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Angiogenesis in mucous retention cyst: a human *in vivo*-like model of endothelial cell differentiation in mucous substrate

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BACKGROUND: Mucous retention cysts contain a mucous pool in the lumina, in which pure angiogenic processes are occasionally observed. By using this unique human material, our aim was to understand the *in vivo* angiogenic process.

METHODS: Fifteen surgical tissue samples of mucous retention cysts of the lip were examined for expression of vascular endothelial markers and extracellular matrix molecules by immunohistochemistry and *in situ* hybridization (ISH).

RESULTS: Endothelial cells forming new vascular channels showed immunopositivities for CD31, CD34, vascular endothelial growth factor (VEGF), and von Willebrand factor (vWF). These newly formed capillaries were surrounded by tenascin-positive matrices and further by a dense infiltration of CD68-positive cells with foamy to epitheloid appearances. Some of these cells were simultaneously positive for CD34, VEGF, and one of its receptors, Flk-1, and they showed definite mRNA as well as protein signals for tenascin. In addition, these cells often tended to be aligned, which suggested tubule formation.

CONCLUSION: The results suggest that monocyte/macrophage lineage cells are a major source for endothelial cells at least in mucous retention cysts and that tenascin produced by those cells plays an important role in differentiation of endothelial cells.

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Keywords: angiogenesis; CD31; CD34; endothelial progenitor; Flk-1; mucous retention cyst; tenascin; vascular endothelial growth factor

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Introduction

Angiogenesis is one of the most important stromal reactions in every kind of pathophysiological event. However, how neovascularization takes place in live tissue has not been fully established, especially in recruitment and differentiation of endothelial cells in terms of whether or not they originate from detached endothelial cells of injured vessels or hematogenous progenitor/stem cells (1, 2). It has been recently hypothesized that endothelial cell progenitors originate mainly from peripheral blood circulation based on *in vitro* studies in which CD34-positive mononuclear blood cells express vascular endothelial growth factor (VEGF) receptors, such as Flk-1 and Tie-2 (3, 4). When these cells are stimulated, for instance, by culture on matrigel, they can be differentiated to endothelium-like cells expressing VEGF and can form vessel-like structures (5). These progenitor cells of hematogenous origin have been shown to be potent to proliferate and to differentiate into endothelial cells in the presence of VEGF (6).

On the contrary, *in vivo* analyses have not yet been widely performed, because it has been difficult to establish reasonable animal models, and because angiogenesis in pathologic conditions is an extremely complicated process in which many other kinds of cells besides endothelial cells are involved (7, 8). Therefore, *ex vivo* studies have been devised in which isolated blood vessels of animals or humans were cultivated to examine capillary-like structures sprouting from the vessel walls (9, 10). Such kinds of investigations have indicated that endothelial progenitor cells originated from undifferentiated mesenchymal cells located in the perivascular stroma (11). Thus, it is still controversial whether endothelial progenitor cells are from hematogenous stem cells or local stem cells.

Mucous retention cysts are one of the most common lesions which are diagnosed during routine surgical pathology service for dental practice (12–14). Oral pathologists usually notice that mucous retention cysts

often are displayed as beautiful examples of endothelial cell sprouting with tubule formation in the background of the mucous substance retained in the cystic lumen. This histologic feature of endothelial cell proliferation is quite similar to *in vivo* angiogenic processes by endothelial cells embedded in gel culture (5), because there is no association of other kinds of cells besides endothelial cells and no pre-existing extracellular matrices produced by different kinds of cells other than endothelial cells within the cystic lumina. Because of this advantage of an unusual process of granulation formation, mucous retention cysts can be considered as a natural human *in vivo*-like material for studying pathologic angiogenesis. The mucin pool without pre-existing collagenous matrices seems to function as a sort of culture medium for endothelial cell growth and differentiation.

In this study, we focused on the process of neovascularization in this particular human lesion, and morphologically analyzed endothelial cell differentiation as well as the role of some extracellular matrix (ECM) molecules in the angiogenetic process by means of immunohistochemical and ISH techniques.

