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Cytokine Profiling in Primary Cicatricial Alopecia: Androgenic Alopecia and Leptin Connections

Surya Suresh, Nikhila Leemon, S. Najeeb and Sreejith Parameswara Panicker*

Department of Zoology, Advanced Centre for Regenerative Medicine and Stem Cell Research in Cutaneous Biology (AcREM-Stem), University of Kerala, Kariavattom, Thiruvananthapuram – 695581, Kerala, India; p.sreejith@gmail.com, psreejith@keralauniversity.ac.in

Abstract

Primary Cicatricial Alopecias (PCA) are a group of autoimmune inflammatory disorder which cause permanent destruction of hair follicles, mainly affected by the inflammatory cells such as lymphocytes, neutrophils, or combination of these. The management of PCA has become one of the most challenging clinical issues among dermatologists. The specific treatment of any form of the PCAs is currently unknown. We aim to identify the cytokine biomarkers in each type of PCA and study cytokine signatures' (role) in alopecia's pathogenesis and therapeutic aspects. Total protein from affected individuals scalp biopsy was extracted using the total protein extraction kit. For the cytokine detection, we used RayBio® C- Series Human Cytokine Antibody Array C5 with the help of a chemiluminescence detector. A total of 42 cytokines were analyzed and found significant differences in the diseased states' ratio compared to normal and unaffected samples. The inflammatory cells and associated cytokines are essential to develop a thorough understanding of alopecia's autoimmune nature. The cytokines can be incorporated with current therapeutics for the better management of alopecia.

Keywords: Central Centrifugal Cicatricial Alopecia (CCCA), Cytokine, Folliculitis Decalvans (FD), Frontal Fibrosing Alopecia (FFA), Lichen Plano-Pilaris (LPP), Primary Cicatricial Alopecia (PCA)

1. Introduction

Cicatricial or scarring alopecias are autoimmune hair disorders, which cause permanent destruction of pilosebaceous units[1]. It includes Primary Cicatricial Alopecia (PCA) and Secondary Cicatricial Alopecia (SCA). Depending on the type of inflammatory cell reaction into the disease's active phase, PCA is classified into lymphocytic, neutrophilic, and mixed types. Lichen Plano-Pilaris (LPP), Frontal Fibrosing Alopecia (FFA), and Central Centrifugal Cicatricial Alopecia (CCCA) are the most common lymphocyte-mediated PCA. Folliculitis Decalvans (FD) is a rare progressive form of neutrophilic scarring alopecia that affects the scalp.

Cytokines and related molecules control cyclical hair growth and have a role in the pathogenesis of alopecia. They are small secreted protein functions in inflammation, hematopoiesis, and information exchange between the immune system and host tissue cells. Deregulation of cytokine production and the corresponding signaling networks are essential for the pathogenesis of autoimmune diseases. Type-1 cytokines include IL-2, IFN-γ, IL-12, and TNF-β, where type-2 cytokines include IL-4, IL-6, IL-10, and IL-13, mediate natural immunity and adaptive immunity. The mediators of innate immunity are TNF-α, IL-1, IL-10, IL-12, IFN-α, IFN-β, IFN-γ, and chemokines, and the mediators of adaptive immunity are IL-2, IL-4, IL-5, TGF-β, IL-10, and IFN-γ.

Monokines include IL-1, TNF-α, IFN-α, IFN-β, and colony-stimulating factors. Chemokines are chemotactic cytokines, which include CC chemokines that include CCL1 to CCL28. CXC chemokines contain CXCL1 to CXCL17. C chemokines contain XCL1 and XCL2. CX3C chemokines contain CX3CL1. Interferons (IFN) are signaling protein molecules that have potent roles in antiviral and immunomodulatory functions. Type 1

*Author for correspondence

includes 13 subtypes of IFN-α and IFN-β, IFN-ε, IFN-κ, and IFN-ω[2-3]. IFN- γ is the only type 2 cytokine with antiviral activities. Type 3 IFN consists of IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4.

