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Immunophenotypical analyses of myofibroblasts in rat excisional wound healing: possible transdifferentiation of blood vessel pericytes and perifollicular dermal sheath cells into myofibroblasts

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Summary. Cutaneous fibrosis after wound is evoked by myofibroblasts capable of producing collagen; the derivation and features remain to be investigated. Immunophenotypical characteristics of myofibroblasts were analysed in excisional rat wound healing, of which samples were obtained on post-wounding (PW) days 1 to 26. Myofibroblasts were characterized for expressions of intermediate cytoskeletons such as vimentin, desmin, and α-smooth muscle actin (α-SMA). To pursue the progenitor, immunolabeling analyses were performed using stromal-/bone marrow-stem cell markers (Thy-1 and A3). Myofibroblasts reacting to vimentin and α-SMA were first seen on PW day 5, then peaked on PW day 9 in granulation tissues, and gradually decreased in remodeling tissues; these immunopositive cells reacted simultaneously to Thy-1. Desmin-reacting cells were limited to newly-formed blood vessels in wound bed. The single/double immunolabelings revealed that pericytes (identified by positive reaction to PDGFR-ß and negative reaction to endothelial markers) in newly-developing blood vessels reacted to vimentin, α-SMA, Thy-1 and A3, and occasionally to desmin, and that perifollicular dermal sheath cells in the wound periphery showed increased expressions for vimentin, Thy-1 and A3. There is considerable immunophenotypical similarity between myofibroblasts (expressing vimentin, α-SMA and Thy-1), pericytes (reacting to vimentin, α-SMA, Thy-1 and A3) in newly-developing blood vessels, and perifollicular dermal sheath cells (reacting to vimentin, Thy-1 and A3). Collectively, myofibroblasts in rat cutaneous fibrosis are characterized by vimentin, α-SMA and Thy-1 expressions, and the cells might be generated from the pericytes or perifollicular dermal sheath cells in the lineage of stroma-/bone marrow-stem cells.

Key words: Wound healing, Myofriboblast, Pericytes, Perifollicular dermal sheath cells

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Introduction

The wound repair process is sequentially divided into three overlapping phases: inflammation, proliferative (granulation tissue), and remodeling phases (Singer and Clark, 1999). During the wound granulation tissue phase, myofibroblasts, so-called contractile cells, play important roles in fibrogenesis through the synthesis and secretion of extracellular matrices such as collagens (Hinz et al., 2001, 2007). The decreased number of myofibroblasts is a hallmark of the transition from the granulation tissue phase to the remodeling phase (Singer and Clark, 1999). The functional and morphological abnormalities of myofibroblasts in fibrotic areas have been reported in aberrant wound repair lesions such as hypertrophic scarring and keloid (Desmoulière et al., 2003). Understanding the cellular characteristics of myofibroblasts would provide better insights into appropriate therapies against fibrocontractive diseases.

The origin of myofibroblasts in cutaneous wound healing is diverse (Eyden, 2001; Hinz et al., 2001, 2007).

The surrounding pre-existing fibroblasts and microvascular cells are the main candidates for the derivation (Desmoulière et al., 2003; Rajkumar et al., 2005; Hinz et al., 2007; Barisic-Dujmovic et al., 2010; Humphreys et al., 2010). In addition, mesenchymal cells located around hair follicles have been regarded as other possible sources of myofibroblasts (Jahoda and Reynolds, 2001). It has recently been reported that myofibroblasts in wound granulation tissues might be derived from circulating bone marrow-stem cells (Mori et al., 2005). The relationship of these possible sources with myofibroblasts, as well as the mechanism of transdifferentiation towards myofibroblasts remain to be clarified.

It is well known that myofibroblasts in fibrotic lesions could variously express intermediate cytoskeletons, such as vimentin, desmin, and α-smooth muscle actin (α-SMA) (Darby et al., 1990; Kohnen et al., 1996; Ide et al., 2004). Additionally, it has been reported that Thy-1 (CD90), a 25-37 kDa GPI-anchored cell surface protein might be expressed during the development of myofibroblasts; Thy-1 is also a marker for mesenchymal stem cells (Dudas et al., 2007, 2009; Crisan et al., 2008). We has recently generated an antibody (A3) using a rat malignant fibrous histiocytoma (MFH)-derived cloned cell line as the antigen, and A3 labeled with rat mesenchymal stem cells around blood vessels and in the bone marrow (Yamate et al., 2007). The relationship in expression patterns between intermediate cytoskeletons and Thy-1 or A3 remains to be investigated in rat wound healing tissues. The immunophenotypical analyses using antibodies to these markers would be beneficial in characterizing myofibroblasts in the wound healing and to pursue the possible derivation of myofibroblasts. The present study investigated detailed characteristics of myofibroblasts in cutaneous wound healing in rats.

Materials and methods

Animals and wound models

The following experiments conformed to the institutional guidelines of Osaka Prefecture University for animal care. Ten-week-old F344/DuCrj male rats (Charles River Japan, Shiga, Japan) were used after a one-week acclimatization period. The rat's dorsal skin was shaved and cleaned with 70% alcohol. Subsequently, three circular wounds were made by using a 5-mm diameter dermal biopsy punch (Maruho Co., Osaka, Japan) in the right and left of the rat's back midline under anesthesia with isoflurane. Day 0 was designated as when wounds were made. After wounding, rats were kept in an individual cage under specific-pathogen-free conditions in an animal room at a controlled temperature of 21±3°C and with a 12-h light-dark cycle; they were allowed free access to a standard commercial diet (MF; Oriental Yeast Co, Ltd, Tokyo, Japan) and tap water *ad libitum*. Wound tissues from three to four rats were each examined on post-wounding (PW) days 1, 3, 5, 7, 9, 12, 15, 20 and 26. Skin tissues from unwounded 10-week-old male rats served as controls (PW day 0).

Tissue preparation and histopathology

Skin tissue samples were collected from the dorsal wound area including the surrounding normal skin. The tissues were immediately immersed into 10% neutral buffered formalin and PLP-AMeX fixatives (Suzuki et al., 2000), and then embedded in paraffin. Fresh tissue samples were excised and embedded in O.C.T. compound (Sakura, Tokyo, Japan) and snap-frozen in -80°C before sectioning with a cryostat. Deparaffinized sections from formalin-fixed paraffin-embedded tissues were cut at 3-4 μm, and routinely stained with hematoxylin-eosin (HE) for morphological observations.

Immunohistochemistry

The following primary antibodies were used for immunolabeling: (i) monoclonal mouse anti-rat Thy-1 (rat CD90; 1:500, Cedarlane, Burlington, NC, USA); (ii) monoclonal Alexa 488-conjugated mouse anti-rat Thy-1 (clone OX-7; 1:200, BioLegend, San Diego, CA, USA); (iii) monoclonal mouse anti-human α-SMA (clone IA4; 1:400, Dako, CA, Carpinteria, USA); (iv) monoclonal mouse anti-vimentin (clone V9; 1:200, Dako, Carpinteria, CA, USA); (v) monoclonal mouse anti-human desmin (clone D33; prediluted, Dako, Carpinteria, CA, USA); (vi) monoclonal mouse anti-rat MFH (clone A3; 1:500, TransGenic Inc., Kobe, Japan); (vii) monoclonal rabbit anti-platelet derived growth factor receptor-beta (PDGFR-ß; 1:200, Cell Signaling, Boston, MA, USA); (viii) polyclonal rabbit anti-human von Willebrand Factor (vWF; prediluted, Dako, Carpinteria, CA, USA); (ix) polyclonal goat anti-rat CD34 (1:1000, R&D Systems, Minneapolis, MN, USA).

