

Human resident CD34+ stromal cells/telocytes have progenitor capacity and are a source of α SMA+ cells during repair

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Summary. We studied the progenitor capacity of human resident CD34+ stromal cells/telocytes (SC/TCs) in the enteric wall affected by inflammatory/repair processes (appendicitis, diverticulitis of large bowel and Crohn's disease of the terminal ileum) at different stages of evolution (inflammatory, proliferative and remodelling). In these conditions, CD34+ SC/TCs are activated, showing changes, which include the following overlapping events: 1) separation from adjacent structures (e.g., from vascular walls) and location in oedematous spaces, 2) morphological modifications (in cell shape and size) with presence of transitional cell forms between quiescent and activated CD34+ SC/TCs, 3) rapid proliferation and 4) loss of CD34 expression and gain of α SMA expression. These events mainly occur in the inflammatory and proliferative stages. During the loss of CD34 expression, the following findings are observed: a) irregular cell labelling intensity for anti-CD34, b) co-localization of CD34 and actin, c) concurrent irregular labelling intensity for α SMA and d) α SMA expression in all stromal cells, with total loss of CD34 expression. While CD34 expression was conserved, a high proliferative capacity (Ki-67 expression) was observed and vice versa. In the segments of the ileum affected by Crohn's disease, the stromal cells around fissures were α SMA+ and, in the transitional zones with normal enteric wall, activated CD34+ SC/TCs were observed. In conclusion, human

resident CD34+ SC/TCs in the enteric wall have progenitor capacity and are activated with or without differentiation into α SMA+ stromal cells during inflammatory/repair processes.

Key words: CD34+ stromal cells, Telocytes, α SMA+ stromal cells, Bowel, Crohn's disease

Introduction

Resident CD34+ stromal cells/telocytes (SC/TCs) (Popescu and Fausone-Pellegrini, 2010; Fausone-Pellegrini and Popescu, 2011; Popescu, 2011a,b), located in both perivascular and stromal positions in the connective tissue, act in the control and regulation of other cell types, mainly stem/progenitor cells (control of growth and differentiation) in stem cell niches ("nurse" progenitor cells) (Popescu, 2011a,b; Popescu et al., 2011a,b; Gherghiceanu and Popescu, 2012). Moreover, SC/TCs facilitate neoangiogenesis (physical contacts and chemical signalling, e.g., VEGF and NO) (Manole et al., 2011; Ceafalan et al., 2012; Popescu and Nicolescu, 2013) and, like mesenchymal stem cells, express PDGFR α and PDGFR β (Pieri et al., 2008; Suciu et al., 2012; Vannucchi et al., 2013). CD34+ SC/TCs therefore play an important role in repair (Popescu, 2011a,b; Popescu et al., 2011a,b; Popescu and Nicolescu, 2013; Manole et al., 2011; Ceafalan et al., 2012; Gherghiceanu and Popescu, 2012; Díaz-Flores et al., 2014a) and CD34 expression is the best available immunohistochemical choice for their identification (Fausone-Pellegrini and

Popescu, 2011; Díaz-Flores et al., 2013).

From a broad perspective, repair includes two types of processes: regeneration (damaged cells are replaced by other cells of the same type) and repair through granulation tissue (with neovascularization, abundant fibroblasts/myofibroblasts and recruitment of macrophages) (Díaz-Flores et al., 2009a, 2011a). In a previous work, we assessed the behaviour of human adipose-tissue-resident CD34+ stromal cells when activated *in vivo* during inflammatory/repair processes, at different stages of evolution. Our observations point to CD34+ stromal cells as a source of α smooth muscle actin (SMA)+ cells (myofibroblasts) during repair through granulation tissue (Díaz-Flores et al., in press). Therefore, in addition to functioning as a nurse of progenitor cells, CD34+ SC/TCs could also have a progenitor capacity *per se*. This capacity needs to be assessed in a specific tissue or organ in which previous studies have confirmed that CD34+ stromal cells are TCs, as occurs in the enteric wall (Pieri et al., 2008; Faussone-Pellegrini and Popescu, 2011; Milia et al., 2013; Vannucchi et al., 2013).

Given the above, this study was undertaken to assess the behaviour of human enteric tissue-resident CD34+ SC/TCs when activated during inflammatory/repair processes at different stages of evolution.

Materials and methods

Tissue samples

Specimens from the enteric wall affected by inflammatory/repair processes (appendicitis, diverticulitis of large bowel and Crohn's disease of terminal ileum) were obtained from the archives of the Department of Anatomy, Histology, Pathology and Radiology of the Faculty of Medicine/University Hospital of the Canary Islands and Department of Pathology Hospiten® Hospital (Spain). Specimens from the appendix and large bowel were selected following the criteria outlined in a previous work in which the peri-intestinal adipose tissue affected by inflammatory/repair processes was studied (Díaz-Flores et al., 2015). Depending on morphological characteristics, we chose the following stages/phases: inflammatory (vasodilation, hydration with formation of oedematous spaces, leukocyte margination and infiltration of leukocytes, predominantly neutrophils), proliferative (angiogenesis, stromal cell proliferation and granulation tissue formation with fewer neutrophils and a higher number of macrophages) and maturational/remodelling (tracts of spindle-like stromal cells between a collagen-rich matrix). Six samples were selected for each stage. In Crohn's disease (n: 4), specimens of terminal ileum presenting typical features, including narrow fissures, were selected. In general, patients were all Caucasian, 13 men and 9 women, aged from 12-68 years. Normal enteric wall was obtained from surgical specimens in which the enteric wall was not affected by pathological

processes. All protocols were performed in accordance with international ethical guidelines.

Light microscopy

Specimens were fixed in a buffered neutral 4% formaldehyde solution, embedded in paraffin, and cut into 4 μ m-thick sections, which were stained with hematoxylin and eosin (H&E).