Materials and methods

Materials

A total of 232 surgical samples of mucous retention cysts (mucoceles) diagnosed in the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences during the period between 1995 and 2001 were reviewed, and 15 typical samples with neovascularization within the cystic lumen were selected. Surgical materials were fixed in 10% formalin routinely processed and embedded in paraffin. Serial sections were cut at 5 μm thickness, and one of each set of the sections was stained with hematoxylin and eosin (HE) and alcian blue. Another set of sections was used for immunohistochemical staining for ECM molecules, such as tenascin, type IV collagen; for endothelial cell markers, such as VEGF and its receptor, Flk-1, von Willebrand factor (vWF), CD31 and CD34; and for the monocyte/macrophage marker, CD68. The other set of serial sections was used for *in situ* hybridization (ISH) for tenascin mRNA.

Antibodies

Rabbit polyclonal antibodies against tenascin were purchased from Chemicon International Co. (Temecula, CA, USA; diluted at 1:500). Rabbit polyclonal antibodies against type IV collagen were purchased from ICN Pharmaceuticals Inc. (Aurora, OH, USA; 1:100). A mouse monoclonal antibody against VEGF (C-1) was obtained from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA; 1:50). A mouse monoclonal antibody against human CD31 (JC-70A) was purchased from Dako Ltd (Copenhagen, Glostrup, Denmark; 1:50). A mouse monoclonal antibody against human CD34 (NU-4A1) was purchased from Nichirei Co. (Tokyo, Japan; 1:100). Rabbit polyclonal antibodies against human vWF were purchased from Dako (1:200). A mouse monoclonal antibody against human CD68 (PG-M1) was also obtained from Dako (1:50). A mouse monoclonal antibody against Flk-1 (A-3) was purchased from Santa Cruz (1:50).

Immunohistochemistry

Immunohistochemical experiments were performed by using the Envision peroxidase system for rabbit and mouse antibodies (Dako). After deparaffinization, sections were treated in 0.3% H_2O_2 in 0.01 M phosphate buffered saline (PBS) for 30 min at room temperature to block endogenous peroxidase activity. The sections were then rinsed in 0.01 M PBS and pretreated to restore antigenicity. For ECM molecules, the sections were, digested with 3 mg/ml hyaluronidase (type I-S, 440 U/g; Sigma Chemical Co., St Louis, MO, USA) in PBS for 30 min at 37°C. For CD31, CD34, and CD68, the, sections were treated with 0.1% (w/v) trypsin (type II; Sigma) in 0.05 M Tris-HCl (pH 7.6) containing 1% $CaCl_2$ for 30 min at 37°C. For type IV collagen, the sections were digested with 4 mg/ml pepsin (Sigma) in 0.01 M HCl for 30 min at 37°C. After enzymatic treatments, the sections were incubated in 5% skim milk in PBS containing 0.05% triton X-100 (PBST) for 30 min at 37°C to block non-specific protein bindings, and then the sections were incubated at 4°C overnight with the primary antibodies. After rinsing in PBST, the sections were incubated with goat antibodies against rabbit or mouse immunoglobulin G (IgGs) conjugated with peroxidase-labeled dextgran polymers (EnVision + peroxidase, rabbit/mouse; Dako) for 30 min at room temperature. The peroxidase reaction products were visualized by incubation with 0.02% 3,3′-diaminobenzidine (DAB; Dohjin Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl solution (pH 7.4) containing 0.005% H_2O_2. The sections were then counterstained with hematoxylin.