Inter-Leukins (IL), the secreted proteins, has a role in cell signaling, especially in the immune and hemopoietic systems[4-5]. Interleukin comprises IL-1 to IL-37. IL-1 is involved in lymphocyte activation, and regulates the production of granulocyte-macrophage colony-stimulating factor. T cells are responsible for the activation of IL-2, which impacts B cell proliferation, T cell proliferation and differentiation, and activation of NK cells. Besides, it causes activation of cytotoxic lymphocytes and macrophages[6-7]. Lymphokines are protein mediators, which may influence the pathogenesis of inflammatory diseases. Granulocyte colony-stimulating factor (G-CSF), Macrophage-Colony Stimulating Factor (M-CSF), and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), the secreted glycoproteins, regulate the proliferation of granulocytes and macrophages. Recent studies revealed that GM-CSFs could develop autoimmune diseases and inflammatory diseases besides pro-inflammatory functions[8-9].

Cytokines seem to be a useful indicator of the development of autoimmune and inflammatory disorders. There is little information on the impact of cytokines on the development of alopecia. The present study, therefore, investigated the role of cytokines in the pathogenesis of PCA.

2. Materials and Methods

2.1 Human Tissue

The study group consisted of individuals >18 years of age having LPP, FFA, CCCA, and FD. The disease diagnoses were made with clinical experts' help, and 4-mm scalp biopsies were taken from three individuals of each diseased state and from an unaffected area of the diseased individual to understand the earlier disease progression. Scalp biopsy of healthy individuals was considered as control. The declaration of Helsinki principles was performed in the study, and it was reviewed and approved by the Institutional Ethics Committee (ULECRIHS/UOK/2016/1) on research involving human participants. Patients with infectious, inflammatory, or systemic diseases were excluded from this study.

2.2 Protein Isolation

Total protein from scalp biopsy was extracted using the total protein extraction kit (Qiagen) following the manufacturers' protocol, and it was estimated using BCA protein assay kit II.

2.3 Protein Array

For the cytokine detection, we used RayBio® C- Series Human Cytokine Antibody Array C5. We performed the cytokine array according to the manufacturer's instructions. Human cytokine array membranes were blocked with blocking buffer and kept for incubation at Room Temperature (RT) for 30 minutes, followed by incubation with the sample for 5 hours at RT or overnight at 4^0C. The PVDF membranes were then washed three times with wash buffer I and two times with wash buffer II at RT for 5minutes per wash and incubated with the biotinylated antibody at RT for 90 minutes. Then horseradish peroxidase-conjugated streptavidin was used for the second wash and incubated at RT for 2 hours, and the third wash was done with detection buffer for 2 minutes. For the detection of each cytokine, we used a chemiluminescence detector (BIORAD).

3. Statistical Analysis

A statistical test has been developed for protein array data to identify differentially expressed proteins relative to a fold change threshold. Fold change is the measurement of gene expression, describing changes in two sets of arrays' expression levels. The fold change threshold that must be met for a marker can be considered a positive or negative fold change set, and the fold change must be greater than or equal to zero. Statistical analyses were performed using the SPSS software.

4. Results

Our work deals with the importance of cytokines in the pathogenesis of PCA. In the cytokine profiling, the affected individuals of LPP (Table 1), FFA (Table 2), CCCA (Table 3), and FD (Table 4) showed remarkable expression levels compared with the unaffected area of the affected individual. Our study found that all the cytokines' expression levels were significantly higher in the disease states of FD than those in LPP, FFA, and CCCA. The GCSF level was increased in FD patients over