Immunohistochemistry

For immunohistochemistry, three- μ m-thick sections were cut and attached to silanized slides. After deparaffinization and pretreatment for enhancement of labelling (antigen retrieval PT-Link (Dako), Ref. 1012), the sections were blocked with 3% hydrogen peroxide and then incubated with primary antibodies (Dako, Glostrup, Denmark) (10-40 min). The primary antibodies used in this study were as follows: CD-34, (ready to use), code no. IR63261; α smooth muscle actin (α -SMA), (ready to use), code no. IR61161; CD-31, (ready to use), code no. IR61061; CD-68 (ready to use) code no. IR 60961; h-caldesmon, (ready to use), code no. IR05461; Ki-67 (MIB1), (ready to use), code no. IR62661, and CD45 (ready to use) code no. IR751. For the double immunostaining, we used anti-CD34 antibody (DAB - undetected within nucleus) and anti-Ki67 (DAB - detected exclusively within nucleus) as a proliferation marker (marker of all active phases of the cell cycle and mitosis). The immunoreaction was developed in a solution of diaminobenzidine and the sections were then briefly counterstained with hematoxylin, dehydrated in ethanol series, cleared in xylene, and mounted in Eukitt®. Positive and negative controls were used.

Immunofluorescence and confocal microscopy

For immunofluorescence, tissue sections were obtained as described above. For antigen retrieval, sections were deparaffinized and boiled for 20 minutes in sodium citrate buffer 10 mM (pH 6), rinsed in Tris-buffered saline (TBS, pH 7.6, 0.05 M), and incubated with the following primary antibodies diluted in TBS overnight in a humid chamber at room temperature: mouse monoclonal anti-CD-34, code no. IR63261 (ready to use), goat polyclonal anti-actin (1/50 dilution, C-11, sc-1615, Santa Cruz Biotechnology), mouse monoclonal anti-CD68 (ready to use, Clone KP1, IR609, Dako) and mouse monoclonal anti-Iba1/AIF1 (1/200 dilution, MABN92; Millipore). For the double immunofluorescence staining, sections were incubated with a mixture of monoclonal and polyclonal primary antibodies (mouse monoclonal anti-CD34, and goat polyclonal anti-actin). The day after, the slides were rinsed in TBS and incubated for 1 h at room temperature in the dark with the secondary biotinylated donkey anti-goat IgG (H+L) (1:300, Code: 705-065-003, Jackson

Progenitor capacity of CD34+ stromal cells

ImmunoResearch) and Alexa Fluor 488 goat anti-mouse IgG (H+L) antibody (1:500, A11001, Invitrogen), followed by incubation with streptavidin Cy3 conjugate (1:500, SA1010, Invitrogen) for 1 h at room temperature in the dark. Nuclei were detected by DAPI staining (Chemicon International, Temecula, CA, USA). After washing in TBS, sections were exposed to a saturated solution of Sudan black B (Merck, Barcelona, Spain) for 20 minutes to block the autofluorescence. They were rinsed in TBS and then were cover-slipped with DABCO (1%) and glycerol-PBS (1:1). Negative controls were performed in the absence of primary antibodies. Fluorescence immunosignals were obtained using a Fluoview 1000 laser scanning confocal imaging system (Olympus Optical).

Results

Quiescent CD34+ SC/TCs and α SMA+ stromal cells in the enteric wall

Morphologically, quiescent (in unperturbed physiological conditions) enteric CD34+ SC/TCs show a slender body and long, bipolar or multipolar cytoplasmic processes (Fig. 1A-C) (telopodes), presenting thin segments (podomeres) and dilated portions (podoms). These cells are negative for anti- α SMA, anti-h-caldesmon, anti-CD45, anti-CD68 and anti-CD31. CD34+ SC/TCs are observed in the muscularis mucosae (on its surface and around groups of SMCs), submucosa (between collagen and elastic fibres), muscular propria (around fascicles and bundles of SMCs, and between SMCs) and serosa. In all these layers, CD34+ SC/TCs surround vessels of different calibre (Fig. 1D,E) and nerves. CD34+ SC/TCs are also present around ganglia of Meissner and Auerbach's plexuses (Fig. 1F). CD34+ SC/TCs are absent in the lamina propria of the mucosa (Fig. 1G) (except in some of its deep regions adjacent to muscularis mucosae) and in some areas immediately underlying the mesothelium. Conversely, α SMA+ cells are present in the lamina propria of the mucosa, except in some of the deep regions, and immediately underlying the mesothelium. Therefore, where CD34+ SC/TCs are present, α SMA+ stromal cells are absent, and vice versa.

Activated CD34+ SC/TCs in the enteric wall (appendicitis and diverticulitis)

Activated CD34+ SC/TCs are observed during the inflammatory and proliferative stage of repair in the layers of the enteric wall, where they are normally present. Thus, at the inflammatory stage, activated CD34+ SC/TCs conserve the location outlined above (although they separate from the vascular wall and from other cells and interstitial fibrils), increase in size and appear as large, plump, stellate or ovoid cells in oedematous spaces (Fig. 2A-F). The nuclei also increase in size and show one or two prominent nucleoli (Fig. 2F). Inflammatory cells (mainly neutrophils in the inflammatory stage and macrophages in the proliferative

stage) are also present in the oedematous spaces. Transitional cell forms between quiescent and activated CD34+ SC/TCs are also present, conserving more or less evident telopodes (Fig. 2A,F). These phenomena occur most commonly around vessels, regardless of size (Fig. 2A-E). Activated CD34+ SC/TCs show mitoses (Fig. 3A-C), which increase in number at the onset of the proliferative stage. These cells in mitoses adopt a rounded morphology, and expression of CD34 is peripheral, leaving an unstained central zone where the chromosomes are highly evident (Fig. 3A-C). A high proliferative index is demonstrated in sections double-stained with anti-CD34 and anti-Ki-67 (Ki-67 is detected exclusively within nucleus, whereas CD34 is not) (Fig. 3D). None of the activated stromal cells are α SMA+ in the inflammatory stage (positivity for anti- α SMA was only observed in pericytes and SMCs), and few stromal cells are α SMA+ at the onset of the proliferative stage.

Loss of CD34 expression in activated SC/TCs. Progressive differentiation into α SMA+ stromal cells (appendicitis and diverticulitis)

As the proliferative stage progresses, loss of CD34 and gain of α SMA expression occurs during and after mitotic activity in activated CD34+ SC/TCs. Thus, activated CD34+ SC/TCs and α SMA+ stromal cells may coincide in several locations. In some of these locations, both types of cells present different labelling intensity in each type (ranging from high to low expression of CD34 and α SMA) (Fig. 4A,B). Co-location of CD34 and actin is observed in activated SC/TCs by double immunofluorescence (Fig. 4C-E,F-tH). When granulation tissue is formed, the majority of SC/TCs express α SMA, while CD34 expression decreases dramatically (Fig. 5A). α SMA+ stromal cells show a higher expression of this marker in cytoplasmic bundles parallel to their longitudinal surface. α SMA+ stromal cells conserve their increased size, show an elongated morphology and are interrelated with other neighbouring α SMA+ stromal cells. During granulation tissue formation, a high number of macrophages are also detected by immunofluorescence (insert of Fig. 5A).

