Interleukin 1-α: A Modulator of Melanocyte Homeostasis in Vitiligo
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Abstract

Background: Vitiligo is a hypomelanotic autoimmune skin disorder arising from a breakdown in immunological self-tolerance, which leads to aberrant immune responses against melanocytes leading to selective destruction of melanocytes. High levels of Interleukin 1 (IL1) have been reported in various autoimmune disorders including vitiligo. The aim of present study was to explore the effect of IL1-α on melanocyte biology by monitoring the melanocyte viability, IL1R1 membrane expression, melanogenesis (TYR, TYRP1 and MITF-M) and other immune modulatory molecules (IL1A, IL1B, IL1RN, IL1R1, IL8, TNFA, IL6, and ICAM1) upon exogenous stimulation of IL1-α on primary cultured normal human melanocytes (NHM).

Materials and methods: Melanocytes were isolated from normal human skin biopsies and cultured in vitro. The NHMs were treated with IL1-α (0-100 ng/ml) and cell viability was monitored by MTT assay, IL1R1 membrane expression was monitored using flow cytometry and relative gene expression was performed using semi-quantitative RT-PCR.

Results: The dose dependent effect of IL1-α on melanocytes showed ~12% melanocyte death and ~22% increase in IL1R1 membrane expression upon 100 ng/ml IL-1α treatment for 48 hrs. Further, IL1RN, IL1A, IL1B, IL6, TNFA, ICAM1 showed significantly increased expression (p<0.05) and MITF-M showed significantly decreased expression (p<0.05) upon IL1-α (10 and 100 ng/ml, 48 hrs) stimulation on NHM; while TYR, TYRP1, IL8 and IL1R1 showed no difference.

Conclusion: Overall, the present study suggests the crucial role of IL1-α in melanocyte destruction in vitiligo by regulating MITF-M and other immunomodulatory molecules.

Keywords: Vitiligo; Interleukins; Melanocyte; Autoimmunity; Melanogenesis

Abbreviations: NHM: Normal Human Melanocyte; IL: Interleukin; IL1R1: Interleukin 1 Receptor 1; IL1RN: Interleukin 1 Receptor Antagonist; TRITC: Tetramethyl Rhodamine Isothiocyanate; MTT: 3-(4,5-Dimethylthiazolyl-2)-2,5-Diphenyltetrazolium Bromide; TNFA: Tumor Necrosis Factor-A; ICAM1: Intercellular Adhesion Molecule-1; MITF-M: Microphthalmia-Associated Transcription Factor-M; TYR: Tyrosinase; TYRP1: Tyrosinase Related Protein 1; CTLA-4: Cytotoxic T-Lymphocyte Associated Antigen-4; IFNy: Interferon Gamma

Introduction

Vitiligo is an acquired hypomelanotic pigmented disorder characterized by circumscribed depigmented macules in the skin caused due to loss of functional melanocytes. Worldwide incidence of vitiligo ranges from 0.04 to 2.16% [1]. In India, it affects 0.5 to 2.5% of the population and the states of Gujarat and Rajasthan have the highest incidence rate of 8.8% [2,3]. The exact etiology and detailed pathogenesis of vitiligo are not fully understood, but autoimmunity has been strongly implicated in the development of the disease [4-10]. We have earlier reported that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected [11]. In addition, 75% vitiligo patients showed presence of anti-melanocyte antibodies in the sera [12]. There is growing evidence that cytokines are important in the depigmentation process of vitiligo [13,14]. Cytokines such as IL1, IFN-γ or TNF-α which are released by lymphocytes and keratinocytes can initiate apoptosis in melanocytes. Also, TNF-α, IL1-α, IL6 and TGF-β are the potent inhibitors of melanocyte growth [13]. Previous studies including our lab reports have shown increased transcript/ serum levels of TNF-α, TNF-β, IFN-γ, IL4, IL1-β and ICAM1 in vitiligo patients [15-21]. Interestingly, high IL1-α level has been reported in the lesional skin of patients with non-segmental vitiligo [22].

