Expression of macrophage activation-specific factors in hyperplastic scar tissue during hyperplasia phase by antibody array blotting membrane assay and its clinical significance

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Abstract. The expression of macrophage activation-specific factors in hyperplastic scar (HS) tissues during hyperplasia phase was detected by antibody array imprinted membrane method and the role of macrophage activation in the natural evolution of HS was explored. A total of 83 patients with HS admitted to the Affiliated Hospital of Beihua University (Jilin, China) between February 2021 and July 2021 were enrolled. The clinical data of the patients were retrospectively analyzed. These patients were divided into the hyperplasia HS group (n=26) and the decline HS group (the HS tissues ceased to grow and were in regression periods; n=57) according to the time of scar formation and clinical characteristics. The HS tissues were collected from patients in both groups. The contents of IL-12, IL-10, VEGF and basic fibroblast growth factor (bFGF) were detected by antibody array imprinted membrane method and the contents of IL-12, IL-10, VEGF and bFGF in tissues with various groups of tissues and clinical features were compared. The connection between macrophage activation-specific factors with VEGF and bFGF was analyzed using Pearson correlation analysis. The contents of IL-10 (9.48±1.06), VEGF (24.15±2.64) and bFGF (37.48±2.56) were much lower and IL-12 levels (16.45±0.85) were strongly higher in hyperplasia HS group compared with those in the decline HS group (14.56±1.26 for IL-10, 27.85±2.63 for VEGF, 43.15±3.16 for bFGF and 10.46±0.75 for IL-12, P<0.001). In the hyperplasia HS group, the contents of IL-10, VEGF and bFGF were obviously higher and the IL-12 levels were markedly lower in patients with age ≥30 years, protuberance height <2 mm, soft flexibility, low hyperemia degree and no concomitant symptoms than those in the patients with age <30 years, protuberance height ≥2 mm, hard flexibility, high hyperemia degree and concomitant symptoms (P<0.001). Pearson correlation analysis showed that IL-12 was negatively correlated with VEGF and bFGF (r=-0.328, 0.600, P<0.01). IL-10 was positively correlated with VEGF and bFGF (r=0.486, 0.684, respectively, P<0.001). In conclusion, macrophage activation-specific factors were abnormally expressed in hyperplasia HS, mainly M1 macrophages, accompanied by severe inflammatory reaction. The transformation of M1 macrophage into M2 macrophage usually occurred during the declining HS phase, which accelerated scar formation by promoting the formation of fibroblasts and angiogenesis. Detection of macrophage activation-specific factors may contribute to evaluate the clinical stage of HS.

Introduction

Hypertrophic scar (HS) is a pathological scar that continues to proliferate after local epithelization of wounds and is a common clinical manifestation of excessive tissue repair after burns, wounds or lesions. The clinical manifestations are often pruritus and pain, which can lead to local deformity or dysfunction, seriously affecting the normal work and daily life of patients (1,2). At present, the etiology of HS is still unclear, but may be related to skin lesions, inflammatory reaction at the trauma site and gene mutation. Inflammatory response is recognized as the initial stage of wound healing. Excessive inflammatory reaction can induce abnormal accumulation of extracellular matrix (ECM), resulting in the formation of HS (3). IL-1β produced by scar cell activation is a key step in immune inflammatory response after trauma. IL-1 β not only directly affects the human body by activating lymphocytes and inflammatory factors, but also regulates the body's autoimmune system by directly stimulating the production of abundant inflammatory factors and affects the wound surface through autoimmune response (4).

