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Stimulated human melanocytes express and release interleukin-8, which is inhibited by luteolin: relevance to early vitiligo

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Summary

Vitiligo is a disorder of depigmentation, for which the pathogenesis is as yet unclear. Interleukin (IL)-8 is a key inflammatory chemokine. We investigated the regulation of IL-8 production in human melanocytes, and the IL-8 serum levels and skin gene expression in patients with vitiligo and in controls. Cultured melanocytes were stimulated for 24 h with tumour necrosis factor (TNF) 100 ng/mL and IL-1 β 10 ng/mL, with or without pretreatment with luteolin 50 μ mol/L for 30 min, and IL-8 release was measured by ELISA. Serum cytokines were measured by a microbead array. Skin biopsies were taken from healthy subjects ($n = 14$) as well as from marginal lesional and nonlesional skin from patients with vitiligo ($n = 15$). IL-8 gene expression was evaluated by quantitative reverse transcriptase PCR. Both TNF and IL-1 β stimulated significant IL-8 release ($P < 0.01$) from melanocytes, whereas pretreatment with luteolin significantly inhibited this effect ($P < 0.01$). IL-8 gene expression was significantly increased in vitiligo compared with control skin ($P < 0.05$). IL-8 may be involved in vitiligo inflammation. Inhibition by luteolin of IL-8 release could be useful for vitiligo therapy.

Vitiligo is a cutaneous disorder of depigmentation, characterized by absence of functional melanocytes. It affects 1–3% of the general population,¹ and its exact pathogenesis remains

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obscure. However, recent evidence suggests that oxidative stress, auto-immunity and melanocyte apoptosis are involved.¹

T-helper (Th)-1 cells can increase angiogenesis through the expression of vascular endothelial growth factor (VEGF) by both Th-1 cells and mast cells.² The release of inflammatory cytokines, especially interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF) after skin trauma (Koebner phenomenon) might lead to the recruitment of T cells in the skin and exposure to new antigen-expressing melanocytes. TNF has been shown to induce IL-8 (CXCL8) mRNA expression in a melanoma cell line³ and to upregulate IL-8 receptor expression in normal melanocytes.⁴

IL-8 is a chemokine important in inflammatory skin diseases, and is produced by monocytes, mast cells, fibroblasts, endothelial cells, dendritic cells and keratinocytes.⁵ IL-8 is chemotactic to neutrophils, T-lymphocytes, basophils and keratinocytes.⁵

We studied the effect of TNF and IL-1 β on IL-8 release from human cultured primary melanocytes, before and after treatment with the natural flavonoid luteolin. We also studied the gene expression and serum levels of IL-6, IL-8 and VEGF in biopsies taken from controls and from the affected and nonlesional skin of patients with vitiligo.

Report

Human primary melanocytes from ATCC (PCS-200-013) were cultured in cell medium [Dermal Basal Cell Medium; PCS-200-030; American Type Culture Collection (ATCCP, Manassas, VA, USA) supplemented with 100 U/mL penicillin/streptomycin (Life Technologies, Grand Island, NY, USA) and 50 μ L phenol red (PCS-999-001; ATCC).

Incubation of melanocytes with TNF 100 ng/ml (210-TA-010, R & D Systems, Minneapolis, MN, USA) for 24 h induced significant ($P < 0.01$) IL-8 gene expression (Fig. 1a) as measured by quantitative reverse transcriptase (qRT)-PCR, and significant ($P < 0.01$) release into the supernatant fluid as measured by ELISA (R & D Systems) (Fig. 1b). Because our previous evidence had indicated that the naturally occurring flavonoid luteolin can inhibit the effect of TNF on cultured T cells,⁶ we treated the melanocytes with luteolin 50 μ mol/L (Sigma-Aldrich, St. Louis, MO, USA) for 30 min before stimulation with TNF 100 ng/mL. This treatments significantly inhibited ($P < 0.01$) both IL-8 gene expression (Fig. 1a) and IL-8 release into the supernatant (Fig. 1b).

Similarly, incubation of melanocytes with IL-1 β 10 ng/mL (201-LB-005, R & D Systems) for 24 h also induced significant ($P < 0.01$) IL-8 gene expression (Fig. 2a) and release (Fig. 2b). Treatment with luteolin 50 μ mol/L for 30 min before stimulation with IL-1 β 10 ng/mL significantly inhibited both IL-8 gene expression ($P < 0.01$, Fig. 2a) and release ($P < 0.05$, Fig. 2b) from melanocytes.

Biopsies were obtained for tissue experiments. The scientific/ethics committee of the A. Sygros Hospital (Athens, Greece) approved the sample collection, and all subjects gave signed informed consent.

Full thickness marginal punch skin biopsies (4 mm³) were obtained from patients with nonsegmental vitiligo ($n = 15$; Table 1), who had new-onset and active disease, and either had not received any systemic medication and were free of any systemic inflammation, or had received topical therapy only. In the latter case, skin was collected from previously untreated areas. Nonlesional biopsies were also obtained (at least 150 mm away from any lesions) from each patient. Biopsies were also obtained from healthy volunteers ($n = 14$; Table 1) who met the same exclusion criteria. In all cases, biopsies were taken from nonexposed areas in order to avoid visible scars.

The biopsy tissues were placed in RNA stabilization reagent (RNAlater; Ambion, Inc. Austin, TX, USA), and stored at -80°C . They were transported on dry ice for analysis at Tufts University (Boston, MA, USA). Samples carried no identifiers except for subject age and gender. There was no significant difference in age or gender between patients with vitiligo and controls (Table 1). Calculating that one palmar area equals approximately 1% of total body surface, the patients were grouped according to percentage body area affected: $< 5\%$ ($n = 2$), $5\text{--}10\%$ ($n = 9$), $10\text{--}25\%$ ($n = 3$), $25\text{--}50\%$ ($n = 0$) and $> 50\%$ ($n = 1$).

