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Keloid Nodule Metabolic Activity for Continuous Expansion

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Background: We previously reported that keloid nodules had such specific structures that higher expression of autophagy proteins and glycolytic markers was observed in the central zone fibroblasts than in marginal zone fibroblasts. The purpose of this study is to investigate how keloid nodules play a role in metabolic activity for continuous expansion.

Methods: A total of 57 nodules were randomly chosen from seven keloid samples and divided into four groups of disease duration (2, 4, 6, and 17 years). Immunohistochemical and immunofluorescent analyses were performed.

Results: Immunohistochemical analysis with anti-CD-31 confirmed that the nodules had a structure with a greater number of vessels in the marginal zone than in the central zone. The density of fibroblasts in nodules (190.29±64.45) was significantly higher than that of surrounding connective tissue (140.18±63.94) ($P < 0.05$). The area of each nodule increased for 2 to 4 years, then decreased, graphically represented by an approximately horizontal line, to 17 years. The ratio of total nodule area/dermis area increased as disease duration lengthened. The maximum ratio was the 17-year group at 48.01%. The nodule number/dermis area ratio rose approximately with advancing disease duration.

Conclusions: Instead of increasing the size of the nodules, their number and total area increased within the keloid lesions. We believe that the keloid nodules must play an important role in energy metabolic activity for continuous growth by increasing in number and total area. *(Plast Reconstr Surg Glob Open 2022;10:e4492; doi: 10.1097/GOX.0000000000004492; Published online 24 August 2022.)*

INTRODUCTION

We have continued research to investigate how keloid tissue obtains energy for continuous growth and expansion because the mechanism has not been known very well, and it has been previously reported that keloid tissue exhibited high adenosine triphosphate (ATP) levels even after around 10 years[1] and had high lactate levels, perhaps due to anaerobic glycolysis caused by hypoxia-related blood vessel flattening and crushing specifically in the central zone (CZ) of the keloid.[2,3] Another study of ours showed that keloids have distinct central hypoxic and marginal normoxic zones based on expression of CD31.[4]

We considered whether keloid nodules played an important role in obtaining energy for metabolism and found that the CZ of the nodule had few blood vessels, and the surrounding marginal zone (MZ) had a circular layer of collagen bundles rich in blood vessels.[5] Higher expression of autophagy proteins and glycolytic markers was observed in fibroblasts in the central hypoxic zone of keloid nodules than in MZ fibroblasts.[5]

The histological nodular structures were traditionally considered as characteristic of hypertrophic scars and to be absent from keloids.[6] However, many researchers reported that the keloids were histologically characterized by the constant presence of abnormally thick, hyalinized collagen fibers and collagenous cellular nodules.[7,8,9] Chong et al[10] reported that whirling hypercellular fibrous nodules (WHFNs) were composed of whirling collagen bundles with numerous activated young fibroblasts, so

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that these might serve as a proliferating center of keloid collagen structure. We think that keloid nodules with such specific structures play an important role in maintaining the energy metabolism of keloids and have thought that to understand the changes in keloid nodules may result in the discovery of a new therapy method.

What course will the keloid nodule follow if the keloids continue expansion and proliferation gradually for a long duration? Will the sizes and numbers of the nodules increase? The purpose of this study is to investigate the course through which the keloid nodules proceed in the long term.

Takeaways

Question: The purpose of this study is to investigate how keloid nodules with specific structures develop through the disease duration.

Findings: The density of fibroblasts in nodules was significantly higher than that of surrounding connective tissue. The area of each nodule increased for 2 to 4 years, then decreased, graphically represented by an approximately horizontal line, to 17 years. The ratio of total nodule area/dermis area increased as disease duration lengthened. The nodule number/dermis area ratio rose approximately with advancing disease duration.

Meaning: Keloid nodules with specific structures are thought to play an important role in energy metabolic activity for continuous growth and expansion.