The present study focuses on IL1-α, which is a pro-inflammatory cytokine and it is also known as a B-cell activating factor [23,24]. IL1 affects virtually all cells and organs and is a major pathogenic mediator of auto-inflammatory, autoimmune, infectious, and degenerative diseases [25-27]. The genes of IL1 family reside in close proximity forming a gene cluster (IL1R1–IL1A–IL1B–IL1RN), in the region 2q12-q21 of human chromosome 2, and are derived from gene duplications which occurred 320-400 M years ago (IL-1/IL-1ra divergence) and 270-300 M years ago (IL1A/IL1B duplication) [28-30]. IL1-α and IL1-β also induce expression of their own genes, which serves as a positive-feedback loop that amplifies the IL1 response in an autocrine or paracrine manner [31]. IL1-α is reported to stimulate IL-1, TNF-A, and IL-6 expression in human cardiac myofibroblasts [32]. Pro-IL1-α has a nuclear localization domain which functions as a component of transcription regulation [31]. These reports lead to the hypothesis of the present study where IL1-α might modulate melanogenesis and other immuno-regulatory genes.

Thus, the aim of present study was to explore the effect of IL1-α on melanocyte biology by monitoring the melanocyte viability, IL1R1 membrane expression, melanogenesis (TYR, TYRP1 and MITF-M) and other immune modulatory molecules (IL1RN, IL1R1, IL1A, IL1B, IL6,IL8, TNFA and ICAM1) upon exogenous stimulation of IL1-α on NHM.

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Materials and Methods

Ethics statement

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of our study was explained to all participants and written consent was obtained from all the subjects before performing our studies.

Culture establishment of primary normal human melanocytes (NHM)

Melanocytes were isolated from human skin samples and cultured successfully using the standard protocol with slight modifications [33,34]. Briefly, the epidermis was separated from the dermis after an overnight incubation of skin biopsies in 0.25% Dispase II protease (Sigma-Aldrich, USA) prepared in phosphate buffer saline (PBS) (Himedia, India) at 4°C. In order to separate epidermal cells, the epidermis was incubated at 37°C for 10 min, in a solution of 1X Trypsin Phosphate Versene Glucose (TPVG) Solution (Himedia, India). Cellular suspension was centrifuged at 1,300 rpm for 5 min at room temperature to harvest cells. Melanocytes were selectively cultured in M254 medium with human melanocyte growth supplements (Gibco life Technologies™, Cascade Biologics™, Portland, Oregon) and 1X antibiotic-antimycotic solution (Himedia, India). The cells were maintained in a humidified incubator with 5% CO₂ at 37°C and media replenishment was given after every 2-3 days. Melanocyte-keratinocyte mixed population starts appearing around 4-9 days. Melanocytes were purified from keratinocytes by differential trypsinisation, which is based on the more sturdy and adherent property of keratinocytes. After the removal of spent media, gentle Dulbecco’s Phosphate-Buffered Saline (D-PBS) wash is given and Trypsin is added for detachment of cells and cells harvested in initial 2-3 minutes are enriched in melanocytes and the remaining cells in the culture flask constitute majorly keratinocytes. Further upon one more cycle of differential trypsinization we get complete rid of keratinocytes. In addition, melanocytes were given G418 (Geneticin DisulfateSalt, MP Biomedicals, France) treatment for gradual removal of fibroblasts to obtain primary human melanocyte and further were split at 1:3 ratio, when cell confluency reached 80%. Media was replenished after every 48-72 hrs; melanocytes cultured up to the fifth passage were used for experiments.

Confirmation of melanocytes by DOPA staining

The pure culture of melanocytes was confirmed by L-DOPA (1,3,4-dihydroxy phenylalanine, Hi-Media, India) staining [35]. NHM were trypsinized, plated on coverslips and cultured for ~3 days before detection of tyrosinase activity. For DOPA reaction, culture media was removed and cells were rinsed twice in PBS, fixed for 20 min in 4% formaldehyde solution in PBS, washed three times with PBS and then incubated at 37°C for 18 hrs in the dark with 10 mM L-DOPA. After incubation, the cells were rinsed with distilled water, dehydrated, mounted and number of cells positive for tyrosinase activity were observed using light microscopy.

MTT assay

The number of viable cells was recorded using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium salts], as described by Mosmann [36]. NHM were seeded in 96-well plates at an initial density of 5x10⁴ cells/well and after overnight incubation, cells were treated with IL1-α (Human cell Express™, Human recombinant IL1-α, Biovision San Francisco) in dose dependent manner (0, 10, 50, 100 ng/ml). After 48 hrs of treatment, MTT (Molecular probes’ by Life Technologies™, China) was added to each well and the cells were incubated at 37°C for 4 hrs. The medium was removed and dimethyl sulfoxide (DMSO) (Sisco Research Laboratories, Mumbai) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using ELISA plate reader (Thermo scientific Multiskan EX, Shanghai, China).