Table 1. Cytokine Profiling of LPP

Cytokine array	LPP Ratio Normal	Unaffected Vs Normal Fold Change (LPP)	Affected Vs Normal Fold Change (LPP)
ENA-78	1.00	1.40	2.80
GCSF	1.00	1.44	4.95
GM-CSF	1.00	-1.02	3.15
GRO	1.00	-1.55	1.95
GRO- α	1.00	-1.03	2.35
I-309	1.00	-1.04	1.63
IL-1 α	1.00	-1.47	1.47
IL-1 β	1.00	-1.52	1.42
IL-2	1.00	1.15	2.68
IL-3	1.00	-1.28	1.12
IL-4	1.00	1.00	1.98
IL-5	1.00	1.25	3.65
IL-6	1.00	-1.11	1.79
IL-7	1.00	1.07	2.12
IL-8	1.00	1.14	1.30
IL-10	1.00	-1.45	1.16
IL12p40p70	1.00	1.15	2.47
IL-13	1.00	-1.15	2.96
IL-15	1.00	-1.08	2.34
IFN- γ	1.00	1.53	1.74
MCP-1	1.00	-1.83	-1.17
MCP-2	1.00	-6.12	-3.91
MCP-3	1.00	-4.80	-2.17
MCSF	1.00	-1.85	-1.13
MDC	1.00	-3.20	-1.64
MIG	1.00	-1.44	1.46
MIP-1 δ	1.00	-2.34	1.20
RANTES	1.00	-3.12	-1.80
SCF	1.00	1.04	2.45
SDF-1TA	1.00	-1.52	1.47
RC	1.00	-1.33	1.49
TGF- β1	1.00	1.17	2.45
TNF- α	1.00	-1.49	1.20
TNF- β	1.00	-1.44	1.20
EGF	1.00	-1.84	-1.26
IGF-1	1.00	-1.79	-1.04
Angiogenin	1.00	-3.47	-2.64
Oncostatin-M	1.00	-1.63	-1.31
Thrombopoietin	1.00	1.28	2.24
VEGF	1.00	1.19	2.04
PDGF-BB	1.00	-2.24	-1.19
Leptin	1.00	-2.04	1.18

The cytokine Expression was compared with the normal ratio, unaffected Vs normal fold change, and affected Vs normal fold change. The area that lacks scarring alopecia of affected individuals is considered the unaffected category, which enables an understanding of earlier disease progression. Fold change is the measurement of a gene expression, describing changes in two sets of arrays' expression levels.

Table 2. Cytokine Profiling of FFA

Cytokine array	FFA Ratio Normal	Unaffected Vs Normal Fold Change (FFA)	Affected Vs Normal Fold Change (FFA)
ENA-78	1.00	3.24	5.19
GCSF	1.00	1.45	3.37
GM-CSF	1.00	2.21	3.47
GRO	1.00	2.09	2.79
GRO- α	1.00	2.42	5.17
I-309	1.00	2.08	4.10
IL-1 α	1.00	2.49	2.93
IL-1 β	1.00	2.94	3.30
IL-2	1.00	2.58	4.10
IL-3	1.00	2.53	3.35
IL-4	1.00	2.85	4.10
IL-5	1.00	2.21	4.27
IL-6	1.00	1.85	3.30
IL-7	1.00	1.60	2.97
IL-8	1.00	3.00	2.84
IL-10	1.00	2.06	2.45
IL12p40p70	1.00	2.55	4.61
IL-13	1.00	1.45	2.31
IL-15	1.00	2.31	3.05
IFN- γ	1.00	3.14	3.37
MCP-1	1.00	1.79	2.38
MCP-2	1.00	-1.50	1.94
MCP-3	1.00	-1.23	2.45
MCSF	1.00	2.07	3.25
MDC	1.00	1.53	2.40
MIG	1.00	1.67	2.55
MIP-1 δ	1.00	1.64	2.70
RANTES	1.00	-1.25	1.07
SCF	1.00	2.63	4.34
SDF-1TA	1.00	1.91	2.50
RC	1.00	2.02	2.45
TGF- β1	1.00	2.50	3.02
TNF- α	1.00	1.71	2.91
TNF- β	1.00	1.98	2.82
EGF	1.00	1.45	2.41
IGF-1	1.00	2.10	3.59
Angiogenin	1.00	-1.36	-1.17
Oncostatin-M	1.00	1.44	2.15
Thrombopoietin	1.00	2.03	3.36
VEGF	1.00	1.72	3.11
PDGF-BB	1.00	1.22	1.77
Leptin	1.00	1.56	2.15

The cytokine Expression was compared with the normal ratio, unaffected Vs normal fold change, and affected Vs normal fold change. The area that lacks scarring alopecia of affected individuals is considered the unaffected category, which enables an understanding of earlier disease progression. Fold change is the measurement of a gene expression, describing changes in two sets of arrays' expression levels.

