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Immunohistochemical Evaluation of Dermal Mesenchymal Cells in Relation to the Development of Scleroderma

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Abstract Histopathological and immunohistochemical studies were conducted to investigate the local existence and distribution of mast cells, fibroblasts or dendritic cells, and myofibroblasts in scleroderma, while paying particular attention to the interstices around the adnexa. The skin tissues in 15 cases of systemic scleroderma were classified histopathologically into three stages: early, fully developed, and late stage. Mesenchymal cells were distinguished immunohistochemically by 6 markers: tryptase, CD34, factor XIIIa, alpha smooth muscle actin (αSMA), vimentin, and desmin. In scleroderma, there was an increase in the number of tryptase-positive mast cells in the interstices around the adnexa. With no relation to the interstitial sites, there was a significant decrease in the number of CD34-positive cells in the late stage, and a significant increase in αSMA-positive cells in the fully developed stage, but a decrease in the late stage. Results of the present study brought about the following new findings: it was only in the mast cells that there was a significant difference in the cell distribution between the interstices around the adnexa and the interstices in non-adnexal sites. Secondly, it was suggested that mast cells, CD34-positive dendritic cells, and αSMA-positive myofibroblasts were involved in the fibrosis and shrinkage or disappearance of the adnexa when scleroderma developed.

Key words: scleroderma, mast cell, dermal dendritic cell, CD34, αSMA

Introduction

Histopathological images of the skin observed in scleroderma are characterized by the initiation of fibrosis from the marginal area between the deep part of the dermal plexiform layer and adipose tissue, and from the interstices around the adnexa.[1] However, the pathogenesis of scleroderma remains obscure. It has been suggested that mast cells increase in number in the sclerodermatous lesions and that these mast cells, along with fibroblasts or similar cells and dermal dendritic cells, play an important role.[2-5] To clarify the mechanism of fibrosis in scleroderma, we counted the number of cells and evaluated the cell distribution both histopathologically and immunohistochemically.

Mast cells can be identified because they show metachromatism when stained with toluidine blue and astra blue. Depending on the presence of serine protease, which is contained in granular cells, the mast cells are divided into two types: tryptase-positive mast cells (t+MC) containing only tryptase, and tryptase-positive, chimase-positive mast cells (t+c+MC) containing tryptase and chimase. The t+c+MC type accounts for 95% or more of the mast cells of normal skin. On the other hand, the t+MC type accounts for a major

portion of the cells in the lungs, the respiratory tract, the nasal cavities, and digestive tract epithelia.[6]

Dermal dendritic cells exist in human skin, but their definition is not clear.[7] Morphologically, HE-stained images of the cells show a fusiform or dendritic shape. These cells include fibroblasts, myofibroblasts, histiocytes, macrophages, Langerhans cells, mast cells, and other mesenchymal cells. It has been reported that dermal dendritic cells are immunohistochemically positive for factor XIIIa (FXIIIa) or CD34.[8][9] FXIIIa-positive cells are called type I; and CD34-positive cells are called type II dermal dendritic cells.[32] Vimentin is a representative marker for unspecialized mesenchymal cells in normal skin. Based on these facts, we defined the dermal dendritic cells as immunohistochemically FXIIIa-positive or CD34-positive cells. We excluded FXIIIa-negative and CD34-negative cells even if they were found to be vimentin-positive.

CD34 shows positive reactions with endothelial cells or with dendritic cells around the blood vessels, mainly in the deep dermal plexiform layer or in the interstices.[10][11] CD34 shows positive reactions around the follicles, especially in the bulge portions where it is estimated that follicular stem cells exist, and also around the basal membrane of the eccrine glands.[12-15] It is predicted that CD34-positive dendritic cells, along with the FXIIIa-positive dendritic cells, are involved in the growth of new blood vessels and in tissue repair and show the characteristics of unspecialized fibroblasts.[12-15] There is no CD34-positive cell in the fibrotic lesion (in nodular fasciitis, fibromatosis, keloid, and fibrosarcoma).[13] Fibroblasts are CD34-negative in scars during the period when they produce collagen, but are CD34-positive when they stop producing collagen.[16]

FXIIIa is a cell marker of the monocyte family consisting of macrophages and histiocytes.[17] Similar to dendritic cells such as Langerhans cells, FXIIIa-positive dendritic cells are known to have the ability to present certain antigens and are reported to be involved partially in immunological responses. FXIIIa is not only a marker for inflammatory cells, but also a marker for the stem cells of fibroblasts, and can be found in normal skin as well as in fibrotic lesions.[18]

Muscular tissue markers include alpha smooth muscle actin (αSMA) and desmin. A portion of the cells in the erector muscles of hair in human skin, vascular smooth muscle cells, and pericytes become positive to these markers.[19] These cells that develop positivity to these markers in the dermal interstices are recognized to be myofibroblasts.