Macrophages play an important role in body's inflammatory response. Macrophage activation and its different activation subtypes affect the function of macrophages and wound healing (5,6). Macrophages include M1 and M2 subtypes. M1 macrophages exert a proinflammatory role in secreting pro-inflammatory factors such as IL-12. M2 macrophages play an anti-inflammatory role by secreting some anti-inflammatory factors such as IL-10 (7). Inflammatory response usually exists in HS tissue for a long time and the activation classification of

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macrophages has been implicated in the formation and clinical staging of HS (8). Macrophage activation is an important way to promote scar healing in hyperplasia stage and the content of IL-1β may increase during injury or scar maturation, thereby stimulating wound formation and accelerating the process of wound healing (9). Therefore, exploring the inflammatory factors that promote the formation of hyperplasia phase is of great significance for correctly understanding the characteristics of macrophages in scars and treating the disease (10). The antibody array imprinted membrane method is a determination based on the membrane method and consisting of a pre-spotted membrane with capture antibodies specific to multiple proteins. The sample is spotted onto a membrane and the protein bound to the capture antibody is detected with a second set of labeled antibodies, a method that simultaneously detects the expression of dozens of inflammation-associated factors (10). It is well known that HS tissue has a long-term inflammatory response and there are obvious hypertrophic phases, hypotropic phases and mature phases in clinical manifestations (11). However, whether the process of macrophage activation is involved in and affects the formation and clinical outcome of HS remains unclear.

In the present study, a total of 83 patients with HS admitted to the Affiliated Hospital of Beihua University (Jilin, China) between February 2021 and July 2021 were selected. The current study aimed to analyze the expression of macrophage activation-specific factors in HS tissues during hyperplasia phase by the antibody array imprinted membrane method and to explore the clinical significance of HS. In this way, a possible relationship between HS and scarring was revealed, which provided a new direction for the study of HS mechanism based on macrophages.

Materials and methods

General materials. A total of 118 patients with HS admitted to the Affiliated Hospital of Beihua University (Jilin, China) between February 2021 and July 2021 were selected. The clinical data of the patients were retrospectively analyzed and, according to the inclusion and exclusion criteria (12), 83 patients with HS were finally included as the study subjects. Inclusion criteria: i) All patients had burns and the healing time was within 12 months; ii) it was the first visit after injury for the patient, who had not received relevant drug treatment before enrollment; iii) all patients exhibited by the clinical manifestation of red cicatricial protuberance, pain and itching; and iv) the patients and their families all signed the informed consent and could cooperate with the examination and treatment with good compliance. Exclusion criteria were as follows: i) Patients with important organ dysfunction; ii) patients during lactation or pregnancy; and iii) the patient condition was complicated with infectious diseases or other serious diseases. According to the description of color, thickness, vascular distribution and softness in the Vancouver Scar Assessment Scale (13), combined with the specific time of scar formation and the clinical symptoms of scarring, these patients were divided into the hyperplasia HS group (n=26) and decline HS group (the HS tissues ceased to grow and were in regression periods; n=57). Patients in the hyperplasia HS group were with color (3 points), thickness (2-3 points), vascular distribution (3 points) and softness (2-3 points), while patients in the decline HS group were with color (2 points), thickness (2-3 points), vascular distribution (2 points) and softness (2-3 points). The hyperplasia HS group consisted of 14 males and 12 females, with an average age of 33.16±3.56 years and scar formation time of 86±11 days. The decline HS group was comprised 33 males and 24 females, with an average age of 33.38±6.12 years and a time of scar formation of 192±14 days. The age and sex had no significant difference between groups (P>0.05), while the time of scar formation in the decline HS group was markedly longer than that in the hyperplasia HS group (P<0.05). All procedures performed in studies were in accordance with the ethical standards of the ethics committee of the Affiliated Hospital of Beihua University (approval no. 2021016). The selection process of general data is shown in Fig. 1.

Table I. Quantitative comparison of IL-10 and IL-12 in tissues (mean ± standard deviation).

Groups	Cases, n	IL-10 (pg/ml)	IL-12 (pg/ml)
Hyperplasia HS group	26	9.48±1.06	16.45±0.85
Decline HS group	57	14.56±1.26	10.46±0.75
t		17.861	32.358
P-value		<0.001	<0.001

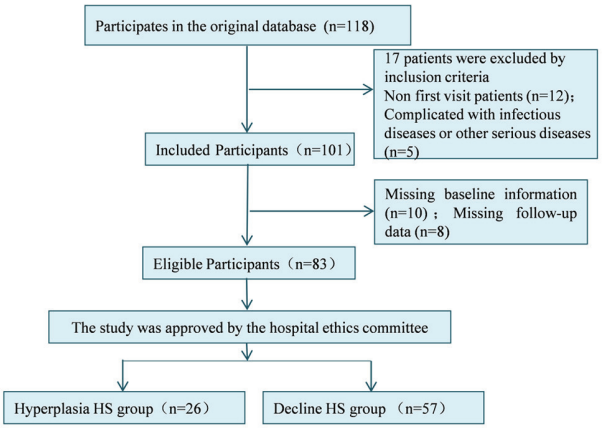

Figure 1. The selection process of general data.