Table 3. Cytokine Profiling of CCCA

Cytokine array	CCCA Ratio Normal	Unaffected Vs Normal Fold Change (CCCA)	Affected Vs Normal Fold Change (CCCA)
ENA-78	1.00	1.84	2.56
GCSF	1.00	1.28	1.76
GM-CSF	1.00	1.36	1.87
GRO	1.00	1.10	1.67
GRO- α	1.00	1.45	2.16
I-309	1.00	-1.04	1.51
IL-1 α	1.00	-1.21	1.16
IL-1 β	1.00	1.16	-1.03
IL-2	1.00	1.52	2.23
IL-3	1.00	1.52	2.04
IL-4	1.00	1.27	2.02
IL-5	1.00	1.78	4.09
IL-6	1.00	1.22	3.28
IL-7	1.00	1.13	3.16
IL-8	1.00	1.70	2.57
IL-10	1.00	1.25	1.66
IL12p40p70	1.00	1.43	2.17
IL-13	1.00	-1.11	1.47
IL-15	1.00	1.31	1.37
IFN- γ	1.00	1.37	1.15
MCP-1	1.00	-1.01	2.42
MCP-2	1.00	1.13	1.74
MCP-3	1.00	-1.16	1.37
MCSF	1.00	1.40	1.95
MDC	1.00	-1.06	1.89
MIG	1.00	1.20	1.69
MIP-1 δ	1.00	-1.36	1.11
RANTES	1.00	-1.82	-1.42
SCF	1.00	1.70	2.25
SDF-1TA	1.00	1.26	1.59
RC	1.00	1.02	1.15
TGF- β1	1.00	1.18	1.39
TNF- α	1.00	1.18	1.69
TNF- β	1.00	1.34	1.92
EGF	1.00	1.06	1.57
IGF-1	1.00	1.35	2.81
Angiogenin	1.00	-2.02	-1.33
Oncostatin-M	1.00	1.79	2.25
Thrombopoietin	1.00	4.33	7.25
VEGF	1.00	1.35	2.16
PDGF-BB	1.00	-1.40	1.18
Leptin	1.00	-1.15	1.23

The cytokine Expression was compared with the normal ratio, unaffected Vs normal fold change, and affected Vs normal fold change. The area that lacks scarring alopecia of affected individuals is considered the unaffected category, which enables an understanding of earlier disease progression. Fold change is the measurement of a gene expression, describing changes in two sets of arrays' expression levels.

Table 4. Cytokine Profiling of FD

Cytokine array	FD Ratio Normal	Unaffected Vs Normal Fold Change (FD)	Affected Vs Normal Fold Change (FD)
ENA-78	1.00	9.12	12.59
GCSF	1.00	16.92	22.83
GM-CSF	1.00	8.58	11.82
GRO	1.00	5.52	11.36
GRO- α	1.00	9.18	13.38
I-309	1.00	9.77	13.83
IL-1 α	1.00	6.67	9.15
IL-1 β	1.00	5.80	8.05
IL-2	1.00	9.35	12.21
IL-3	1.00	3.65	4.85
IL-4	1.00	14.28	19.73
IL-5	1.00	14.06	19.90
IL-6	1.00	11.55	17.31
IL-7	1.00	11.86	17.22
IL-8	1.00	4.08	8.84
IL-10	1.00	4.50	6.30
IL12p40p70	1.00	8.76	12.53
IL-13	1.00	11.04	15.89
IL-15	1.00	7.49	10.57
IFN- γ	1.00	6.68	9.28
MCP-1	1.00	3.57	7.16
MCP-2	1.00	2.00	3.02
MCP-3	1.00	3.45	4.53
MCSF	1.00	3.84	5.04
MDC	1.00	2.33	3.60
MIG	1.00	6.71	10.04
MIP-1 δ	1.00	4.70	7.34
RANTES	1.00	1.21	1.96
SCF	1.00	7.37	10.86
SDF-1TA	1.00	5.07	6.96
RC	1.00	5.33	7.35
TGF- β1	1.00	6.26	8.46
TNF- α	1.00	5.96	8.57
TNF- β	1.00	6.11	9.00
EGF	1.00	3.35	4.90
IGF-1	1.00	4.33	5.80
Angiogenin	1.00	-1.13	-1.08
Oncostatin-M	1.00	1.01	1.41
Thrombopoietin	1.00	7.00	10.70
VEGF	1.00	8.92	13.14
PDGF-BB	1.00	2.85	4.57
Leptin	1.00	4.38	6.41