There is no finding on the precise locations and functions of these cells that act in sclerodermatous lesions. Scleroderma is characterized by the fibrosis of interstices around the adnexa together with the shrinkage and disappearance of the adnexa. There have been reports on the development of lesions in which mast cells were investigated by the depth of dermis and studies in which dendritic cells were investigated at each stage of the disease.[5][20] Moreover there have been no reports of studies that distinguished the surroundings of the adnexa from non-adnexal interstices.

In this study we classified the skin tissues of scleroderma based on the Ackerman's disease stage classification.[27] We examined the numbers and locations of these cells at each stage histopathologically and immunohistochemically, paying particular attention to the interstices around the adnexa, and discussed the relationship between these cells as well as the relationship between the interstitial fibrosis and adnexal shrinkage. We also conducted electron microscopic observations to investigate the mast cell activities.

Materials and Methods

1) Sample selection and preparation

Fifteen patients, who were clinically diagnosed from 1988 to 2002 as having systemic scleroderma (SSc) based on the American College of Rheumatology's preliminary criteria for SSc,[34] were randomly selected. Fifteen volunteers (who underwent removal of the pigmented nevus including normal skin) were selected as site-match controls. The patient and control group ages ranged from 29-70 years (mean ± SD = 51±14) and 12-69 years (22±20), respectively. The male to female ratios were 6:9 and 6:9, respectively. Skin

specimens from SSc patients were obtained from the extensor surface of the forearms in 7 cases, from the dorsal hand in 2 cases, and from the dorsal finger, upper limb, thigh, leg, upper back, or abdomen in one case each. In the SSc patients, the skin biopsies were taken from the central portion of the lesion. Normal skin biopsies were obtained from various body sites as controls.

Archival paraffin-embedded tissues from sclerodermatous lesions and normal skin samples were utilized for this study. The skin biopsy samples were processed with formaldehyde fixation and paraffin embedding, and then cut into 4-μm-thick sections. These sections were stained with haematoxylin and eosin or subjected to immunohistochemical studies.

2) Histopathological study and classification

Sections from the sclerodermatous lesions were histologically classified into three groups: early, fully developed, and late stage in accordance with the classification of A.B. Ackerman.[27] The early stage shows edema and/or marked inflammatory deep dermal sclerosis, and a normal subcutis. The fully developed stage shows an uneven distribution of the appendages within the mid-dermis, in addition to edema and/or moderate inflammation, deep dermal and subcutaneous sclerosis. The late stage shows minimal inflammation, marked dermal sclerosis, liposclerosis, and an uneven distribution, atrophy or loss of the appendages.

3) Immunohistochemical studies

Formalin-fixed, paraffin-embedded, 4-μm-thick sections were mounted on to MAS-coated slides and then stained with an avidin-biotin peroxidase technique using the DAKO-ENVISION kit/HRP (DAB) kit. They were stained with mouse monoclonal and rabbit polyclonal antibodies: anti-tryptase (DAKO), anti-CD34 (Nichirei), anti-FXIIIa (Biogenesis), anti-αSMA (DAKO), anti-vimentin (DAKO) and anti-desmin (DAKO) (Table 1). Briefly, the sections were deparaffinzed in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was removed by immersion in methanol with 3% hydrogen peroxide for 10 min, followed by washes in PBS 3 times. Non-specific binding was blocked by incubation for 5 min at room temperature with non-immune goat serum. The primary antibodies were applied to the sections and incubated for 45 min at room temperature, then washed with PBS 3 times. A secondary rabbit antimouse immunoglobulin was applied for 45 min at room temperature, and then washed with TBS for 15 min. Finally, they were developed with 3,3'-diamino benzidine solution and 1% hydrogen peroxide and then counterstained with Mayer's hematoxylin.