Methods. Protein extraction: The HS tissue samples were placed in an ice box and then were cut into 2x2-mm pieces using ophthalmic scissors. Subsequently, 0.5 g of protein sample was added to 1 ml PBS solution and the mixture was placed in a grinding tube. Then three grinding balls was put into the grinding tube and the mixture was ground for 60 sex with a homogenizer at a speed of 8 m/s. After grinding, the tissue homogenate was centrifuged for 20 min at the speed of 10,000 x g at 4˚C. The supernatant was collected and frozen at 80˚C. The original standard protein sample in BCA kit was diluted to prepare a standard protein solution with the concentration of 1.0 mg/ml. Standard gradient protein samples with concentrations of 0, 1, 5, 10 and 20 mg/ml were prepared. The protein samples obtained in the aforementioned steps were aliquoted into EP tubes, preferably double-welled, 1 μl sample and 19 μl water were added to the sample wells, for

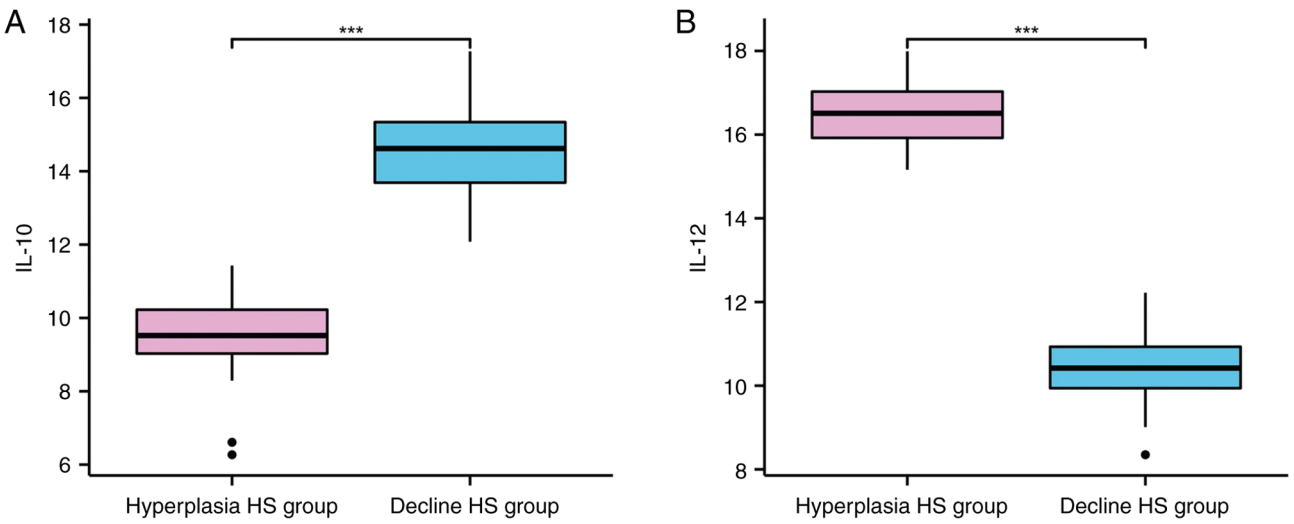

Figure 2. Quantitative comparison of IL-10 and IL-12 in tissues. Quantitative comparison of (A) IL-10 and (B) IL-12 between groups. ***P<0.001. HS, hyperplastic scar.

a 20-fold dilution. The standard gradient protein sample and the protein sample to be tested were added to a 96-well plate and the BCA working solution added from the kit (cat. no. PA115-01; Tiangen Biotech Co., Ltd.). After standing at room temperature for 10 min, the 96-well plate was placed on the microplate reader and the optical density (OD) of all samples were measured at 595 m. The outer diameter of each well was recorded. The standard curve was drawn with the protein content of the standard as the abscissa and the OD value as the ordinate. The OD value of the sample was substituted into the standard curve to obtain the corresponding protein content. Finally, the actual total protein concentration of the sample was calculated according to the specific dilution ratio.