For the single immunohistochemistry, 5 μm of PLP-AMeX- or formalin-fixed paraffin embedded sections were deparaffinized. Epitope retrieval for paraffin-embedded sections was performed at 80°C for 6 hours in 10 mM citrate-buffer pH 6.0. Subsequently, slides were blocked with 5% skimmed milk in phosphate-buffered saline (PBS) for 30 minutes. The sections were incubated with unconjugated primary antibodies for 12 - 14 hours at 4°C, followed by the application of horseradish peroxidase-conjugated secondary antibody (Histofine simplestain MAX PO®; Nichirei, Tokyo, Japan). Positive reaction was visualized with 3,3'-diaminobenzidine (DAB; Vector Laboratories Inc., Burlingame, CA. USA) and sections were then lightly counterstained with hematoxylin.

Frozen sections (10 μm in thickness) were fixed in ice-cold acetone for 5 minutes. Blocking was performed with 10% normal goat serum for 1 hour. In addition, blocking for sections incubated with goat antibody was carried out with 3% bovine serum albumin in PBS for 1

hour. Subsequently, sections were reacted with unconjugated primary antibody for 1 hour. The visualization of specific antibody bindings was performed with appropriate fluorochrome-conjugated secondary antibodies. Thereafter, sections were incubated for 1 hour with conjugated or unconjugated second primary antibody for 1 hour. The sections incubated with unconjugated antibodies were finally treated with appropriate fluorochrome-conjugated secondary antibodies for 30 minutes. Goat-anti-rabbit-Alexa 488 (Invitrogen, Carlsbad, CA, USA) was used for rabbit mono- or poly-clonal antibodies. Mouse monoclonal antibodies were visualized with goat-anti-mouse-Cy3 (Jackson Immunoresearch, West Grove, PA, USA) or goat-anti-mouse-Alexa 488 (Invitrogen, Carlsbad, CA, USA). The antibody for vimentin was directly labeled with HyLyte fluor 555 (Dojindo Laboratories, Kumamoto, Japan). Isotype specific secondary antibody, goat-anti-mouse IgG2a-Alexa 488 (Invitrogen, Carlsbad, CA, USA) was used for α-SMA antibody. Goat polyclonal antibody was visualized with rabbit-anti-goat FITC (Jackson Immunoresearch, West Grove, PA, USA). Slides were mounted with mounting medium including 4',6-diamino-2-phenylindole (DAPI; VECTASHIELD®; Vector Laboratories, Burlingame, CA, USA) for nuclei. Fluorescence signal and co-localization were examined by a laser scanning confocal imaging microscope (C1Si, Nikon, Japan).

Negative control immunostainings were performed, either by omission of the primary antibody or by treatment with non-immunized rabbit or mouse IgG.

Immunohistochemical evaluation

The intensity of immunopositive reactions in wound beds or hair follicles in the surrounding wound bed were assessed semiquantitatively with scoring criteria (number and intensity) as follows : - = negative, ± = very faint positive staining, 1+ = faint positive staining, 2+ = moderately positive staining, 3+ = strongly positive staining. The scoring was evaluated in comparisons with those of normal rat (10-week-old) dermis used as controls.

Results

Wound morphology

In HE-stained sections, the inflammation phase was noted on PW days 1 to 3, followed by the granulation phase on PW days 5 to 12; the tissue remodeling phase was from PW day 15. The inflammation tissue phase was characterized by loss of dermal tissue and increasing number of macrophages in the surrounding tissue. The granulation tissue consisted of infiltrating macrophages, spindle-shaped fibroblastic cells (myofibroblasts as mentioned below), and newly-formed blood vessels (BVs) in the wound bed, as well as re-epithelialization of epidermis. In the tissue remodeling phase, there was decreased number of cellular components and abundant collagen fibers; the wound was almost healed until PW day 26.

Distribution of cells immunoreactive against cytoskeletal markers such as vimentin, α-SMA, and desmin

In the wound area, the number of cells reacting to vimentin, α-SMA, and desmin were seen on PW day 3 in the inflammation phase; the number and expression intensity peaked on PW days 5 to 9 in the granulation tissue, and then gradually decreased on PW days 15 to 26 in the tissue remodeling phase (Table 1).

On PW days 1 and 3, cells immunoreactive to these cytoskeletal markers were mainly seen in and around the

Table 1. Immunohistochemical reactivity to mesenchymal markers during wound healing.

Region/Antibody to	Post-wounding (PW) day									
	0	1	3	5	7	9	12	15	20	26
Wound bed										
Vimentin	1+	±	2+	2+~3+	2+~3+	3+	2+~3+	2+	2+	1+~2+
α-SMA	±	±	±	2+	2+~3+	3+	2+	1+	±	±
Desmin	±	±	1+	3+	3+	2+	1+~2+	1+	±	±
Thy-1	±	±	+	2+	2+~3+	3+	1+~2+	1+	±~1+	±
A3	±	1+	2+	3+	2+~3+	2+~3+	1+~2+	1+	1+	±
Surrounding hair follicle*										
Vimentin	1+	1+	1+	+2	+2	2+~3+	+2	1+~2+	1+~2+	+1
α-SMA	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
Thy-1	±~1+	1+	1+	2+	2+	2+~3+	1+~2+	1+~2+	1+	±
A3	-	2+	3+	2+~3+	2+~3+	2+~3+	2+~3+	2+	1+	-

The reactivities for mesenchymal markers were assessed semiquantitatively by comparing with grade of controls (PW day 0) as follows: - = negative; ± = very faint positive staining; 1+ = faint positive staining; 2+ = moderately positive staining; 3+ = strongly positive staining; see more detailed evaluation methods in "Materials and Methods". Desmin and A3 expressions in wound bed were limited in newly-formed blood vessels throughout wound healing process. *, the reactivity in the surrounding hair follicle was seen mainly in the perifollicular dermal sheath cells.

newly-formed BVs at the wound margin; in addition, oval or fusiform cells reacting to vimentin were sporadically seen in the wound bed. On PW days 5 to 9, the distribution of cells reacting to vimentin and α-SMA began to gradually expand from the wound margin; besides newly-formed BVs, cells immunopositive to vimentin (Fig. 1a, inset; arrows) and α-SMA were frequently seen in the wound bed, and interestingly, these positive cells were present below the epidermis (under re-epithelialization) at the wound margin; on PW day 9 onwards, such vimentin- and α-SMA-positive cells (Fig. 1b, inset; arrows) were diffusely distributed in the wound area. The distribution and kinetics of vimetin- and α-SMA-immunopositive cells were similar to each other (Table 1); these cells were oval and spindle-shaped in morphology.

On the other hand, desmin-positive cells were found only in the newly-formed BVs in the wound bed (Fig. 1c); due to an increase in newly-formed BVs on PW days 5 to 12, the desmin-positive cell number increased. The distribution of the desmin-positive cells was different from that of vimentin- and α-SMA-positive cells (Table 1, and Fig. 1a-c).