All stromal cells are α SMA+ (absence of CD34+ SC/TCs) at onset of maturational/remodelling stage (appendicitis and diverticulitis)

Vessel regression and decrease in the number of inflammatory cells occur at the onset of the maturational/remodelling stage. Abundant spindle-like stromal cells are present. All these cells are α SMA+ (Fig. 5B) and no CD34+ stromal cells are observed. In some areas between fascicles of the muscle layers, α SMA+ stromal cells are also present (Fig. 5C).

Activated CD34+ SC/TCs and α SMA+ cells in the ileum affected by Crohn's disease

The peculiar distribution ("skip" lesions), transmural

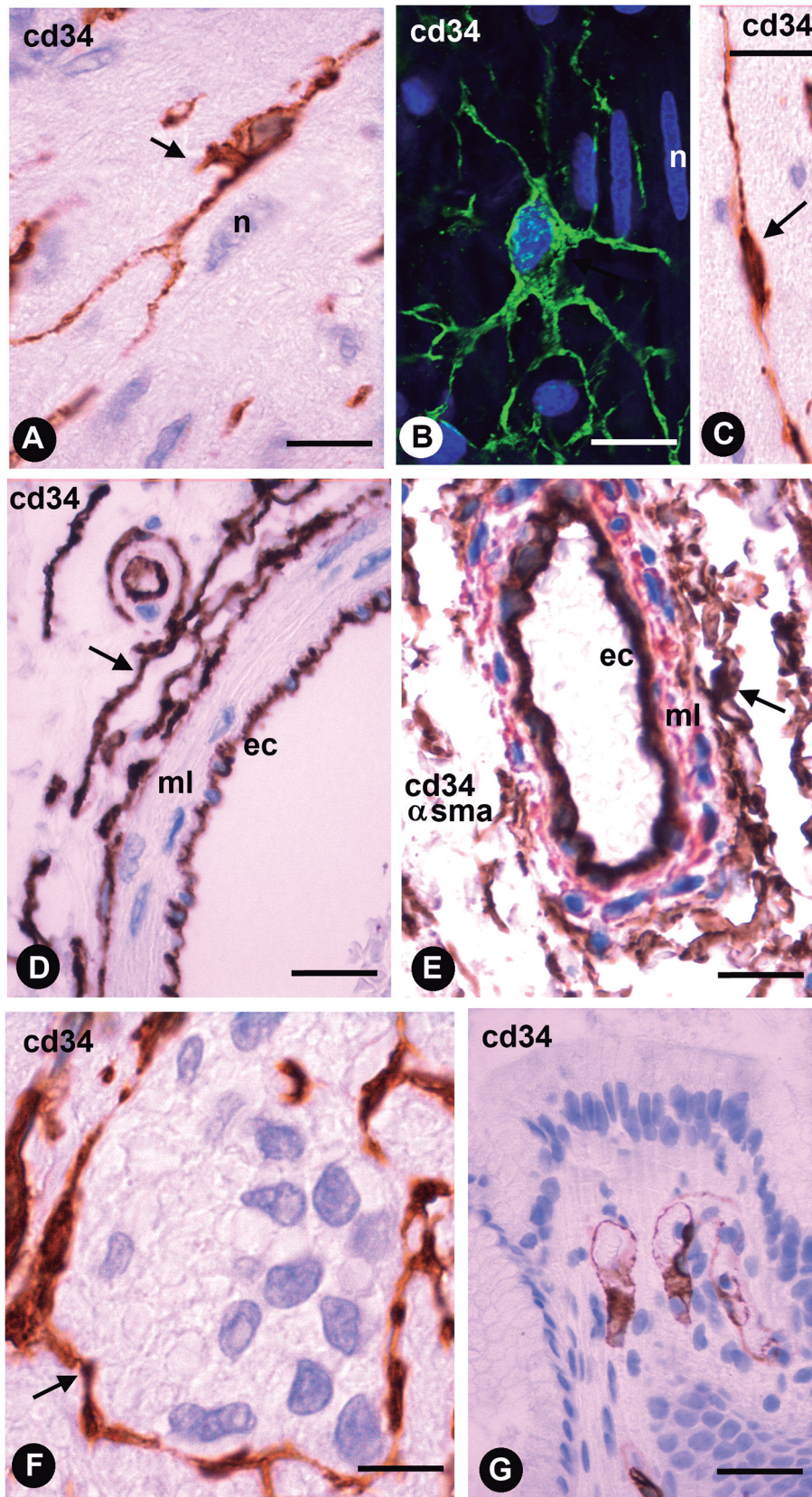


Fig. 1. CD34+ SC/TCs (arrows) in normal enteric wall. **A-C.** Bipolar and multipolar CD34+ SC/TCs between SMCs of muscular propria. **D, E.** CD34+ SC/TCs around vessels. In D, one medium-sized and one small vessel are observed. Endothelial cells (in the intima) and SC/TCs (in the adventitia), both stained with CD34, sandwich the unstained media layer. In E, a medium-sized vessel double-stained with anti-CD34 (expression in ECs and CD34+ SC/TCs, black) and anti-αSMA (expression in SMCs of the media layer, red). **F.** CD34+ SC/TCs envelope a ganglion of the myenteric plexus. **G.** Vessels of the mucosa do not show CD34+ SC/TCs. Nuclei of the SMCs of the lamina propria: n; Endothelial cells: ec; media layer: ml. **A, C, D, F, G.** CD34 immunoperoxidase labelling with haematoxylin counterstain. **B.** Immunofluorescence labelling for CD34. (Nuclei are counterstained with DAPI). **E.** Double CD34 (black) and αSMA (red) immunoperoxidase labelling with haematoxylin counterstain. Bar: A-C, 10 μm; D, E, G, 20 μm; F, 8 μm.