The levels of IL-12, IL-10, VEGF and basic fibroblast growth factor (bFGF) were detected using antibody array blotting membrane method. The specific steps were as follows: The appropriate concentration range of total protein in the sample was determined according to the instructions of the kit (cat. no. SHC-9823; Shanghai Chunmai Biotechnology Co., Ltd.) and the total protein amount of sample measured by the kit was adjusted according to the total protein concentration of different samples measured above to make it consistent. Then, the antibody array imprint membrane (cat. no. AA0135; Wuhan Aimejie Technology Co., Ltd.) was treated with pretreated solution and was then incubated with samples in a shaker at 4˚C for 12 h. The sample was washed for three times using cleaning solution for at least 5 min each time. Then, the poly-HRP-conjugated goat anti-human antibody (cat. no. GAHHRP-050; Guangdong Gukang Biotechnology Co., Ltd.) was added and incubated at room temperature for 1 h. After washing with cleaning solution three times, the special fluorescent developer (cat. no. G9590; Shanghai Shengqizhao Biotechnology Co., Ltd.) was added and cultured at 20-25˚C. Finally, the termination solution was added. The imprint film was put under the Odyssey infrared laser imaging system (Core Qidian Gene Technology (Beijing) Co., Ltd.) for detection. The value of the standard reference point on the antibody array imprint membrane and the value of the reaction point of the specific factor of each group of reactions were measured using the image analysis software provided with the imaging system. This value was compared with the average value of the standard reference point value to obtain the final quantitative value of the factor expression. The final data was recorded. IL-10 and IL-12 kits were purchased from Beijing BioDee Biotechnology Co., Ltd. VEGF and bFGF kits were purchased from Beijing Baiolaibo Technology Co., Ltd.

Outcome measures. The expression levels of IL-10, IL-12, VEGF and bFGF were compared among the groups. The patients' age, height of hypertrophic protuberance, flexibility, degree of hyperemia and concomitant symptoms were collected. The relationship between the expression levels of IL-10, IL-12, VEGF, bFGF and the clinical symptoms of hypertrophic scar was analyzed.

Statistical analysis. SPSS 20.0 software (IBM Corp.) was used to analyze the experimental data. Measurement data such as the age, IL-10, IL-12 were shown as the mean ± standard deviation and compared using an independent-samples t-test between groups. Enumeration data such as the sex were shown as percentage and compared using χ^2 test. Pearson correlation analysis was used to analyze the correlation between macrophage activation specific factor, VEGF and bFGF. P<0.05 was considered to indicate a statistically significant difference.

Results

Quantitative comparison of IL-10 and IL-12 in tissues. The content of IL-10 was lower and IL-12 levels were higher in hyperplasia HS group compared with those in the decline HS group (P<0.001; Table I and Fig. 2).

Comparison of quantitative levels of IL-10 and IL-12 in HS tissues of patients with different clinical characteristics in hyperplastic stage. In the hyperplasia HS group, the content of IL-10 was higher and the content of IL-12 was lower in patients aged ≥30 years, with protuberance height <2 mm, soft

Table II. Comparison of quantitative levels of IL-10 and IL-12 in HS tissues with different clinical characteristics during hyperplastic stage (mean ± standard deviation).