Thy-1-positive cells in wounded skin

In the control skin, Thy-1 immunoreactivity was seen in perivascular cells (pericytes) (Fig. 2a) and in perifollicular dermal sheath (PDS) cells (Fig. 4a), although the number was quite few. In the wounded skin on PW day 3, cells constituting newly-formed BVs at the wound margin began to express Thy-1 (Table 1). On PW days 5 and 7, Thy-1-immunoreaction was seen not only in newly-formed BVs, but also in oval or spindle-shaped cells below the reconstituting epidermis at the wound margin. On PW days 9 and 12, Thy-1-positive cells were observed diffusely throughout the wound bed (Fig. 2b); on PW days 15 to 26 the number was gradually decreased and distribution returned to that of normal skin of the control (pericytes and PDS cells only). The distribution and kinetics of Thy-1-positive cells were similar to those of vimentin- and α-SMA-positive cells (Table 1).

A3-positive cells in wounded skin

In the control skin, A3 immunoreactivity was seen in the pericytes and endothelial cells of pre-existing BVs; the reactivity was faint (Fig. 3a). There were no cells reacting to A3 around the hair bulb (Fig. 4c).

On PW day 3, in the wounded skin, the greater immunoreactivity to A3 was found in the newly-formed BVs at the wound margin. Thereafter, with neovascularization, A3-reacting cells were gradually increased on PW days 5 to 9, showing the greatest expression; on PW days 15 to 26, A3-positive cells were decreased gradually (Table 1).

On PW days 3 to 12, the A3-immunoreactivity was limited to the newly-formed BVs (Fig. 3b); as described

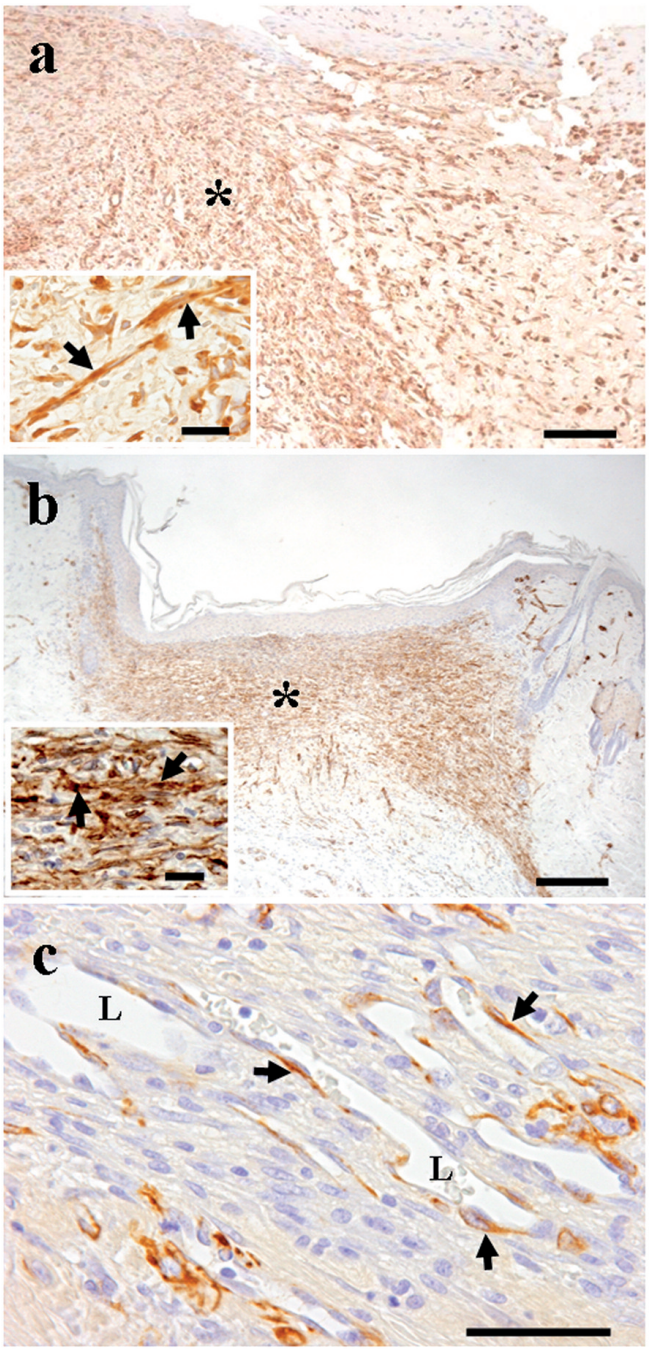

Fig. 1. Immunoreactivity for intermediate cytockeleton markers in the wound bed. Vimentin-reacting cells are seen below the regenerating epidermis on PW day 5 (**a**, asterisk). Cells reacting to α-smooth muscle actin (α-SMA) are extensively distributed in the wound area on PW day 9 (**b**, asterisk). Cells positive for α-SMA and vimentin are elongated or spindle-shaped, indicating myofibroblasts (**a and b**, insets; arrows). Desmin-positive cells are confined only in newly-forming blood vessels (**c**, arrows). L: newly-formed blood vessel lumen. Immunohistochemistry, counterstained with hematoxylin. Bars: a, 100 μm; b, 50 μm; c, 200 μm; insets, 20 μm.

below, these cells were identified as pericytes and endothelial cells. On PW days 15 to 26, besides the BVs, spindle-shaped mesenchymal cells surrounding the hair bulb, apparently regenerating hair follicles, showed a positive reaction to A3 (Table 1).

Immunoreactivity for vimentin, α-SMA, Thy-1, and A3 of hair follicle-constituting cells in the wound bed-surrounding skin tissue

Vimentin- and Thy-1-positive cells were found as a thin single layer in the perifollicular area in the control skin (Fig. 4a,c, respectively). As mentioned above, due to an increased number of vimentin- and Thy-1-positive cells in the wound bed, cells reacting to Thy-1 (Fig. 4b) and vimentin (Fig. 4d) began to increase around hair follicles in the wound bed-surrounding skin tissue; the number of these positive cells was most prominent on PW day 9 (Table 1); these cells were regarded as PDS cells, based on their anatomical location. The Thy-1- and vimentin-positive cells around hair follicles tended to expand into the wound bed (Fig. 4b,d). Although A3-