Double immunostainings were performed for VEGF vs. CD68, CD31 vs. CD68, and tenascin vs. CD68. In the first step, CD68 and tenascin (for the first two double immunostainings) were localized with the Envision system and the reaction products were visualized in brown with DAB. Then, the sections were washed with 0.1 M glycine-HCl (pH 2.2). In the second step, VEGF, CD31, and CD68 were immunolocalized with the ABC method using avidin-alkaline phosphatase ABC kits purchased from Vector Laboratories (Burlingame, CA, USA) and visualized in blue with Nitro blue tetrazolium chloride (NBT) and 5Bromo-4-chloro-3-indolyl phosphate, toludine salt (BCIP) purchased from F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

In situ *hybridization*

Tenascin RNA probes were also prepared with DIG RNA labeling kits (Roche) using SP6/T7 RNA polymerases (Roche). A template of cDNA corresponding to a fragment of EGF-repeats of human tenascin (834–1037 bp) was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using RNA samples of squamous epithelial cells of human oral mucosa and sense (5′-CCCAA GCTTT GGAGT CTGCA-3′, including a *Hind*III site) and antisense (5′-GGAAT TCCAC

ACGCA CTCAT-3′, including an *Eco*RI site) primers. The cDNA was digested with *Hind*III and *Eco*RI, and the resultant 204 bp fragments were ligated into pSPT18/19 vector (Roche). The vector plasmids were linearized with *Hind*III and then transcribed with SP6 RNA polymerase as antisense probes or were linearized with *Eco*RI and then transcribed with T7 RNA polymerase as sense probes.

In situ hybridization was performed as described elsewhere (15). In short, paraffin sections cut at 5 μm were used. After deparaffinization, they were washed in three changes of 2X standard saline citrate (SSC), and treated with 5 μg/ml of proteinase K (Sigma) for 20 min at 37°C. They were then washed with 0.2% glycine in PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5) for 30 min, dehydrated with a series of ethanol (70–100%), and air-dried. Hybridization was performed at 45°C for 16 h in a moist chamber. The hybridization solution contained 10 mM phosphate buffer (pH 7.4), 10% dextran sulfate, 1X Denhardt's solution, 100 μg/ml of salmon sperm DNA, 125 μg/ml of yeast tRNA, 3X SSC, 50% formamide, and 500 ng/ml of probes. After hybridization, the sections were rinsed in 2X SSC and then the hybridized probes were detected with DIG detection kits (alkaline phosphatase Roche). The sections were counterstained with methyl green.

Results

Histopathology

All the 15 cases of mucous retention cysts selected after our histologic review were of the extravasation type. Histologically, the lesions were composed of basically immature granulation tissues, which were located in the lamina propria to the submucosal layer of the oral mucosa (Fig. 1a). The granulation tissues contained pools of extravasated saliva, which were alcian blue positive, in the center. At the boundary of the mucous retention, granulation tissues became occasionally thickened by a dense proliferation of fibroblastic or endothelial cells with varying degrees of neutrophils and macrophages infiltration. The neutrophils and foamy macrophages were in the process of being segregated from the granulation tissues into the retained mucous pool. In addition to these extravasated inflammatory cells, there were definite numbers of newly formed blood capillaries or their extending postcapillary venules within the mucous pools (Fig. 1b). In close proximity to these small vascular channels, mononuclear and round to polygonal or spindle-shaped cells with eosinophilic cytoplasm were scattered in addition to such inflammatory cells as neutrophils or foamy macrophages (Fig. 1c). They were focally packed in a dense fashion but were also sparsely distributed around short and