Indicator	Cases, n	IL-10 (pg/ml)	*t*	P-value	IL-12 (pg/ml)	*t*	P-value
Age, years			49.036	<0.001		46.649	<0.001
<30	13	6.23±0.34			19.52±0.45		
≥30	13	11.89±0.24			12.45±0.31		
Protuberance height, mm			65.519	<0.001		66.046	<0.001
<2	19	11.76±0.16			11.95±0.24		
≥2	7	6.45±0.24			18.75±0.21		
Flexibility			54.948	<0.001		51.258	<0.001
Hard	16	6.37±0.21			18.79±0.34		
Soft	10	11.56±0.27			12.46±0.24		
Hyperemia degree			56.096	<0.001		61.161	<0.001
Low	12	10.99±0.24			12.07±0.31		
High	14	6.85±0.12			18.67±0.24		
Concomitant symptoms			45.843	<0.001		121.639	<0.001
Yes	18	6.96±0.20			19.24±0.12		
No	8	11.23±0.26			12.37±0.16		

Table III. Quantitative comparison of VEGF and bFGF in tissues (mean ± standard deviation).

Groups	Cases, n	VEGF (pg/ml)	bFGF (pg/ml)
Hyperplasia HS group	26	24.15±2.64	37.48±2.56
Decline HS group	57	27.85±2.63	43.15±3.16
t		5.938	8.019
P-value		<0.001	<0.001

bFGF, basic fibroblast growth factor.

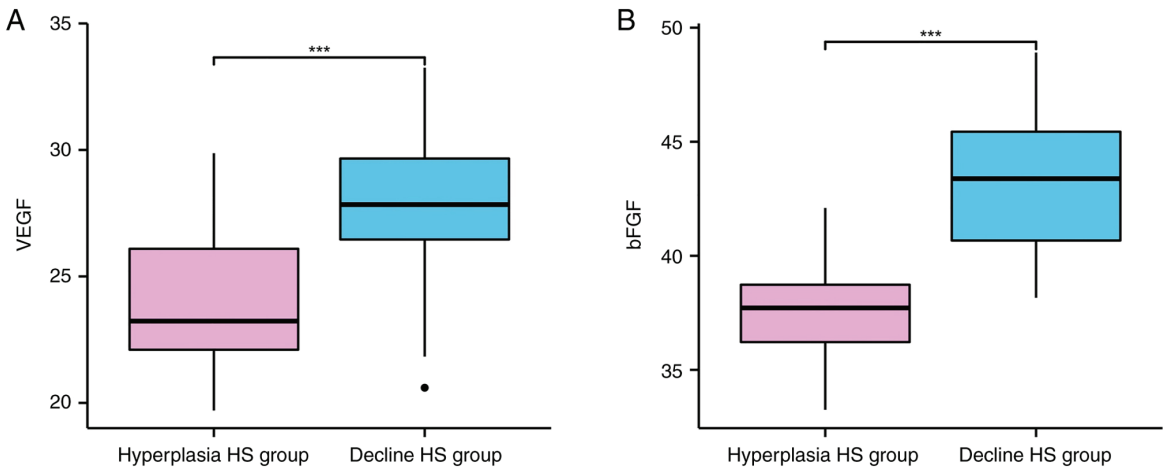

Figure 3. Quantitative comparison of VEGF and bFGF in tissues. Quantitative comparison of (A) VEGF and (B) bFGF between groups. ***P<0.001. bFGF, basic fibroblast growth factor; HS, hyperplastic scar.

flexibility, low hyperemia degree and no concomitant symptoms compared with the patients aged <30 years, protuberance height ≥2 mm, hard flexibility, high hyperemia degree and concomitant symptoms (P<0.001; Table II).

Quantitative comparison of VEGF and bFGF in tissues. The content of VEGF and bFGF was markedly lower in hyperplasia HS group compared with those in the decline HS group (P<0.001; Table III and Fig. 3).

Table IV. Comparison of quantitative levels of VEGF and bFGF in HS tissues with different clinical characteristics during hyperplastic stage (mean ± standard deviation).

