected 24 hours after IL-1β stimulation and IL-6 and TNF-α concentrations were determined by ELISA. The values indicate the mean ± S.D. (n = 6). *$P < 0.05$ *vs* control.

Statistical analysis. Data represent the mean ± standard deviation (S.D.) from six independent experiments. Comparisons between groups were made by a one-way analysis of variance with post-hoc Tukey's test using Statistica version 8 software (StatSoft Inc., USA). P values less than 0.05 were considered significant.

RESULTS

Evaluation of melanocyte viability following exposure to LPS or IL-1β

Effects of LPS and IL-1β on melanocyte viability were evaluated on the basis of intracellular ATP levels and LDH release after 24-h exposition to the stimuli. LPS or IL-1β did not display any significant cytotoxic effect on melanocytes. LDH levels in media were unaffected following exposure to these stimuli. Also, ATP content assay revealed that the viability of melanocytes exposed to LPS and IL-1β in the concentration range tested was comparable to that of cells incubated with medium only.

Modulation of cytokine secretion by LPS or IL-1β

We observed differences in the constitutive production of the cytokines analyzed by melanocytes. IL-1β and TNF-α were detected in the cell supernatants at concentrations of 43.2 ± 6 pg/ml and 26.1 ± 8 pg/ml, respectively, while the constitutive secretion of IL-6 was minimal (1.2 ± 0.5 pg/ml).

Stimulation of melanocytes with LPS induced dose-dependent increase in IL-1β secretion (Fig. 1). At the highest dose of LPS (20 μg/ml) the secretion was approximately 270% of control (i.e., 115.8 ± 24 pg/ml). The effect of LPS and exogenous IL-1β on TNF-α and IL-6 production is presented in Figs. 2 and 3. Incubation of melanocytes with increasing concentrations of LPS had no effect in the production of IL-6. In contrast, stimulation of melanocytes with IL-1β resulted in a significant rise in IL-6 secretion (approximately 75-fold at 0.01–1 ng/ml and 110-fold at 10 ng/ml, compared with the control). The both stimuli showed a similar TNF-α-secretion profile. However, in response to LPS the TNF-α secretion increased 2.5-fold while in response to IL-1β only 1.5-fold. In the both cases, the largest response was observed when the stimulus was applied at low doses.

DISCUSSION

Reports on the production of proinflammatory cytokines by normal human melanocytes are scarce, and the described results are contradictory. Smit *et al.* (1993) demonstrated by immunohistochemistry that human melanocytes constitutively synthesized IL-1β, IL-6 and IL-2. Swope *et al.* (1994) found melanocytes able to produce IL-1α and IL-1β using immunocytochemical staining and the PCR technique. In studies by Mattei *et al.* (1994) cultures of normal melanocytes were positive for RNA transcripts for IL-6 and its receptor (IL-6R), IL-8, IL-10, TNF-β and TNFR (TNF receptor), but negative for IL-1α, IL-1β and TNF-α. In our study, the quantitation of cytokine production at the protein level was based on immunoenzymatic assay (ELISA). The obtained results showed that IL-1β, TNF-α and IL-6 were constitutively secreted by melanocytes, but the amount of IL-6 released was approximately 40- and 20-fold lower than the amount of IL-1 and TNF-α, respectively.

LPS is a well-known endotoxin which elicits a variety of inflammatory responses. In humans, LPS binds the CD14/TLR4/MD2 receptor complex, which triggers the signaling cascade for many cell types to secrete proinflammatory cytokines and nitric oxide (Hari *et al.*, 2010). LPS-treated melanocytes were found to respond to the endotoxin by activation of TLR4 and TLR2, expression of adaptor molecules CD14 and MyD88, promotion of NF-κB subunit p65 translocation to the nucleus (Ahn *et al.*, 2008a; Yu *et al.*, 2009; Jin & Kang, 2010) and enhanced expression of some proinflammatory cytokines and chemokines (IL-6, IL-8, CCL2, CCL3 and CCL5) (Yu *et al.*, 2009). We examined changes in the secretion of IL-1β, IL-6 and TNF-α by cultured human melanocytes in response to LPS. Additionally, the potential of IL-1β to induce TNF-α and IL-6 release in melanocyte cultures was assessed. Our results showed that LPS triggered the secretion of IL-1β and TNF-α by normal epidermal melanocytes. Moreover, we observed differences in TNF-α and IL-6 secretion by melanocytes after exposure to LPS and IL-1β. The incubation of melanocytes with increasing concentrations of LPS did not affect the production of IL-6, while stimulation with IL-1β resulted in a significant growth of IL-6 secretion. These differences may be due to the fact that fewer than ten ligand-occupied IL-1RI receptors are required per cell to induce a strong response and this potency is in contrast to the Toll-like receptor system, which requires a 10- to 100-fold higher receptor occupancy (Dayer, 2002). Besides, secretion of IL-1, IL-6 and TNF-α need not always occur in parallel (Burbach *et al.*, 2001) and the cytokines are capable of cross-regulating one another (Radtke *et al.*, 2010). In this context, IL-1 and IL-6 released by activated melanocytes could form a positive feedback loop in upregulating TNF-α and possibly others cytokines of inflammation (Van Der Meer *et al.*, 2005).

It has been reported that IL-1, IL-6 and TNF-α inhibit human melanocyte proliferation and melanogenesis, and TNF-α induces apoptosis of these cells (Swope *et al.*, 1991; Shang *et al.*, 2002). On the other hand, recent studies have demonstrated that LPS increases melanin synthesis in human melanocytes and induces pigmentation of cultured skin (Ahn *et al.*, 2008b; Jin & Kang, 2010). Other mediators of inflammation, such as nitric oxide, histamine and eicosanoids, have also been shown

to stimulate melanogenesis (Romero-Graillet *et al.*, 1997; Sasaki *et al.*, 2000; Slominski *et al.*, 2004a). Furthermore, postinflammatory hyperpigmentation of the skin is frequently observed in clinical practice (Pandya & Guevara, 2000; Brajac *et al.*, 2009). These date indicate that epidermal melanocytes can modulate their pigmentation in response to inflammatory conditions. As intermediates of melanogenesis and melanin itself are able to inhibit activity of immune cells (Slominski & Goodman-Snitkoff, 1992; Mohagheghpour *et al.*, 2000; Slominski *et al.*, 2009), the process of melanogenesis could have immunomodulatory functions. Interestingly, inhibition of melanogenesis has been proposed as an adjuvant strategy in the treatment of melanotic melanomas (Slominski *et al.* 1998; 2009).

Overproduction of IL-6, TNF-α and IL-1 in the skin is considered a mediator of transition from acute to chronic inflammation and can be implicated in the development of some melanotic disorders (Imokawa, 2006). It is hypothesized that aberrant production of cytokines by melanocytes may be associated with pigmentary changes (Abdel-Malek & Kadekaro, 2006; Hasegawa *et al.*, 2008; Jin & Kang, 2010) and play an important role in the pathogenesis of human diseases, such as lichen planus, psoriasis, vitiligo and melanoma (Clark, 2001; Moretti *et al.*, 2002; Brajac *et al.*, 2009; Hari et al., 2010).

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