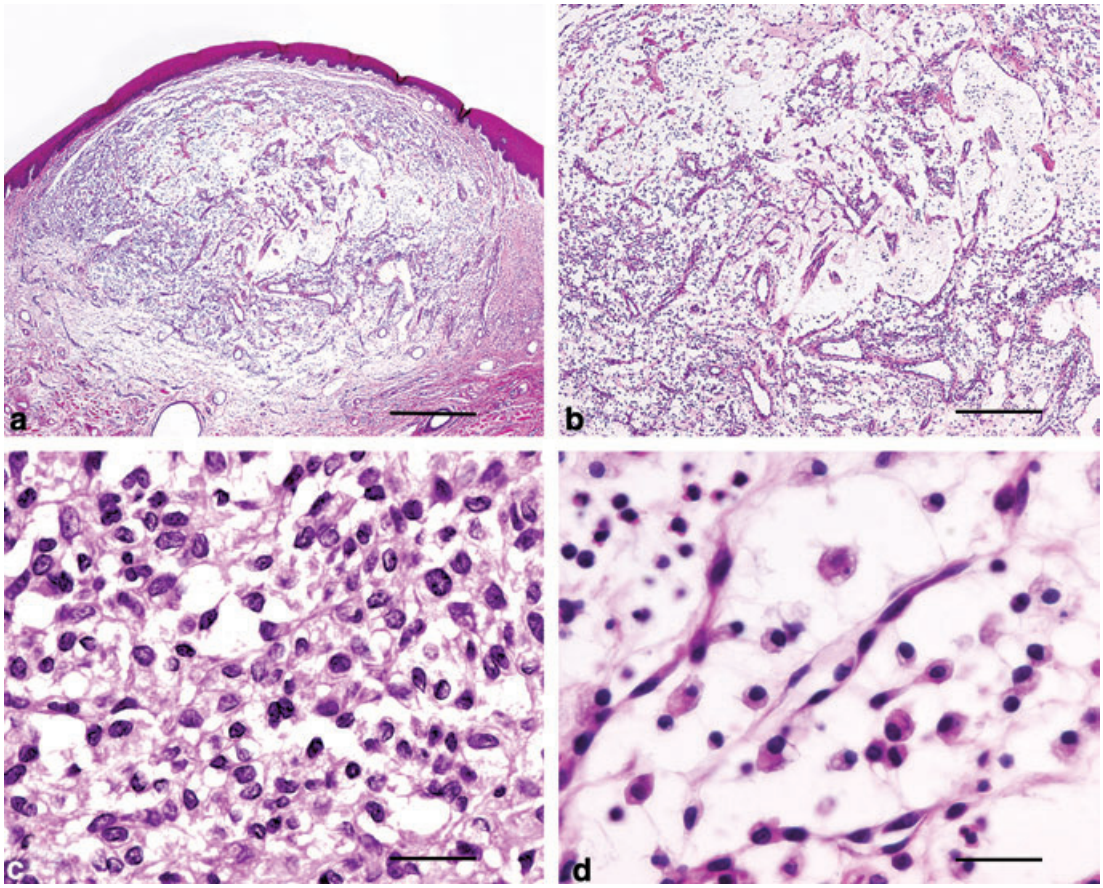

Figure 1 Histology of mucous retention cyst. (a–d) Hematoxylin and eosin (HE). Bars indicate 500 μm (a), 300 μm (b), and 50 μm (c and d), respectively. Mucous retention cyst of the extravasated type is located in the lamina propriae to the submucosal layer. It consists of an outer wall of thick granulation tissue and an inner mucous pool (a and b). The mucous pool contains neutrophils as well as foamy macrophages in densely packed fashions (c) in addition to capillaries, which are newly formed or are in the process of formation (d).