Indicator	Cases, n	VEGF (pg/ml)	*t*	P-value	bFGF (pg/ml)	*t*	P-value
Age, years			49.036	<0.001		46.649	<0.001
<30	13	25.15±1.45			40.15±1.06		
≥30	13	31.26±2.15			48.52±0.39		
Protuberance height, mm			12.294	<0.001		18.006	<0.001
<2	19	30.59±1.24			48.75±1.06		
≥2	7	24.38±0.78			40.37±1.03		
Flexibility			12.727	<0.001		21.074	<0.001
Hard	16	24.89±1.35			41.06±1.34		
Soft	10	31.25±1.05			49.27±0.85		
Hyperemia degree			13.946	<0.001		21.326	<0.001
Low	12	31.04±1.28			49.85±0.48		
High	14	24.75±1.02			41.08±1.35		
Concomitant symptoms			15.810	<0.001		13.204	<0.001
Yes	18	23.59±1.34			41.67±1.06		
No	8	32.07±1.05			48.33±1.45		

bFGF, basic fibroblast growth factor.

Comparison of quantitative levels of VEGF and b FGF in HS tissues of patients with different clinical characteristics in hyperplastic stage. In the hyperplasia HS group, the content of VEGF and bFGF were higher in patients aged ≥30 years, with protuberance height <2 mm, soft flexibility, low hyperemia degree and no concomitant symptoms compared with the patients aged <30 years, with protuberance height ≥2 mm, hard flexibility, high hyperemia degree and concomitant symptoms (P<0.001; Table IV).

Correlation analysis of macrophage activation specific factor with VEGF and bFGF. Pearson correlation analysis showed that IL-12 was negatively correlated with VEGF and bFGF (r=-0.328, 0.600, P<0.01). IL-10 was positively correlated with VEGF and bFGF (r=0.481, 0.684, respectively; P<0.001; Table V and Fig. 4).

Table V. Correlation analysis of macrophage activation specific factor with VEGF and bFGF.

	VEGF		bFGF	
Indicator	*r*	P-value	*r*	P-value
IL-10	0.328	0.003	0.600	<0.001
IL-12	-0.481	<0.001	-0.684	<0.001

bFGF, basic fibroblast growth factor.

Discussion

Scaring is the natural repair reaction of skin during wound healing and pathological scar is the abnormal hyperplasia of scar, of which HS accounts for 91.40% of pathological scars. HS can be accompanied by pain, itching and other symptoms and not only affects the appearance and aesthetics, but also can lead to dysfunction and affect the quality of life of patients (14). Early evaluation of the pathological process of HS and targeted intervention can help to improve the symptoms and prognosis of patients. HS in hyperplastic stage is characterized by hyperplasia of the dermis or subcutaneous tissue, with a high degree of fibrosis. The dermis is gray-white or gray-black in color and there are obvious hyperplastic cells in the dermis fiber bundle (cicatricial multiple tendinous scar) (15). HS tissue in the hyperplastic stage can also occur on any part of the body surface, such as the scar area of the wound after burns or the scar area after skin grafting (16). During the formation of HS in hyperplastic phase, two key factors are particularly important. One is the accumulation of inflammatory mediators in scar tissue and the other is the thickening of scar caused by the proliferation of fibroblasts in dermis. Local capillary congestion and collagen metabolism can lead to wound inflammation and the proliferation of microorganisms such as bacteria, fungi and other microorganisms, resulting in the killing of abundant cells around the wound. At the same time, the above toxic substances could not be absorbed by the surrounding tissues and organs in time, thus causing inflammatory reaction and inducing HS (17). Therefore, the occurrence and development of HS are closely related to inflammatory response.

Previous studies demonstrated that the lesion tissue may have a relatively severe inflammatory reaction when dermal tissue is seriously injured (18,19). In order to neutralize the inflammatory response, the tissue may evoke excessive accumulation of ECM, ultimately leading to the occurrence of HS. IL-10 and IL-12 are specific factors for macrophage activation, whose variant content reflect the changes in the proportion of macrophage activation