Flow cytometry

IL1R1 membrane expression on NHM was monitored by flow cytometry using monoclonal Rabbit anti-IL-1 receptor 1 antibody (ab40774, Abcam, USA). NHM were seeded (0.4x10⁶ cells/well) in a 6 well plate and incubated overnight at 37°C with 5% CO₂ in a humidified incubator. After overnight incubation, IL1-α (0, 10, 50, 100 ng/ml) treatment was given along with media replacement and the cells were further incubated for 48 hrs. Upon treatment, cells were trypsinised, washed with PBS and fixed with paraformaldehyde at 4°C for 15 min, followed by blocking with 5% bovine serum albumin (Sisco Research Laboratories, Mumbai) for 1 hr at room temperature. Further, primary monoclonal Rabbit anti-IL-1 receptor 1 antibody (ab40774, Abcam, USA) was added to cells and incubated overnight at 4°C. Upon incubation, cells were washed with PBS + 0.02% Tween 20 (PBST) and secondary antibody (Anti-Rabbit IgG (whole molecule) - TRITC, Sigma-Aldrich™, USA) was added and incubated for 1 hr at room temperature in dark conditions. Cells were washed with FACS buffer (BD FACS Flow sheath fluid) and IL1R1 membrane expression was observed using flow cytometry (BD Biosciences FACS-ARIA III).

RNA extraction and cDNA synthesis

Total RNA from primary NHM was isolated and purified using TriZol® reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by 1.5% agarose gel electrophoresis and 260/280 absorbance ratio. RNA was treated with DNase I (Ambion Inc., Austin, TX, USA) before cDNA synthesis, to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the Verso cDNA Synthesis Kit (Thermo Scientific, US) according to the manufacturer’s instructions in the G Palm Thermal Cycler (Model PTC-200, Watertown, MA, USA).

Second strand synthesis

First strand cDNA was used as a template with gene-specific primers to synthesize second strand DNA by conventional PCR. Expression kinetics of IL1R1, IL1RN, IL1A, IL1B, IL6, TNFA, ICAMI1, MITF-M, IL6, TYR and TYRPI genes were analysed. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control (Table 1). Reactions were performed according to the manufacturer’s instructions (Fermentas, Burlington, ON, Canada). DNA fragments were amplified for 39 cycles and signal intensities were analysed on 3.5% agarose gel stained with ethidium bromide. Densitometric analysis was performed using Alpha Imager software, and mean density for respective genes with respect to GAPDH, was plotted.

Statistical analyses

All experiments were performed at least three times in triplicates independently on different days using different batches of cells. Data are presented as the mean ± standard deviation (SD) and the statistical difference between two groups were analyzed by Student’s t-test using GraphPad Prism version 3.02 software (GraphPad Software Inc. San Diego, CA, USA). P-values less than 0.05 were considered statistically significant.
Dose dependent effect of IL1-α on melanocytes

Primary melanocytes were treated with different doses of IL1-α (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM did not show any difference in morphology and viability up to 24 hrs (data not shown). Also, there was no significant morphological change observed in NHM even after 48 hrs of IL1-α treatment (Figure 2A). However, 100 ng/ml of IL1-α treated NHM showed significant decrease in viability (p=0.0210) after 48 hrs of treatment, as compared to untreated NHM (Figure 2B). However, 10 ng/ml & 50ng/ml doses of IL1-α did not show any significant difference (p=0.6658 and p=0.9301 respectively) in cell viability.

IL1-α regulates melanogenesis and other immuno modulatory genes

IL1-α (10 ng/ml and 100 ng/ml) treated NHM were used for expression analysis of IL1R1, IL1RN, IL1A, IL1B, IL6, TNFA, ICAMI, MITF-M, IL8, TYR and TYRP1 genes. Our results showed significantly decreased expression of MITF-M in 100 ng/ml IL1-α treated NHM as compared to untreated NHM (p=0.0066; Figure 4) however, there was no difference at 10 ng/ml IL1-α treatment (p=0.0694). Also, TYR (p=0.4532, p=0.5659) and TYRP1 (p=0.4395, p=0.8858) genes of melanin synthesis pathway exhibited no significant difference upon IL1-α (10 and 100 ng/ml respectively) treatment. Interestingly, expression levels of IL1RN, IL1A, IL1B, IL6, TNFA and ICAMI were significantly up regulated in 100 ng/ml IL1-α treated NHM as compared to untreated NHM (p=0.0066 for IL1RN; p=0.0066 for IL1A; p=0.0066 for IL1B; p=0.0066 for IL6; p=0.0066 for TNFA; and p=0.0066 for ICAMI).