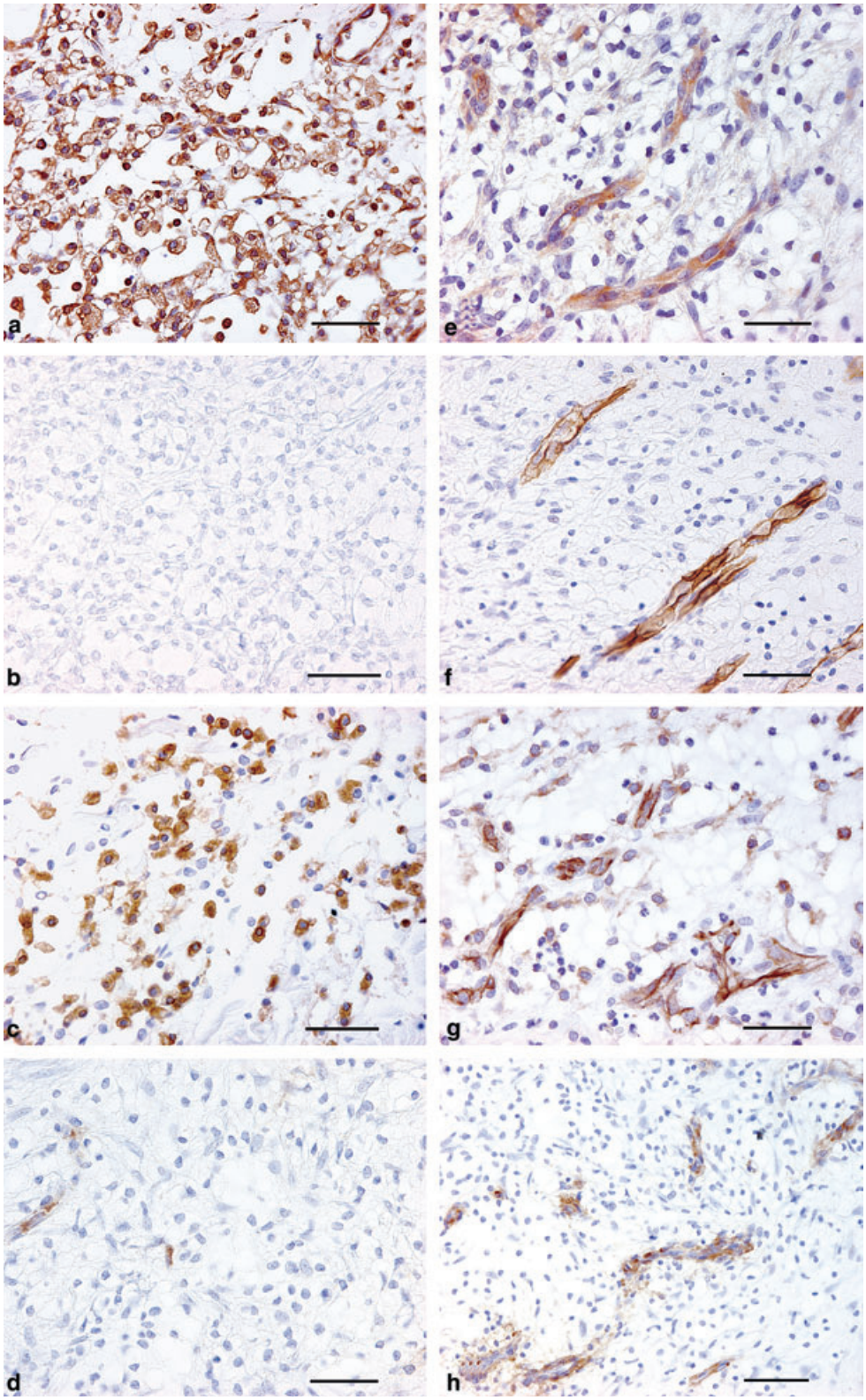

Figure 2 Immunohistochemical demonstration of endothelial cell markers in endothelial progenitor cells in mucous retention cysts. Immunoperoxidase stains for (a and e) (VEGF), (b and f) CD34, (c and g) CD31, and (d and h) type IV collagen. Hematoxylin counterstain; (a–h), bars indicate 100 μm. Densely packed polygonal- or spindle-shaped cells, which were only immunopositive for VEGF and CD31, were considered to be endothelial progenitor cells (a–d). Mature endothelial cells forming vascular channels were strongly immunopositive for different endothelial markers such as CD34 and type IV collagen, in addition to VEGF and CD31 (e–h).