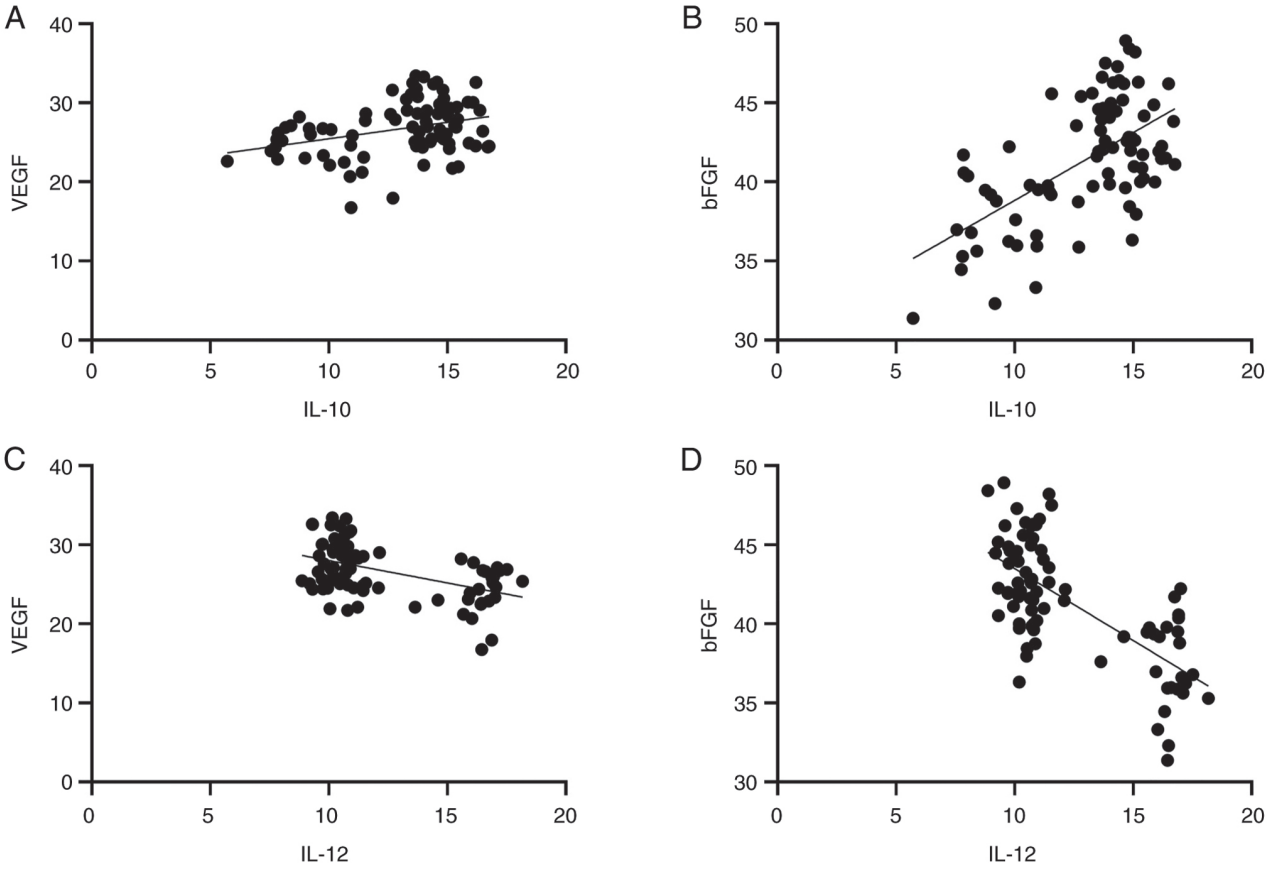

Figure 4. Correlation analysis of macrophage activation specific factor with VEGF and bFGF. (A) Correlation between IL-10 and VEGF, (B) between IL-10 and bFGF (C) between IL-12 and VEGF and (D) between IL-12 and bFGF. bFGF, basic fibroblast growth factor.