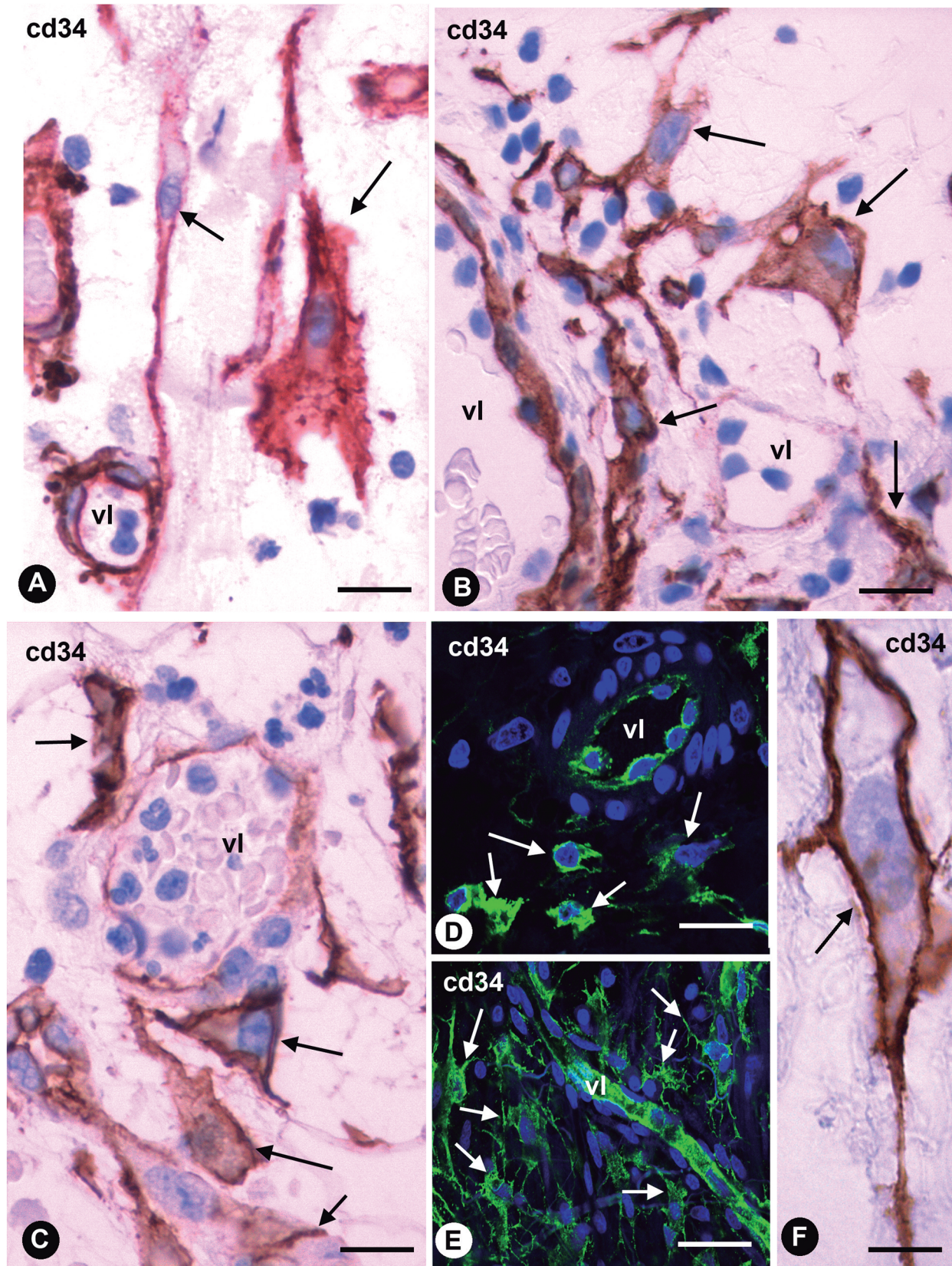


Fig. 2. Activated CD34+ SC/TCs (arrows) during the inflammatory stage of repair (appendicitis) are observed in oedematous spaces around vessels of different calibre. Most CD34+ SC/TCs increase in size and appear as large, plump, stellate or ovoid cells. The nuclei are also increased in size and show prominent nucleoli. Some cells conserve their telopodes (A, F). A-C, D, F. CD34 immunoperoxidase labelling with haematoxylin counterstain. D, E. Immunofluorescence labelling for CD34 (green) (nuclei are counterstained with DAPI). vl: Vessel lumen. Bar: A-C, 10 μ m; D, E, 15 μ m; F, 6 μ m.