MATERIALS AND METHODS

Patient Samples

Keloid tissues were obtained from patients following surgery at the Department of Plastic Surgery, Osaka Medical and Pharmaceutical University (Takatsuki, Osaka, Japan) from 2007 to 2020. All samples were obtained after receiving written informed consent from the patients. This study was reviewed and approved by the institutional review board of Osaka Medical and Pharmaceutical University (acceptance number: 1892), following the tenets of the Declaration of Helsinki. The details regarding patient clinical features are shown in Table 1.

Histopathology

The specimens were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm sections for pathologic and immunohistochemical, and immunofluorescent analyses. Histopathological assessment was based on hematoxylin and eosin (H&E) staining done by an independent pathologist.

Identification of Keloid Nodule

Based on H&E staining, the nodule was defined as a central hypoxic zone with few blood vessels and surrounded by a circular layer of collage bundles rich in blood vessels.[5] We confirmed nodules via CD31 immunohistochemical staining as outlined below according to our previous study.[5]

Immunohistochemical and Immunofluorescent Analyses

Immunohistochemical and immunofluorescent analyses of the keloid samples were performed using paraffin-embedded sections as described above. After deparaffinization and antigen retrieval with 10 mM sodium citrate (pH 6.0), samples were stained with the following primary antibodies: anti-CD31, rabbit polyclonal antibody (1:40; Thermo Scientific, Waltham, Mass.), anti-proliferating cell nuclear antigen (PCNA), and mouse monoclonal antibody (1:500; Proteintech Group, Inc. Rosemont, Ill.). After washing, the samples for immunohistochemical analysis were incubated with a reagent containing goat antirabbit and antimouse immunoglobulins conjugated to peroxidase-labeled polymer (EnVision + System HRP kit, Dako, Tokyo, Japan). After washing, the sections were examined after incubation using ImmPACT DAB Peroxidase Substrate (Vector, Burlingame, Calif.). Counterstaining was performed with hematoxylin. For immunofluorescent analysis, antivimentin, goat polyclonal antibody (1:40; Sigma-Aldrich, St. Louis, Mo.) was used to define fibroblast. Then, samples were incubated with a secondary antibody, Alexa Fluor 594 chicken anti-goat (1:250; Life Technologies, Inc., Rockville, Mo.) and mounted in Vectashield (Vector Lab, Inc. Burlingame, CA 94010) supplemented with 4', 6-diamidino-2-phenylindole (DAPI) to counterstain nuclei.

Measurement Procedure and Selection of Nodules

For analyses depending on the area size of keloid, a total of 57 nodules were randomly chosen from seven keloid samples with previous identification and divided into four groups of disease duration (2, 4, 6, and 17 years). To compare the density of fibroblasts in the nodules with that of the dermis, we selected 17 nodules from the 17-year group. Fibroblasts that showed colocalization with vimentin and DAPI were counted in areas of $367.81 \times 275.86\ \mu m^2$ (× 200; fields under fluorescent microscopy) consisting of keloids and their surrounding connective tissue. Using

Table 1. The Details Regarding Patient Clinical Features

Keloid No.	Age/Sex	Region	Operation History	Disease Duration	Disease Duration Group
1	30 F	Abdomen	Cesarean section	2 y 2 mo	2 y
2	66 F	Epigastrium	Cesarean section	4 y 8 mo	4 y
3		Hypogastrium			
4	63 F	Left shoulder	Localized scleroderma	3 y 6 mo	4 y
5	53 F	Abdomen	Uterine myoma	3 y 2 mo	4 y
6	59 F	Umbilical region	Endoscopic surgery for colorectal cancer	5 y 6 mo	6 y
7	78 F	Abdomen	Chokecytectomy	17 y	17 y

Adobe Photoshop, images of nodules or fibroblasts were captured and saved for computer analysis. We used ImageJ software (NIH; National Institutes of Health, Bethesda, Mass.) for quantitative area analysis of nodules or dermis in keloid sections by setting "threshold," as we previously reported.[5]

Statistical Analysis

Data analysis and graphing were performed using StatMate III software (Graphpad Holdings LLC; Sacramento, Calif.) and GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, Calif.). The Student *t* test was used to determine continuous variables between groups. A value of *P* less than 0.05 was considered statistically significant. We interpolated straight line approximation via Prism software.