types (18,19). M2 macrophages secrete anti-inflammatory mediator IL-10, which inhibits the production of pro-inflammatory factors, blocks inflammatory responses, alleviates inflammatory responses in lesions, and promotes the remodeling and repair of local wound tissues (20,21). M1 macrophages secrete the pro-inflammatory mediator IL-12, which exacerbates the wound inflammatory response and removes tissue pathogens, necrotic cells and tissue debris by activating a variety of inflammatory factors (22,23). The pathological process of HS tissue is closely related to the transformation of M1 macrophages into M2 macrophages. In the present study, the content of IL-10 was lower and IL-12 levels were higher in the hyperplasia HS group compared with those in the decline HS group. Furthermore, in the hyperplasia HS group, the content of IL-10 was higher and the content of IL-12 was lower in patients aged ≥30 years, with protuberance height <2 mm, soft flexibility, low hyperemia degree and no concomitant symptoms. It was hypothesized that the proliferative scar in the clinical stage might have completed the transformation process from M1 macrophages to M2 macrophages, which was consistent with the different inflammatory clinical manifestations of HS hyperplasia and hypoproliferative stages. Thus, monitoring the changes of IL-10 and IL-12 levels can help to evaluate the pathological process of HS.

VEGF is one of the common pro-angiogenic factors in clinical research, which can promote vascular proliferation and facilitate wound healing (24). Activation of VEGF will stimulate endothelial cells to release more angiogenic factors and cytokines, thus promoting wound healing. Neonatal vascular endothelial cells are composed of numerous endothelial cells that can proliferate continuously during cell division. VEGF, which appears during the proliferation of new endothelial cells, interacts with, and activates, inflammatory cytokines *in vivo* to produce inflammatory factors, such as autoantibodies, immunoglobulin A and its receptors (25). Early studies confirmed that abnormal elevation of VEGF in HS tissue might participate in the excessive proliferation of vascular endothelium in the lesion tissue (5,26). bFGF is composed of polypeptide complexes connected by bases or peptides and can stimulate the proliferation and migration of fibroblasts, promote collagen formation and accelerate the degradation of basement membrane and ECM. In addition, bFGF regulates the production and secretion of various cytokines, thereby regulating growth, immunity and local tissue differentiation (26). In the present study, the levels of VEGF and bFGF in the hyperplastic HS group were lower than those in the decline HS group and the levels of VEGF and bFGF were closely related to various clinical features, indicating that VEGF and bFGF may be involved in the occurrence and development of HS. Pearson correlation analysis confirmed that IL-12 was negatively correlated with VEGF and bFGF and IL-10 were positively correlated with VEGF and bFGF. Therefore, the present study hypothesized that HS tissues in the proliferative stage were mostly activated M1 macrophages. When M1 gradually transformed into M2, IL-10 promoted the formation of fibroblasts and angiogenesis by regulating the expression of VEGF and bFGF.

In general, macrophage activation-specific factors were abnormally expressed in hyperplasia HS, most of which were assumed to be M1 macrophages, accompanied by severe inflammatory reaction. The transformation of M1 macrophages into M2 macrophages might occur during the decline HS phase, which accelerates scar formation by promoting the formation of fibroblasts and angiogenesis. Detection of macrophage activation-specific factors could help to evaluate the clinical stage of HS. How to inhibit the continuous activation of M1 macrophages to make the activation of M2 macrophages dominate, so as to make it closer to the temporal transition of M1 and M2 macrophage activation in the process of proliferative scarring after wound healing, may become a new direction for the study on the mechanism of hypertrophic scaring based on macrophages.

The formation of HS is a complex multi-factor process. Based on the current understanding of the mechanism and development process of scar neovascularization, the present study found that macrophage activation-specific factors in HS may be a potential treatment, prognosis and prediction target, which can provide a new method and approach for the prevention and treatment of HS. In the present study, the expression level of macrophage activation-specific factors was determined and analyzed only for clinical surgical removal of scar tissue, but it remains to further verify the correlation between macrophage activation-specific factors and scar development by using rabbit ear and rat tail wound models and to explore and verify the mechanism of action.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and XZ conducted experiments, confirm the authenticity of all the raw data and edited the manuscript. AY designed the study, collected and processed the data. YZ and AY conducted the statistical analysis. XZ reviewed and revised the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All procedures performed in studies were in accordance with the ethical standards of the ethics committee of the Affiliated Hospital of Beihua University (approval no. 2021016). Informed consent was obtained from all individual participants included in the present study. The participating patients all agreed to the publication of the research results.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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