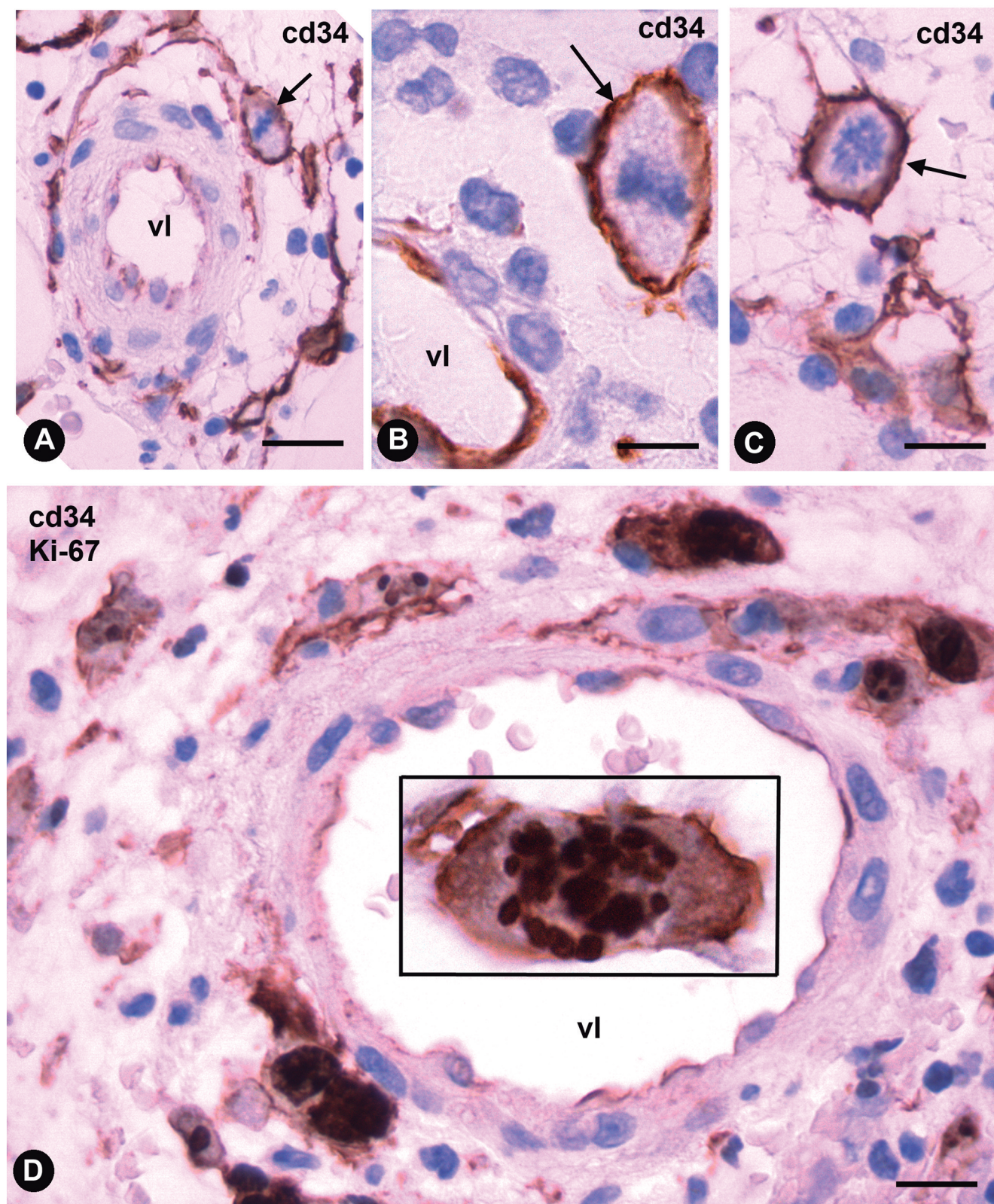


Fig. 3. Activated CD34+ SC/TCs in mitosis (arrows) around vessels of different calibre during proliferative stage (appendicitis) (**A-C**) and with Ki-67 expression (**D**). **A-C.** The cells in mitosis show rounded morphology with peripheral expression of CD34 and chromosomes in the unstained central zone. **D.** High proliferative index in CD34+ SC/TCs is demonstrated in a section double-stained with anti-CD34 (peripheral expression) and anti-Ki-67 (expression in nuclei). In the insert, one activated CD34+ SC/TC in mitosis, showing anti-Ki-67-stained chromosomes. vl: Vessel lumen. Bar: A, 20 µm; B, C, 10 µm; D, 15 µm; insert, 6 µm.

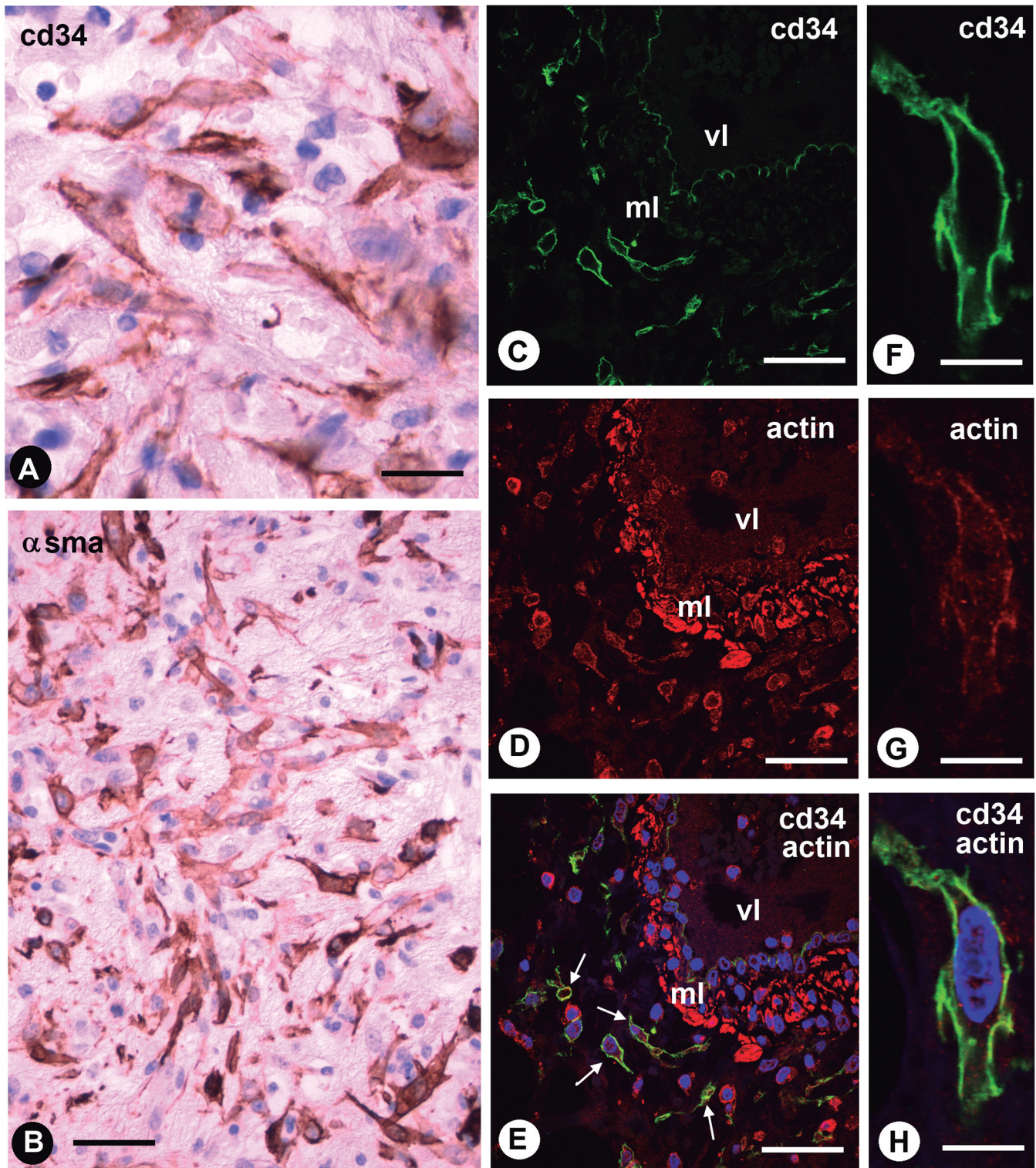


Fig. 4. Activated CD34+ SC/TCs during differentiation into αSMA+ stromal cells (appendicitis). **A.** A zone in which neighbouring CD34+ stromal cells show different CD34 labelling intensity. **B.** A similar zone with αSMA+ stromal cells, which also show different labelling intensity. **C-E.** Co-localization of CD34 and actin (arrows) in stromal cells around a vessel, demonstrated by double-immunofluorescence. Nuclei are counterstained with DAPI. **F-H.** Using a similar procedure, co-localization of CD34 and αSMA in a stromal cell. vl: Vessel lumen. ml: Media layer. Bar: A, 10 μm; B, 15 μm; C-E, 20 μm; F-H, 8 μm.