RESULTS

Detection and Histological Characterization of Nodules

Based on H&E staining of keloids (Fig. 1A), thick hyaline collagen fibers with serious eosinophilia were identified at the dermis layer of the keloid skin layer (marked by asterisks). Immunohistochemical analysis with anti-CD31 (an endothelial blood vessel marker) confirmed the nodule (asterisk in Fig. 1B) in the keloid as a structure, as described in our previous paper, and observed numerous serial vessels in the MZ with a wider lumen than vessels in the CZ (boxed area in Fig. 1B-1). We found a moderate number of nodules in which marginal blood vessels penetrated into the CZ and separated the nodule into two nodules (Fig. 1B-2). A broad distribution of proliferating cell marker (anti-PCNA) positive cells was demonstrated in nodules (Fig. 1C), and strong expression of anti-PCNA was shown in blood vessels of MZ (enlarged area of Fig. 1C). The density of fibroblasts that expressed antivimentin (fibroblast marker) and DAPI (nucleus) in nodules (190.29±64.45) was significantly higher than that of surrounding connective tissue (140.18±63.94) ($P < 0.05$). [**See figure, Supplemental Digital Content 1,** which displays distribution and density of fibroblasts. The cytoplasm of the fibroblast was positive to vimentin (red) and the nucleus was positive to DAPI (blue). Fibroblasts in the nodule (A) and their surrounding connective tissue (B) are indicated by merged images (arrows). Statistical analysis of the density of fibroblasts (C) demonstrated that the relative density of fibroblasts in nodules was significantly higher than that of their surrounding connective tissue ($P < 0.05$). **http://links.lww.com/PRSGO/C147**.]

Statistical Evaluation of Nodules

The area of each nodule of the duration groups increased for the 2- to 4-year groups, then decreased, displaying an approximately horizontal line on the graph, up to the 17-year group (Fig. 2). The ratio of total nodule area/dermis area of a keloid section elevated according to increase of disease duration (Fig. 3). The maximum ratio, 48.01%, was in the 17-year group. The nodule number/