Results

Isolation and culturing of melanocytes

Cells isolated from skin biopsies and cultured in melanocyte growth medium were dendritic in morphology. Different stages of isolation and pure culture of melanocytes from heterogeneous cell population was obtained as shown in Figure 1. Further, we confirmed the pure culture of melanocytes by L-DOPA staining, which showed positively stained melanocytes by DOPA with 80-90% confluency (Figure S1).

Table 1: Details of primers used for gene expression analysis.
to untreated NHM ($p=0.0005$, $p=0.0063$, $p=0.0001$, $p=0.0034$ and $p=0.0017$ respectively). Also, IL1RN, IL1B, TNFA and ICAM1 were found to be significantly up regulated in 10 ng/ml IL1-α treated NHM as compared to untreated NHM ($p=0.0360$, $p=0.0385$, $p=0.0007$, and $p=0.0421$ respectively). However, there was no significant difference observed for IL1A and IL6 in 10 ng/ml IL1-α treated NHM as compared to untreated NHM ($p=0.9001$ and $p=0.0841$ respectively). While, IL8 and IL1R1 transcript levels remained unaltered upon 10 ng/ml IL1-α ($p=0.6838$, $p=0.8063$ respectively) as well as 100 ng/ml IL1-α stimulation ($p=0.7091$ and $p=0.8367$ respectively; Figure 4).

**Discussion**

Melanocytes are neural crest derived cells responsible for pigmentation via producing melanin recognized by presence of melanocyte-specific proteins e.g. TYR, TYRP1, MITF which plays an important role in melanin synthesis pathway [37]. Tyrosinase catalyzes the o-hydroxylation of tyrosine (monophenol) to 3,4-dihydroxyphenylalanine (L-DOPA; diphenol) and the subsequent oxidation of DOPA to dopaquinone. Dopaquinone undergoes autocatalysis to form dopachrome [38]. MITF family has nine isoforms [39], out of which the MITF-M isoform controls tyrosinase and PKC-β transcription in melanocytes [40]. Our results showed significant decrease in MITF-M whereas no significant difference was found in TYR levels upon IL1-α stimulation. Decrease in MITF-M expression is supported by Swope et al., [41] wherein IL-1α was found to show an inhibitory effect on tyrosinase activity at a concentration of $3 \times 10^{-14}$ M on melanocytes. In addition, the MITF transcriptional activity is dependent on its phosphorylation state [42]. Therefore, the inhibitory effect of IL1-α might regulate tyrosinase function rather than its transcription. TYRP1 gene encodes a melanosomal enzyme that belongs to the tyrosinase family and plays an important role in the melanin biosynthetic pathway [43] and similar to tyrosinase there was no significant difference in the TYRP1 levels upon IL1-α stimulation in NHM (Figure 4).

Cytokines are (glyco) proteins that are synthesized and secreted by various cells, which bind to specific receptors on immune as well as non-immune cells and regulate their activation, proliferation and differentiation. Dysregulation of cytokine production or their action is thought to have a central role in the development of autoimmunity [44]. The keratinocytes synthesize cytokines, such as TNF-α, IL-1α, IL-6 which are paracrine inhibitors of melanocyte proliferation and melanogenesis [41]. The epidermal as well as systemic cytokine imbalance has been reported in vitiligo patients including higher TNF-α, IL-6, IL1-α and IL1-β levels [13,15,19,20,45]. Both IL1-α and IL1-β can self-regulate their expression, which serve as a positive-feedback loop that amplifies the IL1 response in an autocrine or paracrine manner [31]. In contrast, IL1-β produced by different cell types including tissue macrophages and skin dendritic cells was reported to activate complement components and other cytokines including IL1 itself [46]. Interestingly, in the present study both IL1-α and IL1-β was found to be significantly upregulated upon IL1-α stimulation in NHM (Figure 4).
IL1 has two receptors, designated as IL1R1 and IL1R2. The IL1R1 and IL1R2 genes are located on the arm of chromosome 2 (2q12–13) and both appear to be driven by multiple promoters [47]. Unlike the IL1-β precursor, the IL1-α precursor is fully active and functions as an "alarmin" by rapidly initiating a cascade of inflammatory cytokines and chemokines, which accounts for sterile inflammation [48,49]. IL1RN is a specific inhibitor of the activity of both IL1-α and IL1-β. IL1RN binds tightly to IL1RI and blocks the activity of either IL1-α or IL1-β. Cell activation by IL1 is believed to be mediated exclusively by IL1RI and requires IL1 receptor accessory protein for cell signaling [50,51].