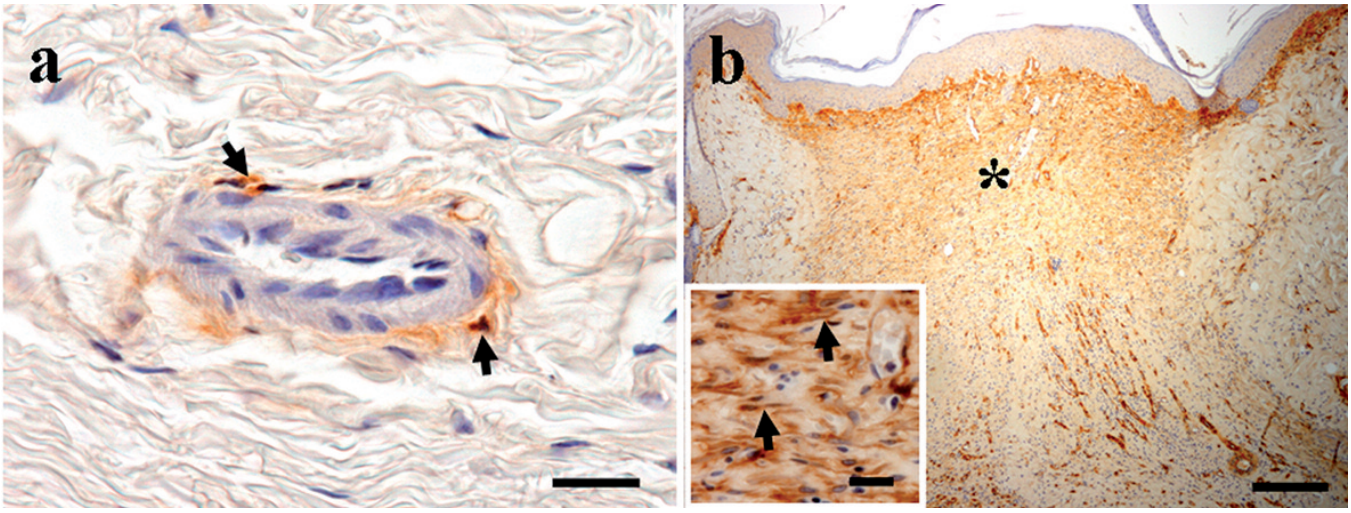

Fig. 2. Immunoreactivity for Thy-1 is seen in pericytes of control rat skin (**a**, arrows). Immunoreactive cells for Thy-1 in wound bed on PW day 9 are diffusely distributed throughout granulation tissue (**b**, asterisk). Elongated or spindle-shaped Thy-1-positive cells are seen in the wound area (**b**, inset; arrows). Immunohistochemistry, counterstained with hematoxylin. Bars: a, 10 μm; b, 200 μm; inset, 20 μm.

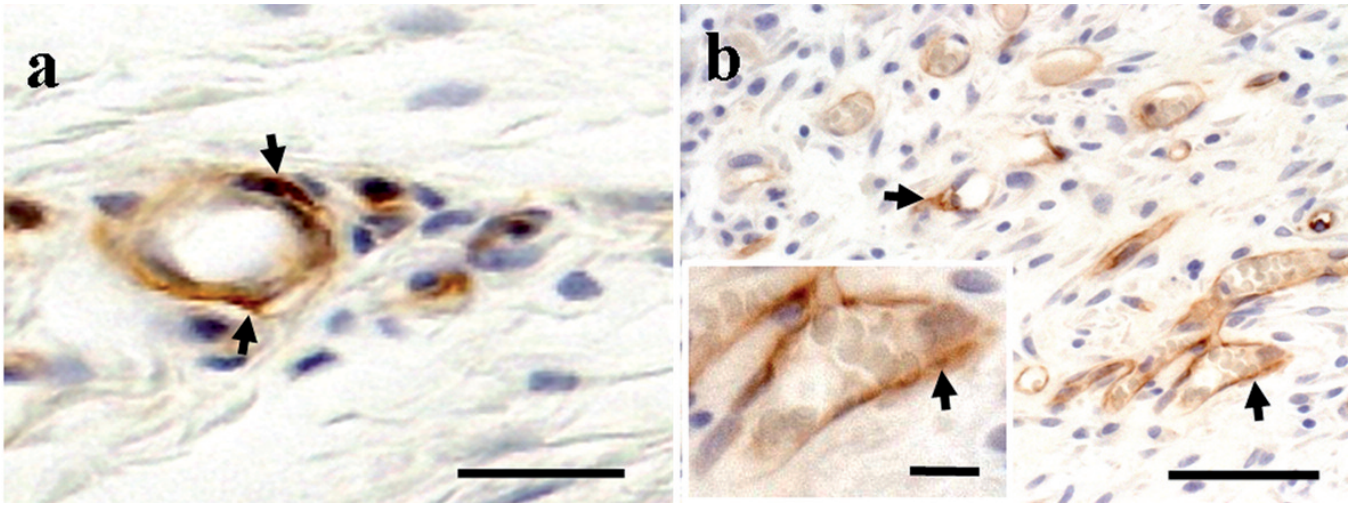

Fig. 3. A3 (a stromal stem cell marker) labels faintly with pericytes (**a**, arrows) and endothelial cells **(a)** of blood vessels in the normal dermis of control rat. In addition, A3-immunoreactivity is exclusively found in the newly-formed blood vessels of wound bed on PW day 7 (**b**, inset; arrows). Immunohistochemistry, counterstained with hematoxylin. Bars: a, 10 μm; b, 200 μm; inset, 20 μm.