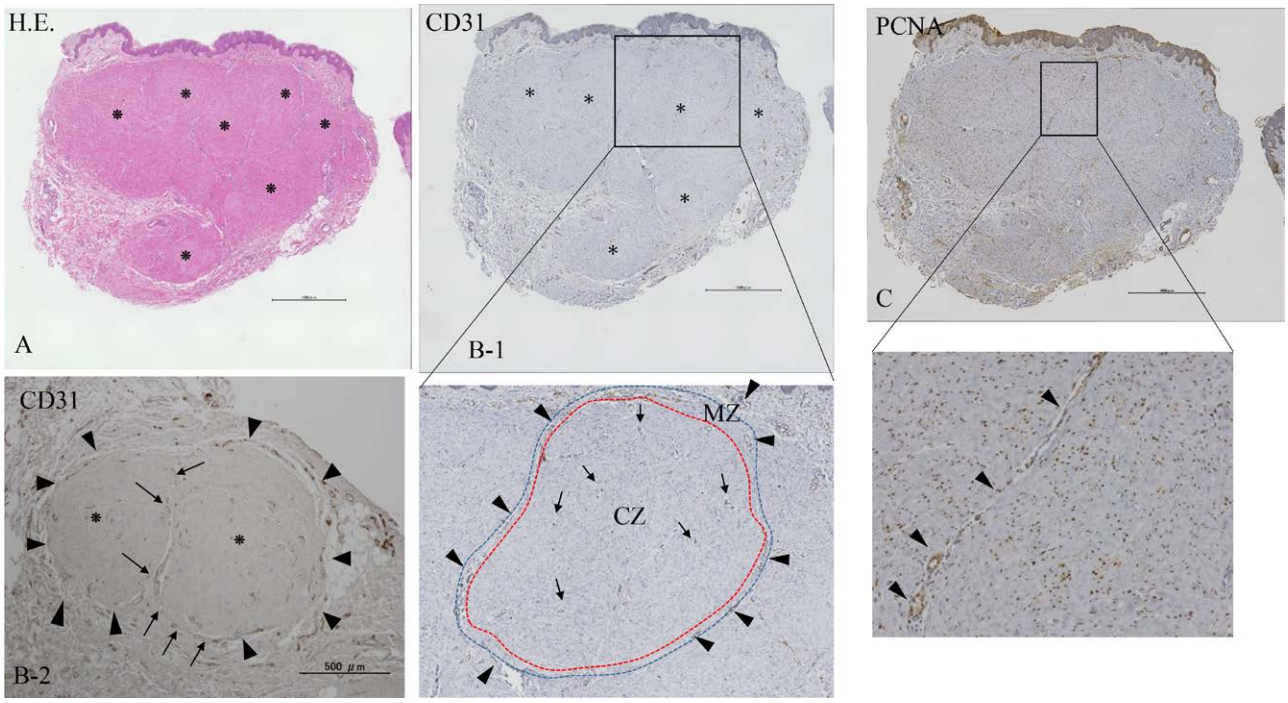

Fig. 1. Histological characterization of nodules. Many thick hyaline collagen fibers (*) are demonstrated in the dermis (A). Nodules (* in B-1) were detected by many vessels (arrowheads showing CD31-positive blood vessels) in the MZ, and small blood vessels were seen in a CZ (arrows) (in boxed keloid area of B-1). Marginal blood vessels in MZ (arrowheads in B-2) showed serial location (hands), penetrating through the CZ, and nodule indicated by arrowheads separated into two nodules (* in B-2). Proliferating cell marker (anti-PCNA) positive cells were distributed in nodules (C), and strong expression of anti-PCNA was demonstrated in vessels of MZ (enlarged area of the square in C).

dermis area ratio in a keloid section rose approximately collinearly with advancing disease duration. Namely, the number of nodules in a keloid increased as disease duration increased (Figs. 2–4).

DISCUSSION

We have focused on the keloid nodules and have investigated them.[5] In this study, the area of each nodule of the keloid duration groups increased for the 2- to 4-year groups, then decreased and displayed an approximately horizontal line on the graph up to the 17-year group. The ratio of total nodule area/dermis area of a keloid section elevated according to increase of disease duration. The number of nodules in a keloid increased as disease duration increased. We also found nodules in which marginal blood vessels penetrated into the CZ (Fig. 1B-2) and separated the nodule into two. Therefore, we propose the hypothesis that the nodule has grown, and the CZ is infiltrated with MZ and separated into two nodules, and each nodule increases in size [**See figure, Supplemental Digital Content 2,** which displays hypothesis that nodules comprise individual keloids. (a) A fibro-proliferative disorder generated in the dermis has grown to a thick hyaline collagen fiber bundle. (b) The nodule structure completed; CZ with few blood vessels; hypoxic zone and MZ with many blood vessels in it; normoxic zone divided. (c) The nodule has grown, CZ is infiltrated with MZ and separated into two nodules (d), and each nodule increases in size (e). **http://links.lww.com/PRSGO/C148**.]

When compared with the clinical presentation of keloid patients, these results resemble the clinical situations. In the early phase, keloids expand rapidly and after that, keloids continue to expand slowly, gradually, and steadily.