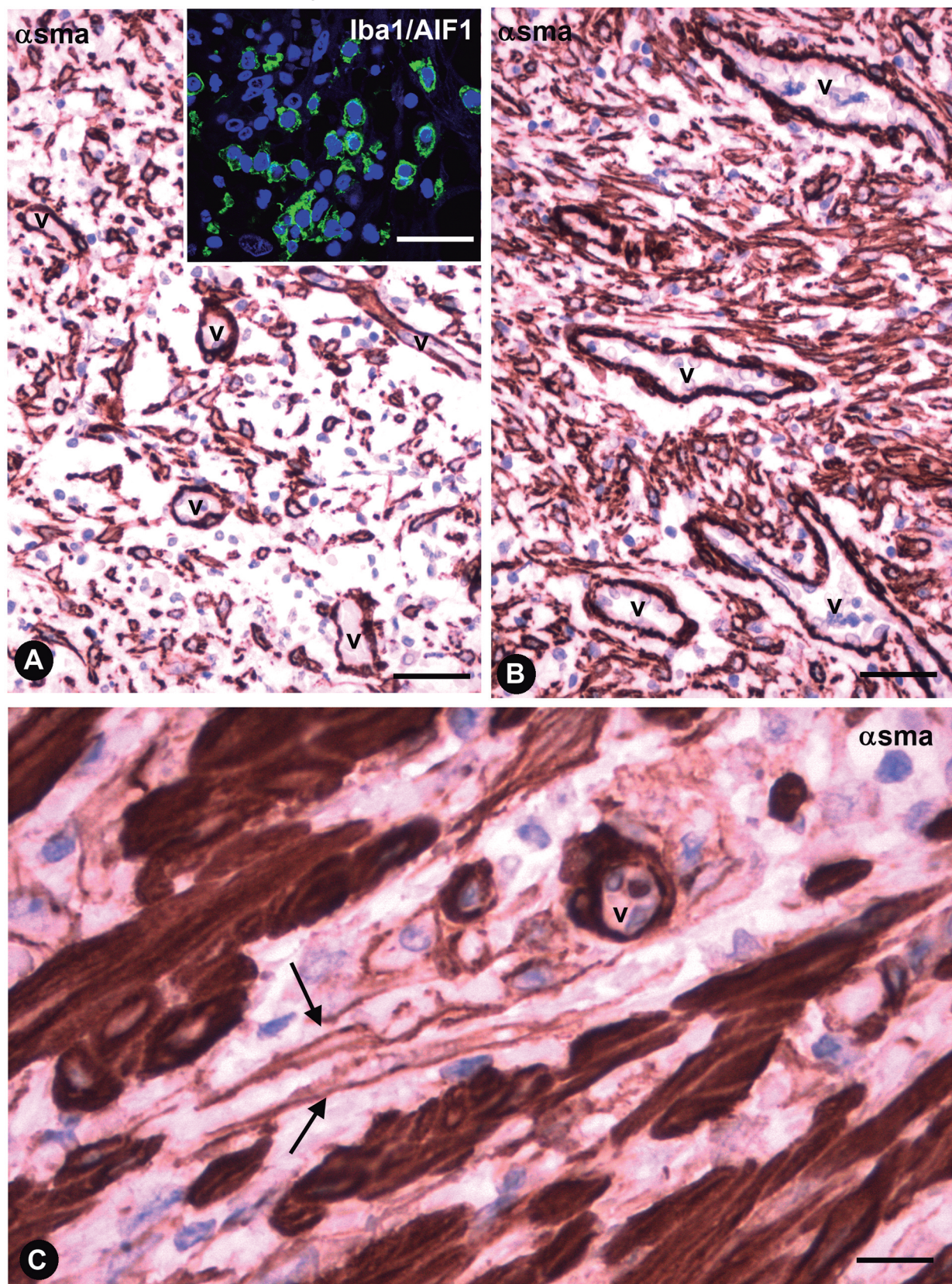


Fig. 5. Stromal cells at the end of proliferative stage (in granulation tissue) and onset of remodelling stage (diverticulitis). **A.** Most stromal cells show αSMA expression at the end of the proliferative stage. Abundant vessels (v) are also observed. In the insert, numerous macrophages express Iba1/AIF1 (detected by immunofluorescence). **B.** All stromal cells show αSMA expression at the onset of the remodelling stage. **C.** Anti- αSMA+ stromal cells (arrows) around small vessels (v) and between fascicles of SMCs in the lamina propria. Bar: A, B, 20 μm; insert, 25 μm; C, 15 μm.