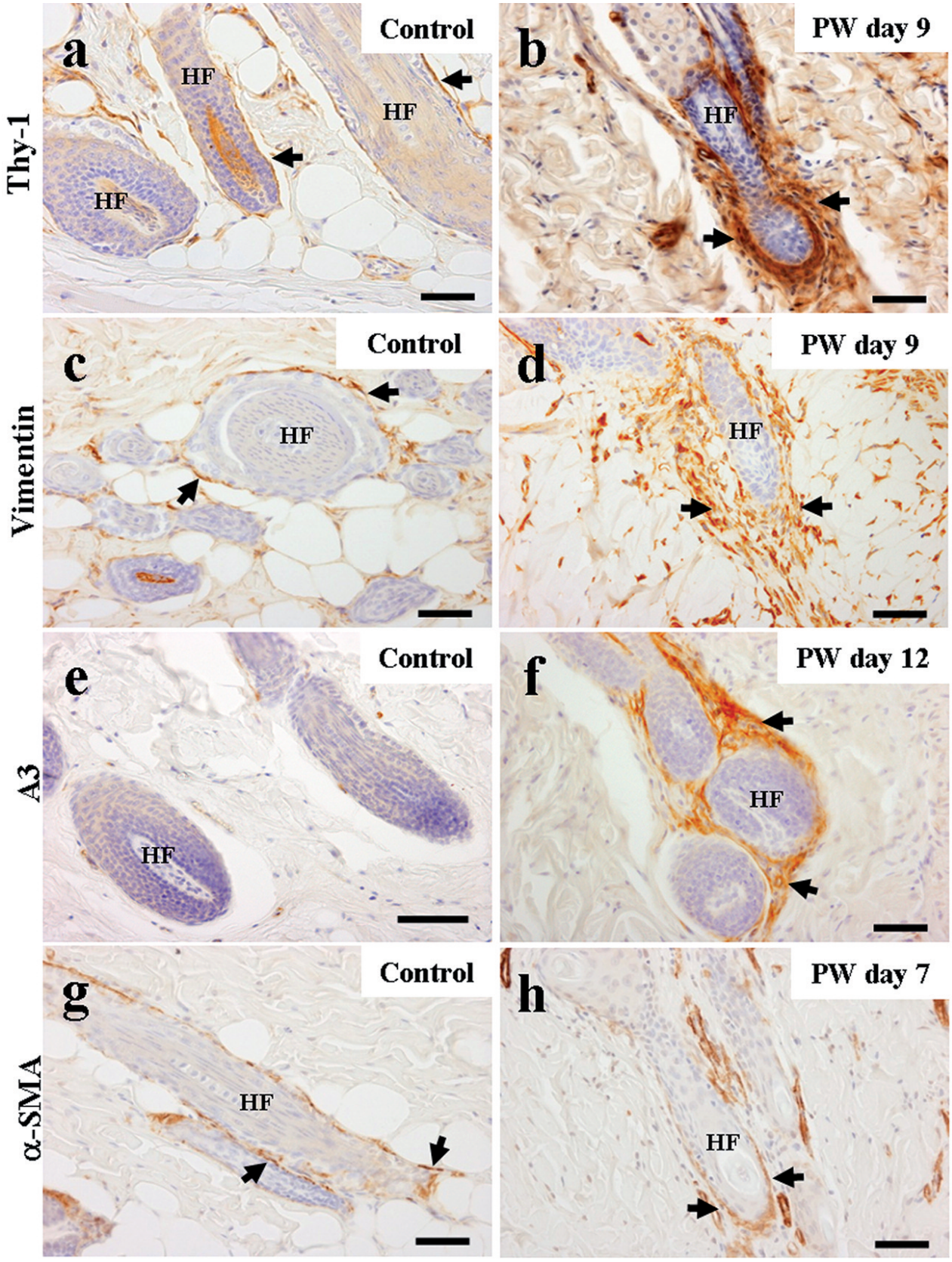

Fig. 4. Immunostaining for mesenchymal cell markers in hair follicles (HF) of normal control skin tissues (**a, c, e, g**), and HFs adjacent to wound area (**b, d, f, h)** in the granulation tissue phase. As compared with reactivity of normal skin HFs for Thy-1 (**a**, arrows; perifollicular dermal sheath cells and dermal papilla cells are positive), vimentin (**c**, arrows; perifollicular dermal sheath cells), A3 (**b**; no positive cells around hair bulb), and α-SMA (**g**, arrows; perifollicular mesenchymal cells), perifollicular dermal sheath cells in HFs adjacent to the wound bed show increased reactivity for Thy-1 (**b**, arrows), vimentin (**d**, arrows), and A3 (**f**, arrows), although α-SMA reactivity does not change (**h**, arrows). Immunohistochemistry, counterstained with hematoxylin. Bars: 50 μm.

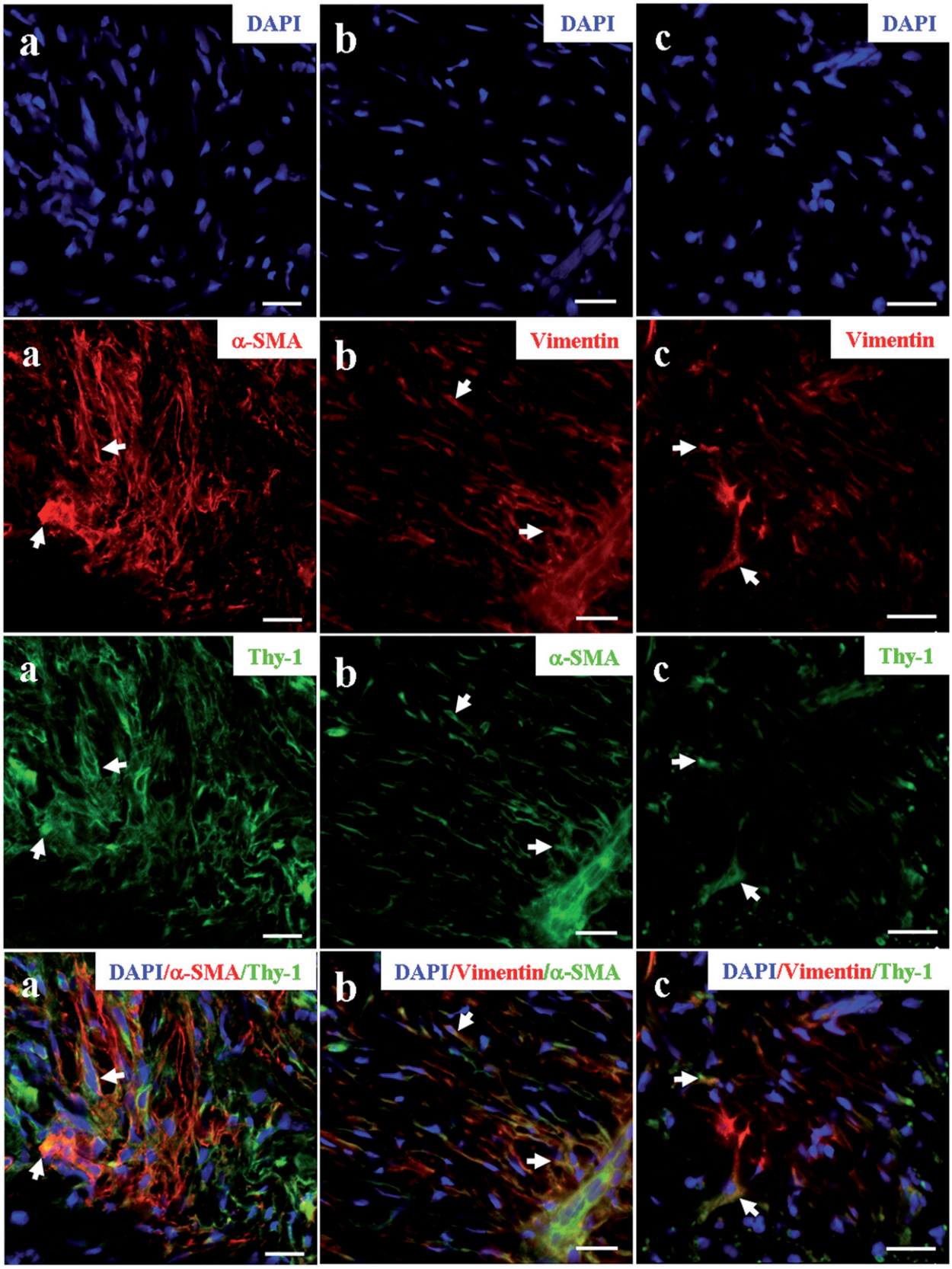

Fig. 5. Double immunofluorescence labelings for α-SMA and Thy-1 **(a)**, α-SMA and vimentin **(b)** or vimentin and Thy-1 **(c)** in the granulation phase on PW day 9. Spindle-shaped or elongated α-SMA-positive myofibroblasts express simultaneously Thy-1 (**a**, merged; arrows) and vimentin (**b**, merged; arrows). Myofibroblasts reacting to Thy-1 also express vimentin (**c**, merged; arrows). DAPI for nuclear stain. Bars: 20 *μ*m.