We wish to consider the advantages obtained from the above-mentioned new findings in this study. These results suggest that the expansion of area of each nodule has a limitation. Instead of increasing in size, the number of the nodules increases, and the total areas of the nodules increase within the keloid lesion. Because the keloid nodules have a specific structure to generate ATP for synthesizing the collagen fibers,[5] increasing their number, while keeping their size moderate, is adequate and reasonable for their function. Keloid nodules with specific structures are thought to play an important role in energy metabolic activity for maintaining and continuous expansion of the keloids. There is a possibility to devise a new therapy by observing the movement of the keloid nodule.

The density of fibroblasts that expressed antivimentin (fibroblast marker) in nodules was significantly higher than that of surrounding connective tissue. The significant high density of fibroblasts in nodules suggested that the fibroblasts in nodular areas have higher metabolic activity than the surrounding area, because there was a highly significant correlation between the number of fibroblasts and the ATP level in our previous study.[1]

A large quantity of ATP is necessary to biosynthesize collagen fibers. In this study, many collagen fibers were found around the keloid nodules.

Lee et al[9] reported WHFNs in auricular keloids. The nodules were composed of whirling collagen bundles with

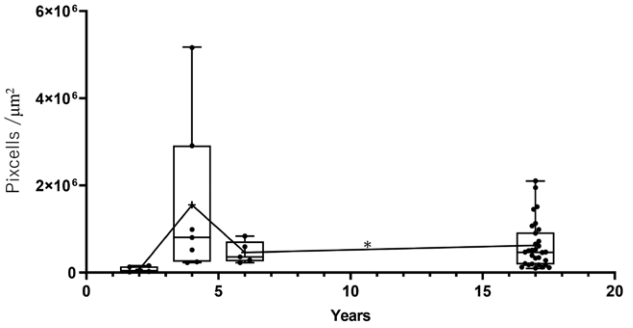

Fig. 2. Statistical evaluation of nodules based on disease duration. Individual nodule area (black dots) showed by box and whisker plot based on disease duration. Straight lines (*) were drawn between the mean nodule areas of the duration groups. The size of each nodule of the duration group increased from 2 to 4 years, then decreased, displaying an approximately horizontal line to 17 years.

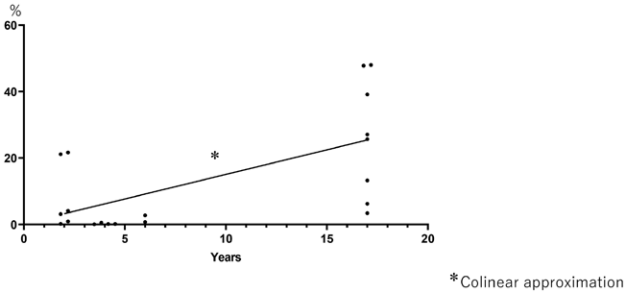

Fig. 3. Total nodule area/dermis based on disease duration. The total nodule area/dermis area of a keloid section elevated according to an increase of disease duration. Individual ratio (black dots), approximation straight line (*).

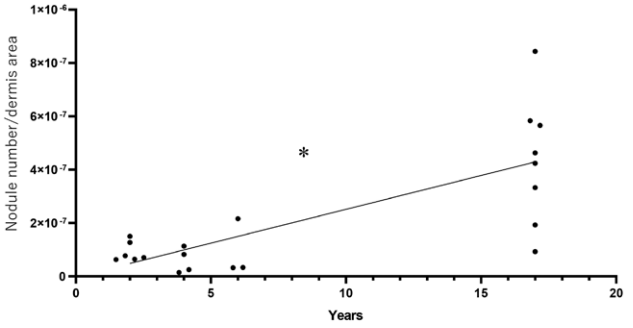

Fig. 4. Percentage of nodule number/dermis based on disease duration. The percentage of nodule number/dermis area in a keloid section showed rising colinear approximation with advancing disease duration. Individual ratio (black dots), approximation straight line (*).

numerous activated young fibroblasts, thus designated as WHFN. WHFN consisted of densely compacted activated young fibroblasts, more than twice the number of other parts, so that these might serve as a proliferating center of the keloid collagen structure. This report shows a result similar to ours.