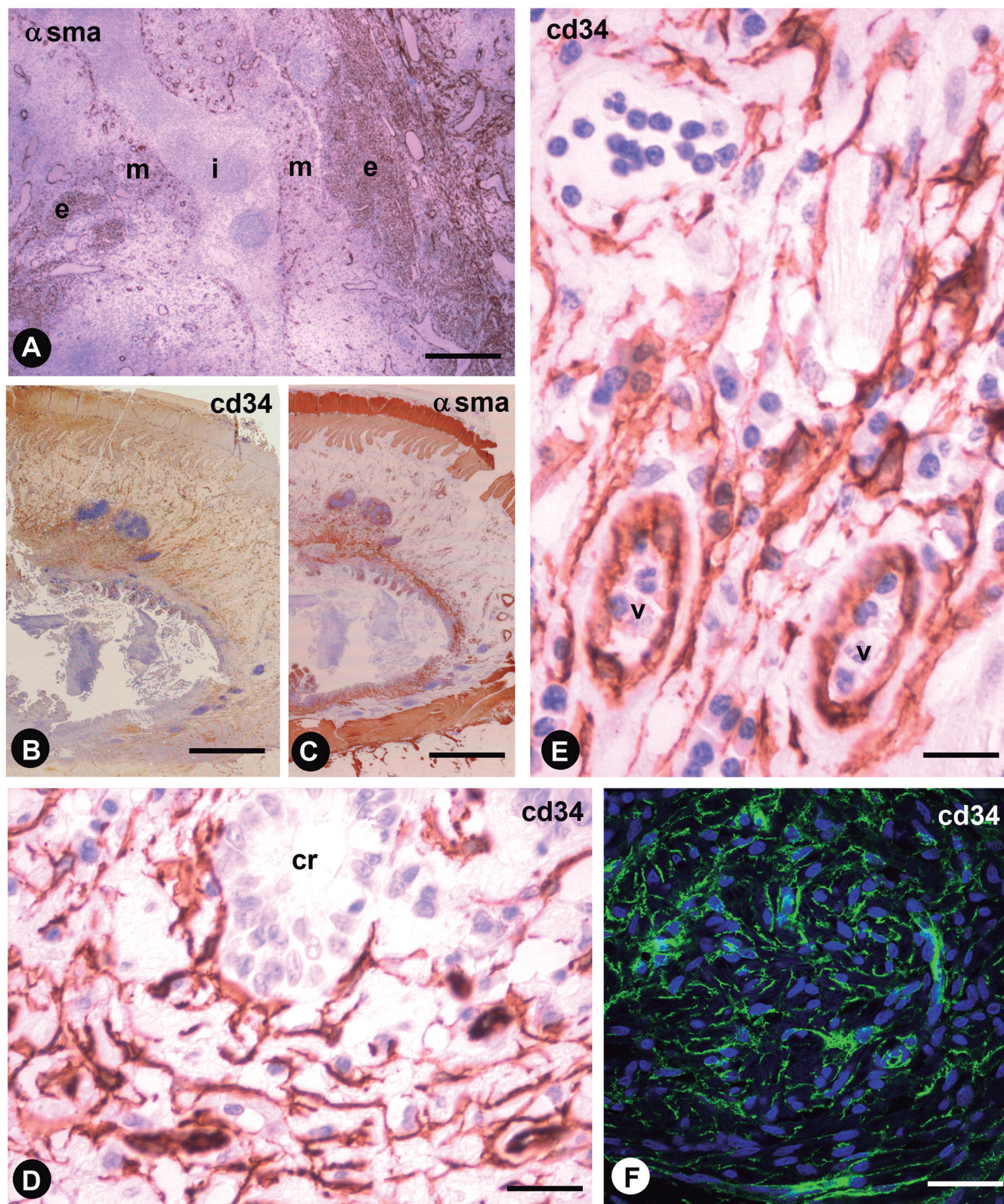


Fig. 6. CD34+ SC/TCs and α SMA+ stromal cells in the terminal ileum affected by Crohn's disease. **A.** A fissure in which three layers are observed: 1) internal (i) with fibrinous exudate, necrotic debris and inflammatory cells. 2) media (m) with inflammatory cells and vessels, and 3) external (e) with abundant α SMA+ stromal cells. **B, C.** Two sequential sections (stained with anti-CD34 and anti- α SMA, respectively) of a transitional zone between an affected segment and the normal bowel. Activated CD34+ SC/TCs are observed in all layers of the enteric wall (except superficial zone of mucosa) (**B**) with absence of α SMA+ stromal cells (α SMA+ cells are smooth muscle cells and vascular SMC/pericytes) (**C**). **D, E.** Activated CD34+ SC/TCs around the base of a crypt (cr) and between smooth muscle cells of muscularis mucosae (**D**) and around vessels (v) of the submucosa (**E**). **F.** Activated CD34+ SC/TCs in a nerve, demonstrated by immunofluorescence (Nuclei are counterstained with DAPI). Bar: A, 35 μ m; B, C, 50 μ m; D, E, 15 μ m; F, 25 μ m.

inflammation with lymphoid aggregates and epithelioid granulomas (in two cases), dilation of lymphatic channels, edema, fibrosis, ulcers and narrow fissures were observed in the ileum affected by Crohn's disease. We pay particular attention to the fissures and the transitional zones between diseased and normal segments of bowel.

Depending on microscopic characteristics, some fissures affected by Crohn's disease show an environment in which three layers can be systematized: 1) internal, formed by a fibrinous exudate, necrotic debris and inflammatory cells (predominantly neutrophils), 2) media, with inflammatory cells and varying number of vessels, and 3) external, whose principal component are spindle-shaped cells, which show α SMA expression (Fig. 6A).

In transitional zones between sharply demarcated diseased segments and normal bowel, activated CD34+ SC/TCs persist in all the layers of the enteric wall, where they are normally present (Fig. 6B). Conversely, α SMA+ stromal cells are not evident (anti- α SMA-stained cells are smooth muscle cells and pericytes) (Fig. 6C). Thus, CD34+ SC/TCs, increased in number and size, are observed in the hypertrophic muscularis mucosae and in deep regions of adjoining mucosa around the base of the crypts (Fig. 6D), in the submucosa (Fig. 6E), muscular propria and serosa. These activated cells frequently appear around vessels and in nerves (Fig. 6F).

Discussion

Our observations during inflammation and repair through granulation tissue in the human enteric wall demonstrate that resident/native CD34+ SC/TCs change from quiescent (unperturbed physiological conditions) to activated stages with phenotypic transformation. Phenotypic modifications include morphological changes (in cell shape and size), rapid proliferation and evolution of CD34 expression towards α SMA expression. This evolution occurs quickly and comprises the following overlapping events in the stromal cells: a) expression of CD34, b) irregular cell labelling intensity for anti-CD34, c) co-localization of CD34 and actin, d) concurrent irregular labelling intensity for α SMA and e) all stromal cells lose CD34 expression and gain α SMA expression. The results concur with those of a previous work, in which we demonstrated that, in similar conditions, native CD34+ stromal cells in adipose tissue can differentiate into α SMA+ stromal cells (myofibroblasts) (Díaz-Flores et al., 2015). In our opinion, CD34+ stromal cells in the adipose tissue may be CD34+ SC/TCs. This being the case, the progenitor capacity outlined here could be considered as a function of CD34+ SC/TCs in general. In this way, a mesenchymal capacity has been suggested for a subset of resident quiescent slow-cycling stromal cells with high proliferative potential. These stromal cells express CD34 and, due to their general characteristics, may be

CD34+ SC/TCs (Popescu et al., 2007; Díaz-Flores et al., 2014a; Sidney et al., 2014).