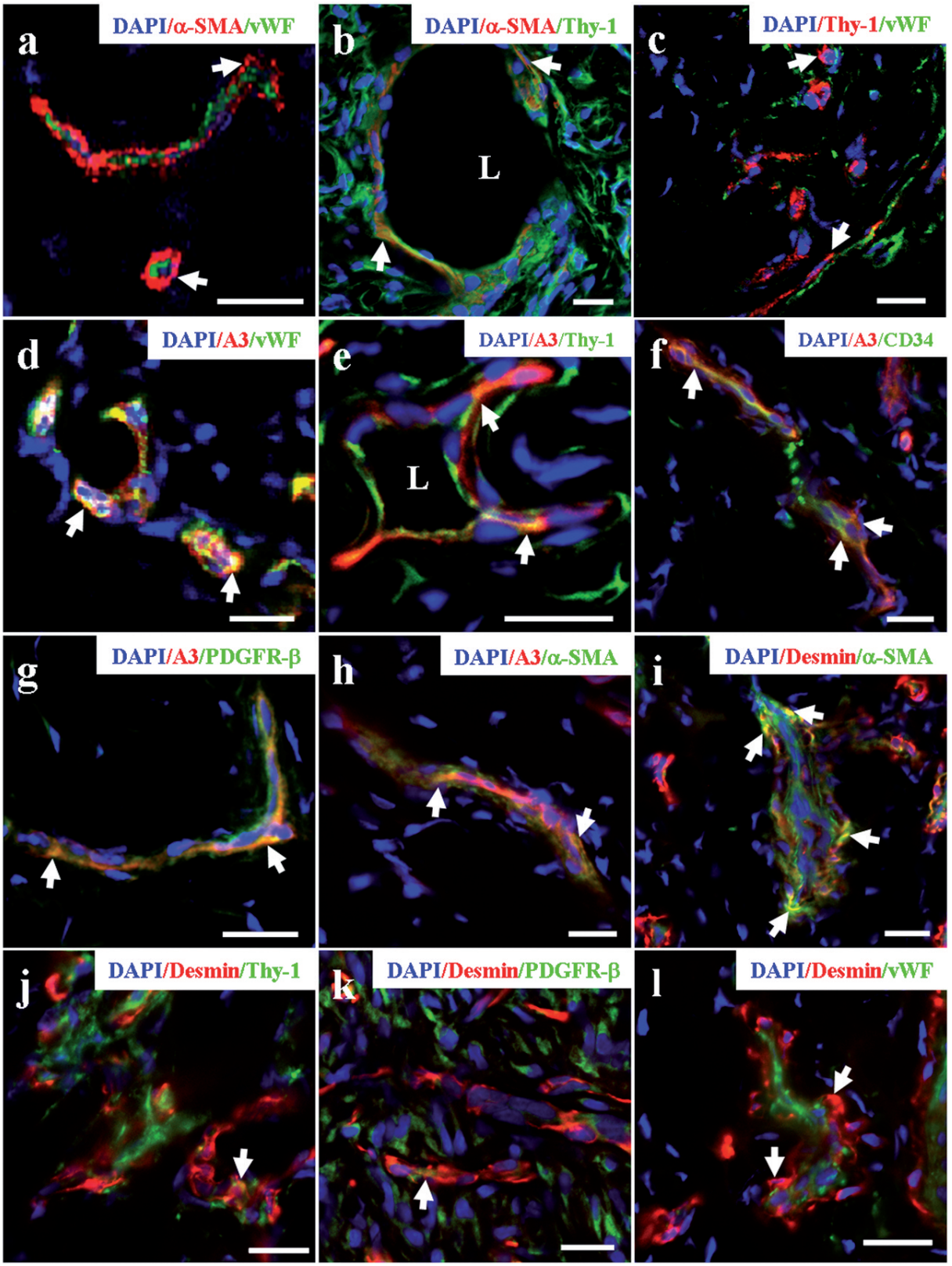

Fig. 6. Double immunofluorescence labelings for mesenchymal cell markers in granulation tissues on PW days 5 to 9 (**a-l**; merged images). Representative double immunopositive cells (yellow to orange) are indicated by arrows. α-SMA-positive cells in newly-formed blood vessels do not correspond to vWF-positive endothelial cells on PW day 7 (**a**, arrows), but correspond to Thy-1-positive perivascular cells (so-called pericytes) (**b**, arrows); L: blood vessel lumen. Thy-1-positive cells do not co-localize with vWF-positive endothelial cells (**c**, arrows). A3-positive cells in newly-formed blood vessels show a co-expression in vWF-positive endothelial cells (**d**, arrows), Thy-1-positive pericytes (**e**, arrows), CD34-positive endothelial cell (**f**, arrows), PDGFR-ß-positive pericytes (**g**, arrows), and α-SMA-positive pericytes (**h**, arrows); A3 is found to label with both endothelial cells and pericytes. Desmin is expressed mainly in α-SMA-positive cells in newly-formed blood vessels (vascular smooth muscles and pericytes) (**i**, arrows), and a small number of desmin-positive cells react to Thy-1 (**j**, arrow) or PDGFR-ß (**k**, arrow); however, vWF-positive endothelial cells do not express desmin (**l**, arrows); besides blood vessel smooth muscles, desmin is expressed in a small number of pericytes which can react to Thy-1 and PDGFR-ß. DAPI for nuclear stain. Bars: 20 μm.

positive mesenchymal cells were not seen around hair bulbs in control skin (Fig. 4e) as mentioned above, PDS cells reacting to A3 appeared in the wound bed-surrounding skin tissue (Fig. 4f); the number increased on PW days 1 to 20, peaking on PW days 3 to 12 (Table 1). The PDS cells reacting to vimentin, Thy-1 and A3 were oval or spindle-shaped in morphology (Figs. 4b,d,f).

There were a small number of α-SMA-positive cells around hair follicles in the control skin (Fig. 4g), but the expression level did not change in the wound bed-surrounding tissues (Fig. 4h) (Table 1).

Localization and mutual relationship of cells reacting to Thy-1, vimentin, α-SMA, desmin, and A3 in wound bed and hair follicles by the double immunofluorescence