Total RNA was extracted from skin (Fibrous Tissue Mini Kit #74704) and cultured melanocytes (Qiagen Mini Kit #74104) (both Qiagen, Valencia, CA, USA), and cDNA synthesis was performed (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA). Using Taqman MGB probes [all Applied Biosystems, Foster City, CA, USA; Hs00174103_m1 for *IL-8*, Hs00985639_m1 for *IL-6*, Hs00900055_m1 for *VEGF* and 4310884E-1102047 for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*,] qRT-PCR was carried out (7300 Sequence Detector; Applied Biosystems), with 40 cycles at 50°C for 2 min, 95°C for 10 min and 95°C for 15 s, followed by 1 cycle at 60°C for 1 min. Results were normalized against GAPDH, and expressed relative to the mean of the control for each gene.

Serum levels of IL-6, IL-8 and VEGF were measured using a microbead array (Human Cytokine Panel I; Milliplex, St Charles, MO, USA).

All *in vitro* conditions were performed in triplicate, and were repeated at least three times ($n = 3$).

The experimental IL-8 release results and the lesional and nonlesional skin IL-8 gene expression results were analysed using the Mann–Whitney nonparametric *U*-test. Power analysis indicated that to show a 60% change in gene expression and a 50% change in SD, 15 patients were sufficient. Data are expressed as the mean $\pm$ SD, and significance was set at $P < 0.05$ and $P < 0.01$.

IL-8 relative gene expression was significantly higher ($P = 0.01$, Fig. 3) in lesional vitiligo skin ($n = 15$) than in control skin ($n = 14$) (Fig. 3). There was no significant difference between lesional and nonlesional skin samples from patients (results not shown).

There were no significant differences in IL-6 or VEGF gene expression between lesional and nonlesional skin of patients, or between patient and control skin or (results not shown).

There were no significant differences between patients and controls for serum levels of IL-6, IL-8 and VEGF as measured by the microbead array.

To our knowledge, this is the first report showing increased release of IL-8 from human melanocytes in response to TNF and IL-1 β , and increased IL-8 gene expression in the lesional skin of patients with new-onset and active nonsegmental vitiligo.

We propose that in the initial stages, where melanocytes are still present in the epidermis, IL-6, IL-1 and TNF released by various cell types⁵ could stimulate high IL-8 skin expression by melanocytes. IL-8 is a powerful chemokine that has been reported to induce oxidative stress, leading indirectly to both keratinocyte and melanocyte apoptosis in vitiligo.¹ Apoptotic cells could in turn release high amounts of pro-inflammatory IL-1 and TNF, further enhancing skin inflammation. We previously showed that both preformed and newly synthesized TNF can be released from mast cells,⁷ the numbers of which seem to be increased in the centre of vitiligo lesions.² A recent paper reported that chemically induced vitiligo led to increased production of IL-6 and IL-8.⁸

We also report for the first time that the natural flavone, luteolin, blocks this TNF- and IL-1 β -induced IL-8 release from melanocytes. Luteolin is a safe, plant-derived polyphenolic compound with antioxidant, anti-inflammatory, antiallergic and cytoprotective properties.⁹ Moreover, the flavonol quercetin, which is structurally related to luteolin, was reported to induce melanogenesis in a human epidermal culture model.¹⁰

Our results suggest the involvement of IL-8 in the initial vitiligo stages, and indicate a possible and a protective role for luteolin in IL-8-induced inflammation.

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Learning points

- Inflammation and oxidative stress are the leading pathogenetic mechanisms of vitiligo.
- IL-8 is the main chemokine in chronic inflammatory dermatoses.
- IL-8 gene expression is increased in vitiligo skin.
- IL-8 gene expression and release is triggered by TNF and IL-1 β in primary human melanocytes.
- Luteolin has potent anti-inflammatory and anti-oxidant actions, but also blocks melanocyte IL-8 gene expression and release.
- Local or oral preparations of luteolin may be useful for the treatment of vitiligo.

Figure 1.

(a) Gene expression and (b) release of interleukin (IL)-8 from cultured melanocytes with or without pretreatment with luteolin (50 μmol/L) for 30 min, before stimulation with tumour necrosis factor (TNF) 100 ng/mL for 24 h ($n = 5$, $**P < 0.01$).

Figure 2.

(a) Gene expression and (b) release of interleukin (IL)-8 from cultured melanocytes with or without pretreatment with luteolin (50 μmol/L) for 30 min, before stimulation with IL-1β 10 ng/mL for 24 h ($n = 5$, $*P < 0.05$, $**P < 0.01$).

Figure 3.

IL-8 gene expression in lesional vitiligo ($n = 15$) and control ($n = 14$) skin samples ($P = 0.01$). There was no clinical information that could explain the two high values in the vitiligo group; however, when these two values were removed from the statistical analysis, the significance ($P < 0.05$) was retained.

Table 1

Subject characteristics.

	Vitiligo n=15), n (%)	Control (n=14), n (%)
Age	39.4 ± 8.6 [*]	45.35 ± 13.77 [*]
Gender		
Female	9 (60)	9 (64.28)
Male	6 (40)	5 (35.75)
Concomitant allergies		
Food allergies	2 (13.33)	–
Drug allergies	2 (13.33)	–
Contact dermatitis	1 (6.66)	–
Rhinitis	2 (13.33)	–
Additional autoimmune disease		
Hashimoto thyroiditis	3 (20)	–

^{*} Values for age are mean ± SD.