The principal objective of our previous work on human native CD34+ stromal/progenitor cells in the adipose tissue was to compare their behaviour *in vivo* during inflammatory/repair processes at different stages of evolution with that outlined in the exponential research on these cells *in vitro*. Indeed, in the freshly isolated stromal vascular fraction (SVF), adipose stromal/stem/progenitor cells (ASCs) are found in the CD34+ population, which is negative for hematopoietic (CD45, CD14), pericytic (40b, 146, α SMA) or endothelial (CD31) markers (Boquest et al., 2005; Sengenès et al., 2005; Mitchell et al., 2006; Lin et al., 2008; Rodeheffer et al., 2008; Suga et al., 2009; Lin and Xu, 2010; Lin et al., 2010; Zimmerlin et al., 2010, 2013; Li et al., 2011; Maumus et al., 2011; Bassi et al., 2012; Bourin et al., 2013; Braun et al., 2013). ASCs have the capacity to adhere firmly to culture plasticware, and to proliferate (provided that CD34 expression is maintained) and differentiate into multiple lineages, such as adipocytes, osteoblasts, chondrocytes and myocytes (Halvorsen et al., 2000; Zuk et al., 2001, 2002; Erickson et al., 2002; Mizuno et al., 2002; Gimble and Guilak, 2003; Hicok et al., 2004; Justesen et al., 2004; Gimble et al., 2007; Daher et al., 2008; Suga et al., 2009). In culture, ASCs lose CD34 expression in successive passages (Mitchell et al., 2006; Lin et al., 2008; Suga et al., 2009; Lin and Xu, 2010; Lin et al., 2010, 2012; Maumus et al., 2011; Bassi et al., 2012; Bourin et al., 2013) and, depending on culture conditions, express other markers, such as α SMA. In our observations during repair *in vivo* in the enteric wall, CD34 expression correlates positively with a high proliferative capacity (double-staining of SC/TCs with CD34 and Ki-67) and differentiation potential, while CD34 expression is lost with successive mitoses and acquisition of α SMA expression (with cell expansion and commitment). Therefore, our previous and current observations concur with the behaviour of stromal cells in culture (Mitchell et al., 2006; Suga et al., 2009; Maumus et al., 2011; Braun et al., 2013; Zimmerlin et al., 2013).

Our results revealing loss of CD34+ SC/TCs and gain of α SMA+ stromal cells in Crohn's disease, mainly in tissues surrounding fissures and in fibrotic areas, concur with those of Milia et al. (2013). Indeed, these authors reported the loss of CD34+ PDGFR α cells and the presence of many α SMA+ spindle-shaped myofibroblasts in some damaged areas of the enteric wall affected by Crohn's disease. Likewise, our observations of the persistence of activated CD34+ SC/TCs in areas adjacent to the lesion agree with previous works (Díaz-Flores et al., 2011b, 2014a,b), in which we describe the activation of CD34+ stromal cells without transformation to α SMA+ stromal cells. In other words, repair after injury may occur with myofibroblast differentiation or without immunophenotypic transformation to myofibroblasts. The distribution of different molecules and the interaction with the

extracellular matrix may condition cell differentiation, the mechanism of which requires further study.

In several processes of longer evolution (e.g., in the stroma of some tumours), in which stromal cells lose CD34 expression and gain α SMA expression (Nakayama et al., 2000, 2001, 2003; Barth et al., 2002a-c, 2004, 2005; Chauhan et al., 2003; Ramaswamy et al., 2003; Kuroda et al., 2005a,b; Ebrahimsade et al., 2007; Nimphius et al., 2007; Wessel et al., 2008), co-localization of CD34 and actin has rarely been reported. We have chosen examples that include the initial stages of the processes, enabling us to observe the rapid phenotypic changes, which occur in a short period of time. Conversely, when the lesion corresponds to a later stage, the period of proliferation and differentiation has already passed, which would explain the absence of cells co-expressing both markers in later stages of the processes.

The phenomena of activation, proliferation and differentiation of CD34+ SC/TCs in the enteric wall are observed predominantly around vessels, in a vascular/perivascular niche, and occur simultaneously with angiogenesis and inflammatory cell recruitment. The vascular/perivascular niche encompasses CD34+ SC/TCs, mural cells (pericytes and SMCs), endothelial cells, cells transmigrating through parietal microvasculature, the extracellular matrix (including the basement membrane) and soluble molecules (Díaz-Flores et al., 2009b). Interactions between these cells and with extracellular matrix constitute a microenvironment where they are controlled in their cell-renewing capacity and differentiation. In our cases, the proliferation and differentiation of CD34+ SC/TCs occur predominantly during the proliferative stage, with angiogenesis and granulation tissue formation. Autocoids, autocrines and paracrines, and mediators present in the microcirculation participate in the mechanisms that intervene in the regulation of quiescent and angiogenic stages of blood vessels (Díaz-Flores et al., 1994, 2009a,b) and probably in quiescent and activated stages of CD34+ SC/TCs. Likewise, SC/TCs may act in angiogenesis by means of physical contacts and chemical signalling, including VEGF and nitric oxide (Manole et al., 2011; Ceafalan et al., 2012; Popescu and Nicolescu, 2013).

In addition to SC/TCs, CD34 is a common marker for diverse progenitors (e.g., hematopoietic progenitor cells, circulating fibrocytes, vascular endothelial cells, muscle satellite cells and hair follicle stem cells) (Sidney et al., 2014). Therefore, CD34+ circulating fibrocytes may also contribute as a source of α SMA stromal cells. Indeed, CD34+ circulating fibrocytes may arise from a subset of circulating monocyte-like cells (Bucala et al., 1994; Abe et al., 2001) and may enter the injured tissues and contribute to myofibroblasts (Abe et al., 2001; Reilkoff et al., 2011). In other words, the participation of native CD34+ SC/TCs as a source of α SMA+ cells during different processes of repair does not exclude a contribution of CD34+ circulating fibrocytes. The extent

to which both types of cells participate in this origin requires further study.

To sum up, in addition to a CD34+ SC/TC function in stem-progenitor cell regulation, widely studied by Popescu et al. (Popescu et al.'s detailed description of these cells in different anatomical sites is particularly interesting) (see www.telocytes.com) (Popescu, 2011a,b; Popescu et al., 2011a,b; Popescu and Nicolescu, 2013; Manole et al., 2011; Ceafalan et al., 2012; Gherghiceanu and Popescu, 2012), we contribute findings revealing resident CD34+ SC/TCs as progenitor cells *per se in vivo* during repair processes.

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