We did not stain chimase for mast cells because all the blocks used were formalin-fixed and paraffin-embedded. Furthermore, mast cells were confirmed by staining with toluidine blue and astra blue.

4) Quantification and evaluation

These cells were expressed as the number of positive cells per 10 high-power fields (×400) for each specimen, obtained separately in the areas of the periadnexal matrix and interstitial portion of the dermis. The area of the matrix is divided into two parts: the adnexa and the non-adnexa in each high power field.

5) Statistical analysis

Statistical analysis of the number of these cells was carried out by paired Student's *t* test.

Table 1 Antibodies used to dermal mesenchymal cells

antibody	type	source	dilution
Tryptase	Mouse	Dako	1:50
CD34	Mouse	Nichirei	1:1
Factor XIII a	Rabbit	Biogenesis	1:50
α SMA	Mouse	Dako	1:50
Vimentin	Mouse	Dako	1:40
Desmin	Mouse	Dako	1:1

6) Ultrastructural studies

Cells from a lesion of the forearm of a patient with SSc were also studied electron-microscopically. Small pieces of tissue from involved skin from the extensor surface of the forearms were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide. The samples were dehydrated in a graded series of alcohol and embedded in Epok 812. Ultrathin sections were cut on a Porter MT 5000 ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and examined under an H-7500 electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 80 kV.

Results

1) Histopathological studies and classification

The sections were histologically classified into three groups: early, fully developed, and late stages consisted of 4 cases (male:female ratio=1:3, mean age±SD=49±7years), 7 cases (4:3, 49±20 years), and 4 cases (1:3, 56±13years), respectively.

2) Immunohistological study

Fig. 1 shows the light-microscopically observed immunohistochemical findings of anti-tryptase (a), anti-CD34 (b), anti-FXIIIa (c), anti-αSMA (d), anti-vimentin (e) and anti-desmin (f), from a case of scleroderma at the fully developed stage. Tryptase-positive mast cells were accumulated in the periadnexa area (a). CD34-positive dermal dendritic cells were seen to be scattered (b). FXIIIa-positive or αSMA-positive or vimentin-positive dermal dendritic cells were seen in abundance in the sclerotic area of the deep dermis(c,d,e). Desmin-positive dermal dendritic cells were not seen (f).

Fig. 2 shows a comparison of the average numbers of positive cells counted in the field of vision at 400x magnifications for the 15

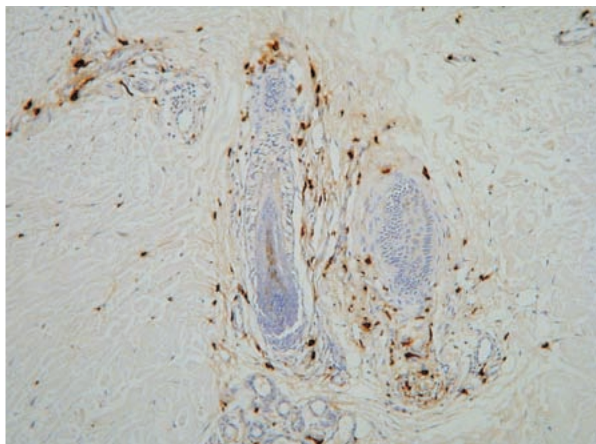


Fig. 1(a)

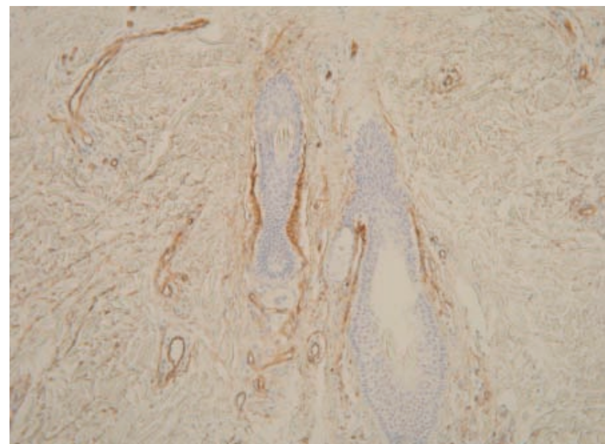
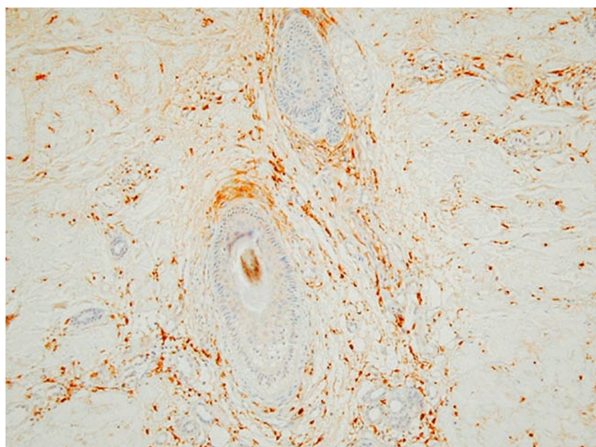