The cytokine Expression was compared with the normal ratio, unaffected Vs normal fold change, and affected Vs normal fold change. The area that lacks scarring alopecia of affected individuals is considered the unaffected category, which enables an understanding of earlier disease progression. Fold change is the measurement of a gene expression, describing changes in two sets of arrays' expression levels.

the maximum average range value, and decreased levels were found in the CCCA patients. FD patients showed an increased level of GM-CSF, and it was reduced in the CCCA patients. The cytokine expression levels were higher in FD patients than those in LPP, FFA, and CCCA. An increased proportion of I-309 was found in FD, whereas in CCCA, it was reduced. Negative values were observed in the unaffected scalp area of the individuals of LPP and CCCA.

Higher levels of IL-1α were found in the FD patients compared to other inflammatory disorders, and negative values were obtained in the unaffected area of the individuals of LPP and CCCA. Similarly, increased IL-1β expression was observed in the FD patients and the affected individuals of CCCA, and the unaffected area of the individuals of LPP showed negative values. The expression profile of IL-2 and IL-12p40p70 was increased in the FD patients, and in CCCA patients, it was reduced. An increased proportion of IL-3 and IL-15 were found in FD patients, whereas the unaffected area of the individuals of LPP showed negative values. The IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, and IL-13 were increased in FD patients over the normal range. Also, the results showed negative values in the unaffected area of the individuals of FFA (IL-8), LPP (IL-10), and CCCA (IL-13). The expression profile of SDF-1TA, VEG-F, and thrombopoietin was increased in FD patients and reduced in LPP patients. FD patients showed increased concentrations of MIG, TNF-α, TNF-β, and negative values were obtained in the unaffected scalp area of the individuals of LPP.

The levels of SCF, RC, and TGF-β1 were higher in FD patients compared to other inflammatory disorders, and also the expression pattern of RC showed negative values in the unaffected area of the individuals of LPP. An increased proportion of EGF and IGF-1 were found in FD patients than those in LPP, FFA, and CCCA. The angiogenin showed negative values in both the affected and unaffected areas of LPP, FFA, CCCA, and FD. The levels of oncostatin-M were highly expressed in CCCA patients; also, negative values were obtained in affected and unaffected scalp areas of the individuals of LPP. In FD patients, PDGF-BB and leptin levels were increased compared with other disorders. The IFN-γ, MCP-1, MCP-2, MCP-3, MCSF, MDC, MIP-1δ, and RANTES were higher in FD patients. Among these IFN-γ, MCP-1, and MCP-3, showed negative values in the unaffected area of the individuals of CCCA, whereas MIP-1δ showed negative values in the unaffected area of the individuals of both LPP and CCCA. The RANTES exhibited negative values in the FFA individuals' unaffected area, both affected and unaffected areas of the individuals of CCCA and LPP. The expression levels of MCP-1, MCP-2, MCP-3, MCSF, and MDC showed negative values in the affected and unaffected scalp area of the individuals of LPP.

In our study, significant differences were evident for all the cytokines evaluated, all of which were increased in the affected individuals of each type of PCA. Our results evidenced that all these cytokines could impart a role in the disease progression of PCA.