Santucci et al[7] and Bux and Madaree [8] reported nodular fibrous areas characterized by keloidal histology. Lee et al[9]

also reported the presence of prominent disarray of fibrous fascicles/nodules in keloid histologic features. Huang et al[11] found dermal nodules with hyalinized collagen in each keloid sample. Dermal nodules scattered hyalinized collagen at the top of the nodule. They reported that large dermal nodules dominated the peripheral dermis and that the dermal nodules became larger with increasing distance from the central areas. They discussed that the changing features of dermal nodules from the periphery to the center of the keloid suggested that inflammation played a role. The dermal nodules in the peripheral region suggest that they may be related to expansion of the keloids. This report shows similarities to our results because the keloid nodule plays a key role in continuous growth and expansion.

We previously reported that keloid tissue exhibited high ATP levels even after around 10 years, perhaps due to anaerobic glycolysis.[1,2] Warburg[12] reported that cancer cells mainly generate energy by nonoxidative glycolysis (Warburg effect). It was thought that the Warburg effect only occurred in cancer cells. However, Vincent et al[13] reported that human skin keloid fibroblasts display similar bioenergetic changes to cancer cells in generating ATP mainly from glycolysis.

Cancer cells are able to induce the Warburg effect in stromal fibroblasts (Reverse Warburg effect).[14,15] In cancer cells, hypoxia and oxidative stress-mediated upregulation of autophagy stimulate glycolysis.[15] Our previous study[5] found specific characteristics: the CZ of the nodules was defined as the part with few blood vessels and the surrounding MZ was defined as a circular layer of collagen bundles rich in blood vessels, and showed greater expression of autophagy proteins (autophagosome marker LC3), lactate dehydrogenase, lactate exporter [monocarboxylate transporter (MCT)4], and hypoxia inducible factor (HIF)-1α in CZ fibroblasts than in the MZ fibroblasts, and greater expression of HIF-2α in MZ fibroblasts and endothelial cells than in the CZ. These results indicated keloid nodules worked as a specific structure by producing energy for metabolism. The schema shows our hypothesis of keloid nodule metabolism (Fig. 5). In the CZ of a keloid nodule, fibroblasts generate energy from glycolysis and autophagy. The autophagy mechanism may work as an antiapototic mechanism in fibroblast survival[16–19] in the CZ of keloid nodules.

However, the current study reported that the enhanced autophagy and glycolysis observed in CZ fibroblasts provided lactate to MCT1-expressing fibroblasts in the MZ via metabolic coupling,[5] allowing their proliferation and resulting in excessive collagen production and fibrogenic activity.[20] Histologically, it was reported that keloid nodules were producing collagen fibers.[10,11] Such a process may play an important role in expanding the keloids by producing the collagen fibers. Recent studies report that