Fig. 1(b)



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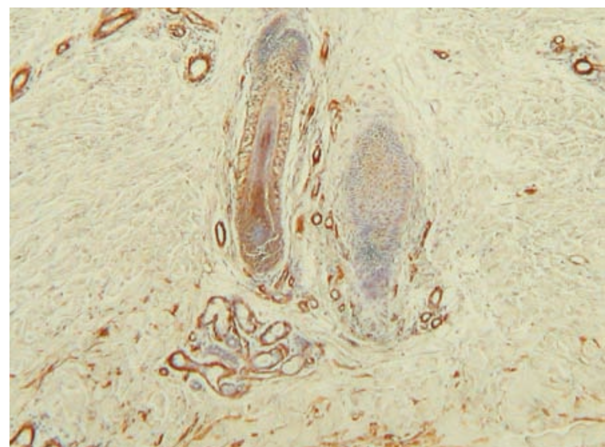

Fig. 1(d)

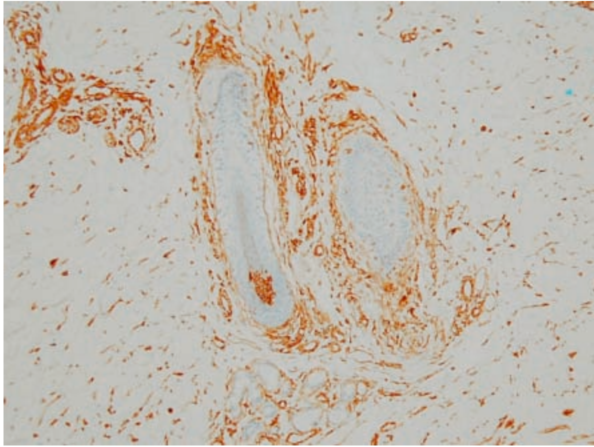
Fig. 1(e)

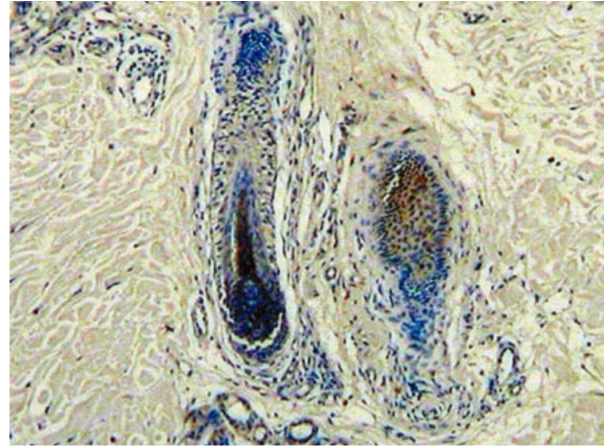
Fig. 1(f)

Fig. 1 Light-microscopically observed immunohistochemical findings from a case of scleroderma at the fully developed stage. Immunohistochemical study of anti-tryptase (a), anti-CD34 (b), anti-FXIIIa (c), anti-αSMA (d), anti-vimentin (e) and anti-desmin (f). (Original magnification: ×100). Tryptase-positive mast cells were accumulated in the periadnexa area (a). CD34-positive dermal dendritic cells were seen to be scattered (b). FXIIIa-positive or αSMA-positive or vimentin-positive dermal dendtitic cells were seen in abundance in the sclerotic area of the deep dermis (c,d,e). Desmin-positive dermal dendritic cells were not seen (f).

cases of SSc and 15 cases of normal skin, by dividing the cell numbers into (A) those in the surroundings of adnexa, and (B) those in the interstices of the non-adnexal parts. Fig. 3 shows population and numbers of positive cells for SSc into (A) and (B). Fig. 4 shows those cell numbers classified by the respective stages of scleroderma.