5. Discussion

This is one of the pioneer studies focused on the potential role of cytokines in the pathogenesis of PCA. Cytokine regulates cyclical hair growth, and the gene expression has been reported in anagen rat hair follicles, and a decisive role for hair cycling has been proposed[10]. Studies suggest that IL-1 can induce hair loss. IL-1α and IL-1β increased with the spontaneous catagen phase during the induced murine hair cycle, and the peak very much increased during the catagen to telogen phase associated with elevated expression of the signal transduction of type IL-1 receptor[11]. The epidermis of transgenic mice has patchy hair loss resembling alopecia areata due to the over expression of IL-1α[12]. The serum levels of IL-1α and IL-4 were significantly increased in localized alopecia areata patients, while in extensive disease states, the serum levels of IL-2 and IFN-γ were elevated. This will be implying that Th1 cytokines may mediate the progression of localized to extensive disease states[13]. An elevated serum level of IL-2 is associated with alopecia areata[14].

MIG is an antiangiogenesis chemokine involved in the infiltration process of T lymphocytes into the inflamed tissues[15]. Research studies showed that the sera of chemokines such as RANTES, IL-8, and MIG were increased in alopecia areata patients[16]. Previous studies revealed that GRO-α is involved in the recruitment of antigen-specific T cells to the inflamed regions, which indicates the involvement of GRO-α in the pathogenesis of autoimmune diseases. Several studies reported decrease of serum levels of GRO-α in alopecia areata patients and explained that the GRO-α might play potent roles in inflammatory conditions. However, it does not seem to play any crucial roles in the pathogenesis of alopecia areata[17]. Nevertheless, in our study, we found elevated level of GRO-α in FD individuals.

IFN-γ was significantly expressed in alopecia areata through a $CD4^{+}$Th1 mediated response[18]. Previous studies showed that the activity of ENA-78 mediated the infiltration of pathogenic neutrophils into the sites of inflammation[19]. The signaling molecule leptin has been involved in hair loss diseases and controls hair follicle morphogenesis[20-21]. Leptin regulates the activity of hair cycle propagation and the growth of hair follicles[22-24]. It is a modifying factor in the pathological process of androgenic alopecia[25]. In our study, TGF-β1 expression was significantly higher in FD patients. Recent studies showed that TGF-β1 is a crucial factor that induces the development of catagen and androgenic alopecia[26]. TGF-β1 can be a crucial down-regulatory modulator of multiple inflammatory processes and a potent cytokine that suppresses keratinocyte hyperproliferation[27-31]. Epidermal Growth Factor (EGF) is critical for the development of skin and hair[32]. EGF is involved in the hair cycle progression and the regulation of hair morphogenesis, and when the expression of EGF occurs continuously, it leads to the production of a wavy phenotype that impedes hair growth. Signaling of growth factor IGF-1 controls the hair growth cycle and the differentiation of hair shafts[33]. Research studies suggest that due to the up regulation of PDGF-A and PDGF-B and the anti-apoptotic effects of IGF-1 promotes hair growth by the action of IGF-1[34]. However, in our data, IGF-1 expression is positively significant in FD patients. This is maybe due to the over expression of EGF and TGF-β, which subsequently abolished the growth and differentiation of a follicle in the telogen and catagen phases. The growth factor VEGF is involved in the anagen phase initiation, and it was highly expressed in FD patients maybe because of the over activity of EGF and TGF-β.

Our data showed a positively significant expression of Platelet-Derived Growth Factor (PDGF-BB) in the FD patients. PDGF-BB is known for its effect on the induction and maintenance of the anagen phase in mice[35]. Other cytokines/chemokines such as IL-7, IL-10, IL-13, IL12p40p70, I-309, GCSF, GMSF, MCSF, SCF, MCP-1, MCP-2, MCP-3, MDC, RC, TNF-β, and MIP-1δ were highly expressed in the affected individuals of folliculitis decalvans. Further studies will be required to determine how these cytokines were highly expressed in the disease states, especially in folliculitis decalvans.

6. Conclusions

The autoimmune nature of PCA can be understood through the study of cytokines. Our findings suggest that the cytokine level was differentially changed in the patients with PCA. It indicates that cytokines may play a role in the pathogenesis of alopecia. In this study, we assume that the control of cytokine production may be essential for managing this disease, and it may provide new therapeutic strategies for patients with PCA.

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