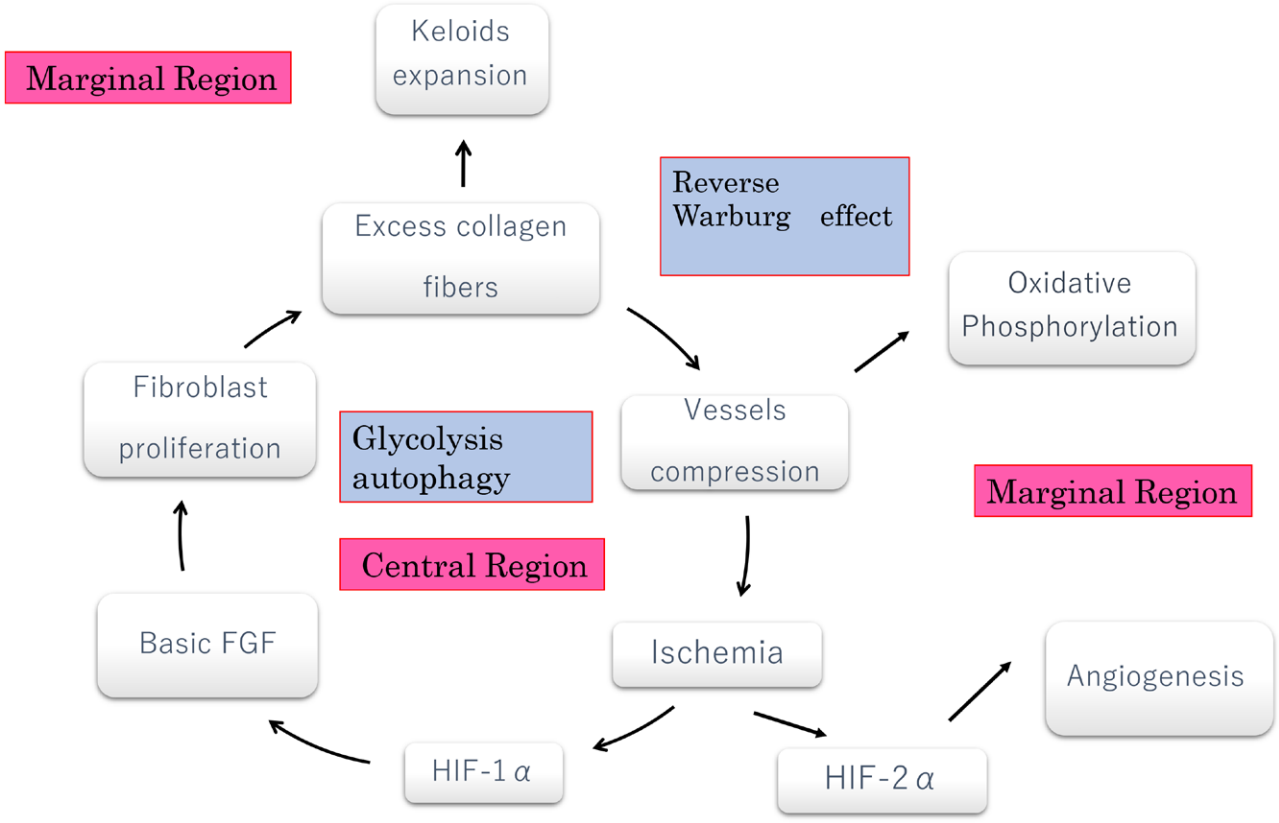

Fig. 5. The schema of our hypothesis of keloid nodule metabolism. In the CZ of keloid nodule, fibroblasts generate energy from glycolysis and autophagy. The enhanced autophagy and glycolysis in CZ fibroblasts provided lactate to fibroblasts in the MZ, allowing their proliferation and resulting in excessive collagen production. The enhanced HIF-2α in the MZ fibroblasts may induce angiogenesis in the MZ of the keloid.

HIF-2α is an essential factor for survival, integrity, and morphology of vascular endothelial cells and hyperproliferation via oxidative phosphorylation.[21,22] The enhanced HIF-2α in the MZ fibroblasts may induce angiogenesis in the MZ of the keloid (Fig. 5).

Autophagy inhibitors and MCT4 blockers[23] may have therapeutic implications for keloid treatment. Injection therapy to the keloid and selectively to the keloid nodule, or ointment therapy is thought to be performed.

In this study, we do not investigate the long process of the keloid nodules in the same patients and investigate the keloid nodules of different phases in different patients. This is the limitation of this study. However, we believe that the keloid nodules with specific structures must play an important role in energy metabolic activity for maintaining and continuing the expansion of the keloids.

CONCLUSIONS

We have investigated how keloid nodules proceed through the disease duration. The area of each nodule increased for 2 to 4 years, then decreased and showed an approximately horizontal line on the graph up to 17 years. The ratio of total nodule area/dermis area elevated according to the increase of disease duration. The nodule number/dermis area ratio rose as disease duration increased. Keloid nodules with specific structures must play an important role in energy metabolic activity for maintaining and continuing the expansion of the keloids.

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