2-1) Scleroderma and normal skin

In the (A) surroundings of the adnexa, the average number of tryptase-positive mast cells in the sclerodermatous lesions (mean±SD = 11.2±6.8) increased significantly as compared to the average number of cells in normal skin (5.2±2.9, $p<0.01$). The average number of CD34-positive cells in the sclerodermatous lesions (2.3±2.4) decreased significantly as compared to the average number of cells in normal skin (9.2±3.3, $p<0.01$). In contrast, the average number of FXIIIa-positive cells (11.4±5.7) in the sclerodermatous lesions significantly increased as compared to the average number in normal skin (9.2±3.3, $p<0.01$). In addition, αSMA-positive cells (5.6±5.1) did not exist in normal skin, and significantly increased in the sclerodermatous lesions ($p<0.05$).

In the (B) interstices of the non-adnexal parts than the surroundings of the adnexa, the average number of CD34-positive cells decreased significantly in the cases of scleroderma (2.4±2.7, $p<0.01$) as compared to the average number in normal skin (9.4±3.0). The αSMA-positive cells were not observed in normal skin, but the number was significantly increased in the cases of scleroderma (6.3±4.2, $p<0.05$) (Fig. 2).

It was only in the tryptase-positive mast cells (A: 11±6.8, B: 8.0±5.1, $p<0.05$) that there was a significant difference between the (A) interstices around the adnexa and the (B) non-adnexal interstices (Fig. 3).

2-2) Respective stages of scleroderma

In the (A) interstices around the adnexa, more of the tryptase-positive mast cells significantly decreased in the late stage (mean±SD=5.5±1.4) than in the early stage (10.2±3.5, $p<0.01$) and the fully developed stage (13.6±6.5, $p<0.01$) (Fig. 4).

The FXIIIa-positive cells significantly increased in the early stage (13.0±4.0) in the (B) interstices of non-adnexa parts than in the

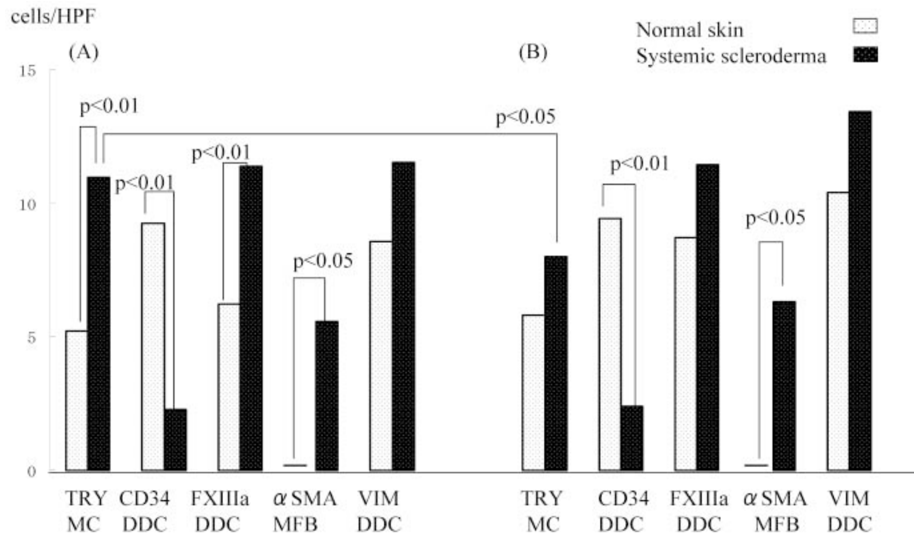

Fig. 2 A comparison of the average numbers of positive cells counted in the field of vision at 400x magnifications for the 15 cases of SSc and 15 cases of normal skin, by dividing the cell numbers into (A) those in the surroundings of adnexa, and (B) those in the interstices of the non-adnexal parts. (TRY: tryptase, VIM: vimentin, MC: mast cell, DDC: dermal dendritic cell, MFB: myofibroblast).