As mentioned previously, the distribution of α-

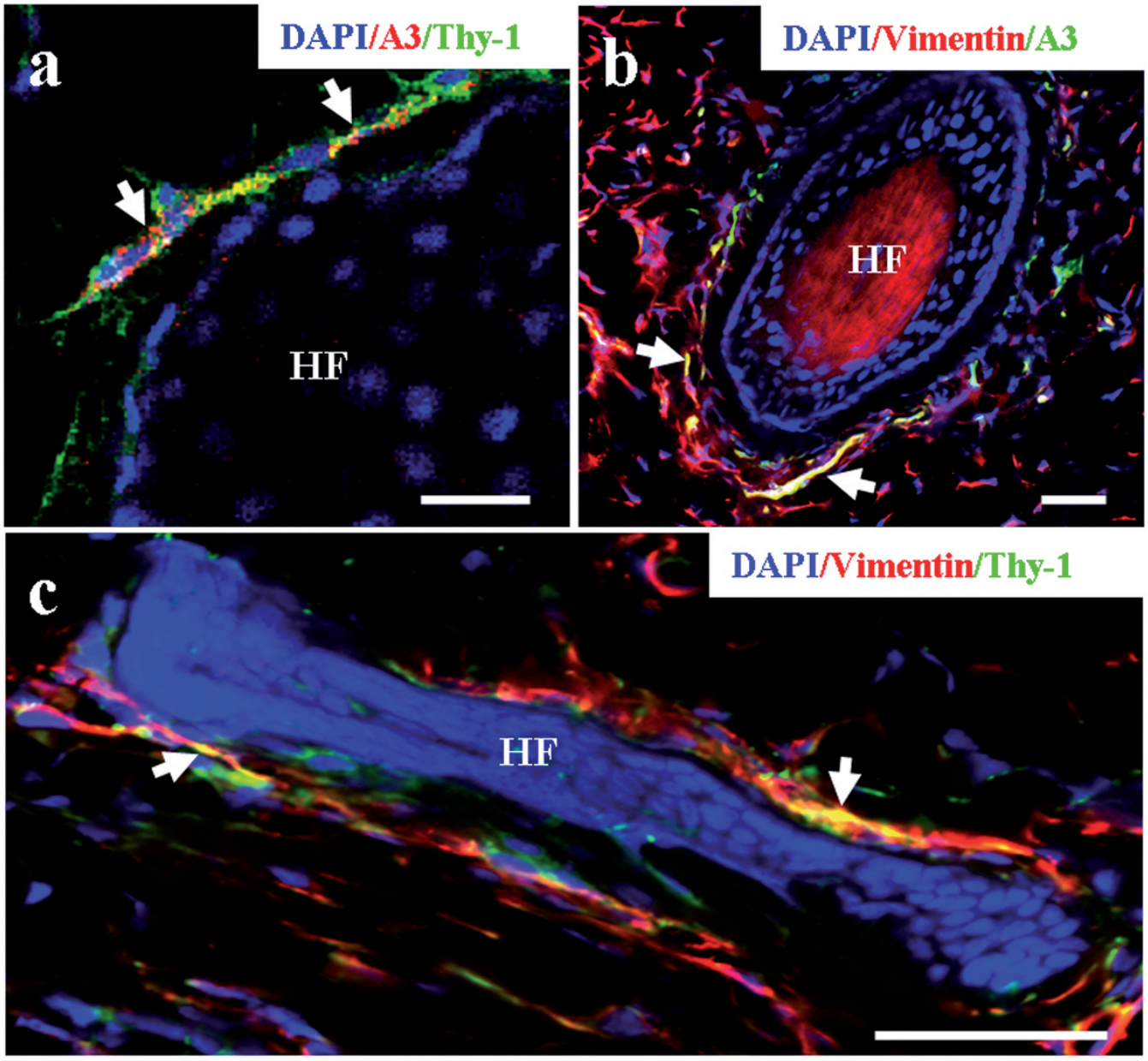

Fig. 7. Double immunofluorescence labelings in wound bed-surrounding hair follicles (HFs) on PW day 9 (merged images shown as yellow to orange for double positive reactions). A3-positive perifollicular dermal sheath cells are also positive for Thy-1 (**a**, arrows) and vimentin (**b**, arrows). Additionally, Thy-1-positive perifollicular dermal sheath cells react to vimentin (**c**, arrows). DAPI for nuclear stain. Bars: a, 20 μm; b, c, 40 μm.

SMA-positive cells seen in the wound bed generally corresponded to that of Thy-1- and vimentin-positive cells. The majority of α-SMA-positive myofibroblasts reacted to Thy-1 (Fig. 5a) and vimentin (Fig. 5b). In addition, Thy-1- and vimentin-positive cells occasionally overlapped each other in the granulation tissue (Fig. 5c).

The endothelial cells of the newly-formed BVs reacted to vWF, but not to α-SMA; α-SMA-positive cells were seen only around the vWF-reacting endothelial cells (Fig. 6a). The α-SMA-positive cells were regarded as pericytes of newly-formed BVs, because the cells showed a positive reaction to Thy-1 (a pericyte marker) (Fig. 6b). There were no cells reacting to both Thy-1 and vWF (Fig. 6c).

As we have seen, the immunoreactivity for A3 was exclusively found in the newly-formed BVs in the wound bed; to identify the cell type, double immunofluorescence labeling was carried out for A3 and vWF or A3 and Thy-1. The vWF-positive endothelial cells reacted to A3 (Fig. 6d); Thy-1-positive pericytes also gave a positive reaction to A3 (Fig. 6e). A3-positive cells reacted to CD34 (an endothelial cell marker) (Fig. 6f) and PDGFR-ß (a pericyte marker) (Fig. 6g). Additionally, the A3-positive pericytes reacted to α-SMA (Fig. 6h). Taking these findings together, it was found that A3-recognizing antigen was expressed in both the pericytes and endothelial cells of newly-formed BVs in the wound bed.

Desmin is expressed in vascular smooth muscles and myofibroblasts (Chaurasia et al., 2009). To clarify the cell type of desmin-positive cells seen in newly-formed BVs in the wound bed, double immunofluorescence labeling was performed for desmin and α-SMA, Thy-1, PDGFR-ß or vWF. α-SMA-positive cells also expressed desmin (Fig. 6i). A small number of cells (fewer than 10%) reacted to desmin and Thy-1 (Fig. 6j), or desmin and PDGFR-ß (Fig. 6k). Contraliry, desmin-positive cells did not correspond to vWF-positive endothelial cells (Fig. 6l). Based on these findings, desmin was expressed not only in the BV's smooth muscles (reacting to α-SMA) but also in pericytes (reacting to α-SMA, Thy-1, and PDGFR-ß).

In the hair follicles in the wound bed-surrounding skin tissue, PDS cells reacting to A3 gave a positive reaction to Thy-1 (Fig. 7a) and vimentin (Fig. 7b). The PDS cells showed double positive reactions to Thy-1 and vimentin (Fig. 7c).

Discussion

Characterization of cutaneous myofibroblasts based on immunoexpressions for intermediate cytoskeletons

Myofibroblasts have been characterized by the immunoexpressions for vimentin (V), α-SMA (A) and desmin (D); these cytoskeletons may be expressed variously depending on developing stages of myofibroblasts and the increased expressions, particularly α-SMA, may be related to the contraction and migration of the cells (Eyden, 2001; Ide et al., 2004; Chaurasia et al., 2009; Desmoulière et al., 2003). Based on the expression levels of these cytoskeletons, myofibroblasts are divided into the following types: cell reacting to vimentin and α-SMA (VA type), cell reacting to vimentin and desmin (VD type), and cell reacting to vimentin, α-SMA and desmin (VAD type) (Chaurasia et al., 2009).

In the wound area, the kinetics of vimentin and α-SMA were similar to each other as demonstrated by the immunohistochemical scoring and double immunolabeling analyses; the immunoexpressions for these cytoskeletons showed the highest level on PW days 7 and 9, in agreement with granulation tissue formation, and then gradually decreased in the remodeling phase until PW day 26. The vimentin- and α-SMA-positive myofibroblasts (VA type) were major constituting cells in the cutaneous granulation tissue (Hinz et al., 2001; Desmoulière et al., 2003; Chaurasia et al., 2009). On PW days 1 to 9 (in the inflammation phase and at the early stage of granulation tissue phase), vimentin- and α-SMA-positive cells were mainly seen around newly-formed BVs and below the reconstituting epidermis at the wound margin; it is likely that myofibroblasts could migrate from these portions into the wound bed.