On the contrary, IL1R2 has no signaling properties, and appears to act as a 'decoy receptor' [52,53]. Bellehumeur et al., [54] have reported the regulation of IL1RI, IL1R2 and IL1RN by IL1-β in human endometrial cells. Our results showed increased IL1RI membrane expression and IL1RN transcript levels upon IL1-α stimulation in NHM (Figures 3 and 4). Though, IL1RI transcript levels remain unaltered in IL1-α treated NHM. In addition, IL1-α is reported to stimulate IL1, TNF-A, and IL-6 expression in human cardiac myofibroblasts [32]. We observed upregulation of IL1RN, IL1A, IL1B, IL6, TNFA and ICAM1 and down regulation of MTF-M upon exogenous IL1-α stimulation in NHM (Figure 4), suggesting its regulatory role in melanocytes homeostasis in vitiligo.

Both TNF-α and IL1-β were reported to stimulate IL8 release from melanocytes [55], whereas in the present study we did not find any effect of IL1-α on IL8 production in NHM (Figure 4). IL8 is an important chemokine produced by monocytes, mast cells, fibroblasts, endothelial cells, dendritic cells and keratinocytes in inflammatory skin diseases. IL8 is chemotactic to neutrophils, T-lymphocytes, basophils and keratinocytes [56]. ICAM1 is important for activating T-cells and recruiting leukocytes [57]. Beyond its classically described function as an adhesion and viral entry molecule, ICAM1 on the surface of T-cells is thought to participate in signal transduction affecting several T-cell functions, including activation, proliferation, cytotoxicity, and cytokine production and thereby, plays an important role in modulating autoimmune diseases [58]. We have previously reported increased expression of ICAM1, IFNG and TNFB, along with decreased cytotoxic T-lymphocyte associated antigen-4 (CTLA4) and decreased T reg cells in vitiligo patients [15-17,59,60]. Increased IFN-γ levels in vitiligo patients lead to increased ICAMI expression which can be a probable link between cytokines and T-cell involvement in pathogenesis of vitiligo [17]. TNF-α is induced by IL1-α, IL1-β and IL-6 synthesis in gloma cells and cardiac fibroblasts [61,62]. In addition, TNF-α in the presence of IFN-γ is known to modulate IL6 and IL6 receptor in human monocytic cells [63]. Interestingly in the present study IL1-α induced IL6 expression in NHM at 100 ng/ml (Figure 4).

Previously, IL1-α was reported to be cytostatic rather than cytotoxic on NHM [41]. In agreement to this the present study showed significant decrease in cell viability of NHM with reduced growth upon exogenous IL1-α stimulation (Figure 2) suggesting the crucial role of IL1-α in melanocyte destruction in vitiligo. The blockade of IL1 by Anakinra; a recombinant IL1RN which blocks the biological activity of naturally occurring IL1 by competitively inhibiting the binding of IL1 to IL1RI, has been implicated to control various autoimmune disorders including inflammation and rheumatoid arthritis, juvenile idiopathic arthritis [64,65]. The blockade of IL1 was also associated with lower levels of C-reactive protein and IL6. Blocking of IL1-β to protect the insulin-producing beta cells has been confirmed in type 2 diabetic patients treated with a monoclonal antibody to IL1-β [66].

Overall, the results of present study along with above reports advocate the therapeutic potential of IL1-α in amelioration of vitiligo. In conclusion, IL1-α might act as an "intracellular" molecule by regulating melanogenesis and cytokines leading to reduced proliferation of melanocytes in vitiligo.

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