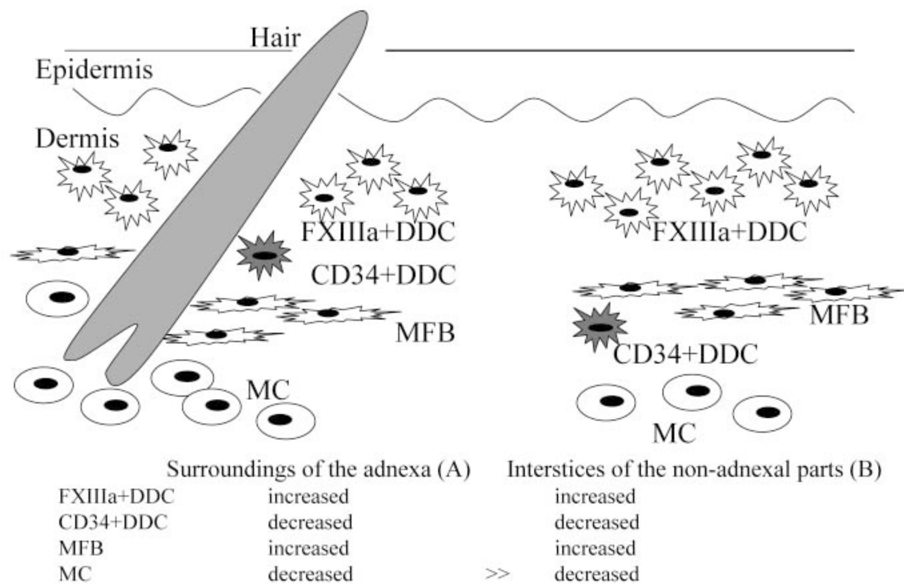

Fig. 3 Population and number of positive cells in SSc, in the surroundings of adnexal (A), and the interstices of the non-adnexal parts (B). (MC: mast cell, DDC: dermal dendritic cell, MFB: myofibroblast).

surroundings of the adnexa, but then decreased significantly in the fully developed stage (9.0±5.1, $p<0.05$), and to a larger extent, in the late stage (6.4±5.4, $p<0.01$). More of the αSMA-positive cells significantly increased in the fully developed stage (8.7±5.2, $p<0.05$) than in the early stage (4.7±3.5), and then more of them significantly decreased in the late stage (6.5±2.5, $p<0.05$) than in the fully developed stage (Fig. 4).

There was no significant difference in the number of these cells in all the stages of

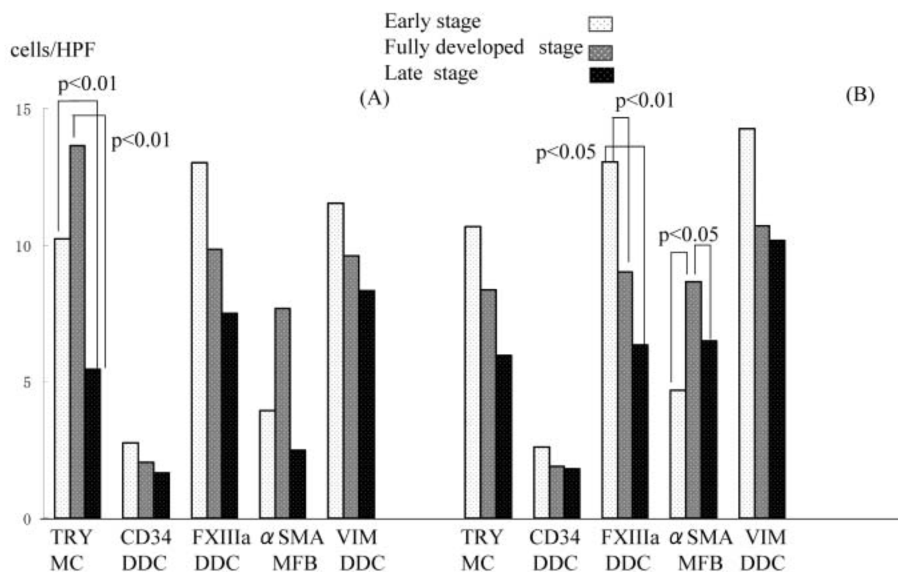

Fig. 4 Number of positive cells in scleroderma according to the stage in the surroundings of adnexal (A), and in the interstices of the non-adnexal parts (B). (TRY: tryptase, VIM: vimentin, MC: mast cell, DDC: dermal dendritic cell, MFB: myofibroblast).

disease between the (A) interstices around the adnexa and the (B) interstices in non-adnexa sites. These mesenchymal cells showed a downward trend with the progress of stages, with no regard to the interstitial sites. However, tryptase-positive mast cells in the (A) interstices around the adnexa and the αSMA-positive cells in both of the (A) interstices around the adnexa and the (B) interstices in non-adnexa sites increased more in the fully developed stage than in the early stage, and again decreased in the late stage.