Interestingly, myofibroblasts expressing vimentin and α-SMA, which were seen in the wound bed, did not show a positive reaction to desmin; in the present wound healing, desmin immunoexpression was limited only to the newly-formed BVs. Previously desmin-positive cells were regarded as vascular smooth muscles formed in the wound bed (Darby et al., 1990; Kohnen et al., 1996; Desmoulière et al., 2003). In double immunofluorescence, desmin-reactivity was seen in α-SMA-positive cells in newly-formed BVs, and the cells reacting to both desmin and α-SMA did not express vWF (an endothelial marker); therefore these cells were considered as newly-formed vascular smooth muscles (Darby et al., 1990; Kohnen et al., 1996; Desmoulière et al., 2003). On the other hand, there were desmin-positive cells reacting to Thy-1 and PDGFR-ß (both, pericyte markers) in the newly-formed BVs, although the number was low. The Thy-1-reacting pericytes were also positive for α-SMA. Taken together, desmin might be transiently expressed in the early stages of myofibroblast development (apparently, as pericytes expressing vimentin, α-SMA and desmin: VAD type), whereas well-developed myofibroblasts (VA type) in the wound bed came to strongly express α-SMA and vimentin by losing desmin. It has been reported that desmin is expressed as a major cytoskeleton in myofibroblasts in pulmonary, hepatic and renal fibrosis (Zhang et al., 1994; Ide et al., 2004; Yamate et al., 2002, 2005). There might be differences in the immunoexpressions of well-developed myofibroblasts between cutaneous fibrosis and fibrosis in other organs (Desmoulière et al., 2003), indicative of heterogeneity of myofibroblasts.

Thy-1 immunoexpression

Thy-1 has roles in cell to cell and cell to matrix

interactions; however the exact functions of Thy-1 remain unknown (Rege and Hagood, 2006). Thy-1 expression may correlate with myofibroblast differentiation (Rege and Hagood, 2006; Dudas et al., 2009). In the wound bed, there were a number of Thy-1-positive cells whose distribution and kinetics were similar to those of vimentin- and α-SMA-positive myofibroblasts, indicating that cutaneous myofibroblasts could also expresss Thy-1 (Dezso et al., 2007; Dudas et al., 2007, 2009).

The mesenchymal stem cells react with Thy-1 antibody (Crisan et al., 2008). Thy-1 immunohistochemistry should be useful in exploring the derivation of myofibroblasts. In agreement with previously reported findings in human skin (Rajkumar et al., 2005), Thy-1 immunoreactivity was seen in the perivascular cells in normal rat skin, as well as newly-formed BVs in the wound bed. Thy-1-positive perivascular cells did not react to vWF (an endothelial marker). The perivascular cells were considered to be pericytes present around BVs. Thy-1-positive pericytes in the wound bed also reacted to vimentin and α-SMA, and occasionally to desmin. The pericytes (probably less differentiated myofibroblasts) in the newly-developing BVs could transdifferentiate into myofibroblasts capable of expressing Thy-1, vimentin and α-SMA in the wound bed following loss of desmin expression (Rajkumar et al., 2005; Yamate et al., 2005, 2007).

More interestingly, vimentin- and Thy-1-positive cells appeared around hair follicles in the wound bed-surrounding skin tissue, and their numbers and reactive intensity increased with granulation tissue formation. In addition to the pericytes, the PDS cells reacting to vimentin and Thy-1 might be a precursor of myofibroblasts in cutaneous wound healing (Jahoda and Reynolds, 2001; Gharzi et al., 2003). It is interesting to note that the α-SMA immunoreactivity was not increased in the perifollicular areas; α-SMA expression might be mediated by factors produced only in the wound bed.

A3 immunoexpression

A3 immunoexpression was seen in immature mesenchymal cells (pericytes) around BVs and bone marrow-stem cells in the fetuses and neonates of rats (Kumagai et al., 2000; Yamate et al., 2007). A3 would be useful for identification of mesenchymal stem cells in pathological lesions of rats, although the functions of antigen recognized by A3 are under investigation. In the wound area, A3-reacting cells were seen exclusively in newly-developing BVs. To identify the cell type, double immunolabeling was carried out; A3-positive cells in BVs reacted simultaneously to vWF and CD34, and the A3 immunoreactivity was also seen in Thy-1- and PDGFR-,-positive pericytes. The present study shows that the endothelial cells and pericytes in cutaneous granulation tissues came to more strongly express antigens recognized by A3, with increased neovascularization. Additionally, increased A3 immunoreactivity was found in the PDS cells which also showed a positive reaction to Thy-1 or vimentin. Based on these findings, it was considered that the A3-positive pericytes and PDS cells might be precursors of myofibroblasts which form granulation tissues. The well-developed myofibroblasts might come to express vimentin, α-SMA and Thy-1 after loss of A3 immunoreaction. It has been reported that pericytes have potential to turn into myofibroblasts under the effects of low level laser therapy (Medrado et al., 2010). However, further in vitro studies are needed to investigate the molecular mechanisms of myofibroblastic trans-differentiation of A3-positive cells, because there is a possibility that the A3-positive cells are a common origin with various different lineages.

The pericytes in the newly-developing BVs and the PDS cells in the wound bed-surrounding tissue might have been regarded as local tissue stem cells (Jahoda and Reynolds, 2001; Liu et al., 2008; Zannettino et al., 2008; Lau et al., 2009; Paquet-Fifield et al., 2009). It has recently been reported that the pericytes are able to proliferate and then migrate into fibrotic skin lesions, resulting in their transdifferentiation to myofibroblasts (Lin et al., 2008; Humphreys et al., 2010). It has been considered that normal skin tissue might contain multipotential stem cells, particularly around hair follicles (Gronthos et al., 2001; Jahoda et al., 2003; Hoogduijn et al., 2006; Liu et al., 2008; Lau et al., 2009). Mesenchymal stem cells in rat bone marrow gave a positive reaction to A3 (Yamate et al., 2007). The pericytes and PDS cells, which both showed increased expression for A3 might be in the bone marrow-stem cell lineage. CD34 is known to be expressed not only in the endothelial cells but also in hematopoietic stem cells (Trempus et al., 2003; Pusztaszeri et al., 2006), and the endothelial cells labeled by CD34 are regarded as endothelial progenitor cells (Trempus et al., 2003). Endothelial cells reacting to both A3 and CD34 might be referred to as endothelial progenitor cells derived from the bone marrow-stem cells.

In conclusion, the present study shows that the granulation tissue formed after cutaneous wound in rats contained myofibroblasts expressing vimentin and α-SMA; it was also found that the myofibroblasts expressed Thy-1. The double immunolabeling revealed that the pericytes in the newly-developing BVs reacted to vimentin, α-SMA and Thy-1, and that the PDS cells in the wound bed-surrounding skin tissue gave positive reactions to vimentin and Thy-1. The pericytes and PDS cells might be precursors of myofibroblasts in cutaneous fibrosis. Furthermore, although A3 immunoreactivity was not seen in developing myofibroblasts in the wound bed, the pericytes and PDS cells reacted to A3 with increased immunointensity and number. It has been considered that myofibroblasts in fibrous tissues might be derived from circulating bone marrow stem cells (Mori et al., 2005). Mesenchymal stem cells in the bone marrow also showed a positive reaction to A3 (Yamate

et al., 2007). The derivation of cutaneous myofibroblasts might be in the bone marrow-stem cell lineage through pericytes and PDS cells. This is the first report on the detailed characterization of myofibroblasts, and the relationship between myofibroblasts and stromal-/bone marrow-stem cells in cutaneous rat fibrosis.

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