3) Ultrastructural findings

Mast cells had some finger-like villous projections (arrows) that were curved and contacted collagen fibers. Mast cells contained abundant granules that showed a lamellar structure in parallel arrangement or dense, amorphous material (Fig. 5).

Discussion

In the present study, our focus of interest was the tryptase-positive mast cells, CD34-

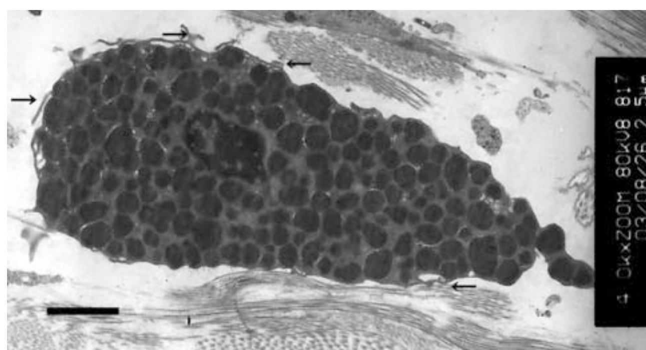

Fig. 5 Ultrastructural findings of mast cell from scleroderma. Mast cell contained abundant granules. These showed small or large, oval or irregular, lamellar structure in parallel arrangement or dense amorphous material. Mast cell had some finger-like villous projections (arrows) that were curved and contacted collagen fibers. Bar, 2.5 μm.

positive dendritic cells, FXIIIa-positive dendritic cells, and αSMA-positive myofibroblasts, all of which are reported to be involved in the pathological conditions of SSc. This is because there has been no report on the difference in the distribution and localization of these cells between the interstices around the adnexa and the interstices in the non-adnexa sites during the development of fibrosis that occurs in sclerodermatous lesions. These cells are involved in the interstitial production and deposition of not only collagen fibers, but also mucin in various inflammatory diseases. For example, in cases of papular mucinosis, a representative disease that is associated with mucin deposition, there were more increases in the number of CD34-positive dendritic cells, FXIIIa-positive dendritic cells, and tryptase-positive mast cells in the surrounding areas, rather than in the mucin deposition area.[33] Accordingly, it can be expected that the distribution and localization of these mesenchymal cells will vary with different disease conditions.

This study revealed that more of the tryptase-positive mast cells, the FXIIIa-positive cells, and the αSMA-positive myofibroblasts showed significant increases in the number of cells in cases of sclerodermatous lesions than in normal skin. On the other hand, CD34-positive cells significantly decreased in number. Although not shown in the results of this study, a similar investigation was conducted on localized scleroderma (n=7), and nearly the same results as those found in the SSc were obtained histopathologically and immunohistochemically. It was only in mast cells that a significant difference was clearly shown between the interstices around the adnexa and the interstices in non-adnexa parts. All of the changes in the number of cells showed more remarkable trends in the interstices around the adnexa than in non-adnexa areas. Although the mechanism remains obscure, the results of the present study suggest that this observed phenomenon is involved in the shrinkage or disappearance of the skin adnexa and in the increase in collagen fibers, which are the histopathological characteristics of scleroderma.

Mast cells coexist with fibroblasts in the dermis. The differentiation and growth of mast cells are affected directly or indirectly by fibroblasts and by various soluble cytokines deriving from keratinocytes.[21][22] It is known that mast cells, along with CD34-positive cells, exist in the connective tissues around the adnexa in normal skin.[29] It also has been pointed out that excessive production of stem cell factor (SCF) from keratinocytes gives rise to an increase in the number of dermal mast cells.[23] The fact that the increase in mast cells in the interstices around the adnexa has been more remarkable than in the non-adnexal interstitial parts, prompted us to predict that keratinocytes and mast cells are closely interrelated. The electron microscopical findings revealed the characteristics of activated mast cells, which were the swelling of granules with changes in their electron-dense contents, fusion of individual granule membranes to form chains of granules, prominent cytoplasmic filaments, empty channels, enlarged Golgi apparatus, and increased amounts of other cytoplasmic organelles.[24][25] Similarly, the sclerodermatous lesions of our case exhibited an increase in the number of mast cells. Electron-microscopic examination revealed morphological changes in activated mast cells.

Dermal dendritic cells are immunohistochemically characterized by the expression of FXIIIa or CD34,[8][9][28] and are distinguished by the existing sites and the difference in the expression of FXIIIa or CD34.[28][32] Dendritic cells were spread widely in the dermal interstices. The dendritic cells were classified depending on their location: they were divided into dendritic cells directly beneath the superficial skin, dendritic cells in the dermal papillary layer, dendritic cells around follicles (especially in the bulge portion), dendritic cells in the follicular papillary area, dendritic cells around eccrine glands, dendritic cells around nerves, and dendritic cells in adipose tissues.[28]

The FXIIIa-positive dendritic cells are found directly beneath the superficial skin, in the dermal papillary layer, and around capillary blood vessels, and are reported to increase in number with inflammatory diseases and tissue repair.[28] It was found from the results of this study that the FXIIIa-positive dendritic cells increased more in SSc than in

normal skin and that there was no significant difference between the interstices around the adnexa and those in non-adnexa sites.

The CD34-positive dendritic cells of normal skin exist in the area around the adnexa, in the deep portion of the plexiform layer, and in the adipose tissues.[28] These CD34-positive dendritic cells are known to decrease in fibrotic lesions, such as a thickened scar or keloid. It is estimated that CD34-positive dendritic cells, along with FXIIIa-positive cells, play a role of some kind in the control of skin fibrosis.[4)16)] In these lesions, the superficial skin is known to thicken, thus leading up to predict that FXIIIa-positive dendritic cells and CD34-positive cells are interrelated with the growth of keratinocytes.[28] In our study, the superficial skin and the adnexa trended to shrink with the fully developed stage. In parallel with this trend, the FXIIIa-positive dendritic cells tended to increase in number and the CD34-positive cells tended to decrease in number. However, there was no clearly significant difference between these two types of cells nor between the interstices around the adnexa and the interstices in non-adnexa areas.

This study showed that FXIIIa-positive cells increased in number, but that CD34-positive cells decreased. Therefore, on the whole, the dendritic cells apparently showed little change in the number of cells. It was discovered, however, that the type of dendritic cells found in sclerodermatous lesions is different from that of the corresponding cells found in normal skin. In addition, the results suggested that these dendritic cells are involved in the fibrosis and consolidation of the skin, with no relation to the interstitial sites (around the adnexa and in non-adnexa areas).

Myofibroblasts are immunohistochemically αSMA-positive. In our light microscopic study, the αSMA-positive cells were found to have increased in number in the surroundings of the adnexa. The appearance of myofibroblast is temporary, and they are reported to disappear or return to the morphology of fibroblasts once they become redundant.[30] It is reported that myofibroblasts exist more abundantly in the papillary areas of the follicles and in the fibrous, connective epidermal sheath, than do dermal fibroblasts.[30)31)] In our experiments, though, none of the αSMA-positive cells (that is, myofibroblasts) were observed in normal skin. According to Gailit et al, myofibroblasts are differentiated by a mediator, such as mast cell tryptase.[26] It is assumed that an increase in the number of tryptase-positive cells in sclerodermatous lesions may subsequently lead to the existence of myofibroblasts.

Mast cells, dendritic cells, myofibroblasts, and keratinocytes are interrelated with one another in their growth, activation, and inhibition. This study was the first attempt to analyze differences in the number and distribution of these cells in the interstices around the adnexa and the interstices in non-adnexa sites. It was only in mast cells that a significant difference was found in the number of cells in sclerodermatous lesions between these two types of interstices. Dendritic cells and myofibroblasts did not show any significant differences. Therefore, the findings of the present study suggested that, except in the case of mast cells, disease conditions, such as fibrosis in the interstices around the adnexa, extended throughout the entire dermis irrespective of the existence of the adnexa.

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