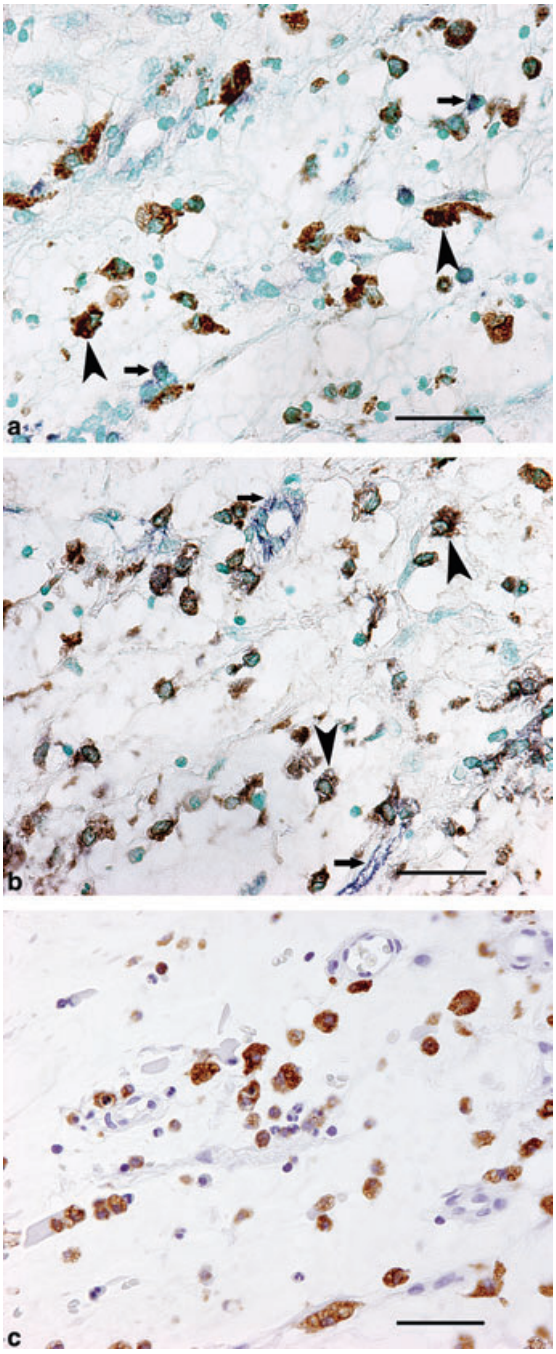

Figure 3 Immuno-colocalization of VEGF/CD31 and CD68 in endothelial progenitor cells in mucous retention cyst. Double immunoperoxidase-alkaline phosphatase stains for (a) VEGF (blue with NBT/BCIP) vs. CD68 [brown with DAB], (b) CD31 (blue) vs. CD68 (brown), and (c) single immunoperoxidase stain for CD68 (brown with DAB). Methyl green counterstain for double immunostainings (a and b), and hematoxylin for single CD68 immunostaining (c). Bars = 100 μm. VEGF (a)- or CD31 (b)-immunopositive polygonal cells, which were considered to be endothelial progenitors, were simultaneously positive for CD68 (large arrowheads), suggesting that the endothelial progenitor cells were of monocytic origin. A small number of cells with only immunopositive for VEGF (a, small arrows), or CD31 (b, small arrows) were also found. However, in panel (c), it was evident that most of the polygonal cells were immunopositive for CD68.

interrupted tubular structures composed of apparently endothelial cells. Such tubular structures were considered to be neovascularization (Fig. 1d), because these immature vascular channels embedded in pale-stained mucous substrates looked like those formed by gel-embedded endothelial cells *in vitro* (16). In the following immunohistochemical experiments, we paid special attention to such newly formed capillaries and the mononuclear cells around them in the mucous pools.

Histochemistry

In the mucous pool of mucous retention cysts, polygonal- or spindle-shaped cells with ovoid nuclei in the cellular granulation tissues were immunopositive for VEGF (Fig. 2a) or CD31 (Fig. 2c), but not for CD34 (Fig. 2b) or type IV collagen (Fig. 2d). In addition to these mononuclear polygonal cells, endothelial cells forming blood vascular channels including capillaries, postcapillary venules, or larger venules were strongly immunopositive for VEGF (Fig. 2e), CD34 (Fig. 2f), vWF (not shown), and CD31 (Fig. 2g). These blood vascular channels were also revealed by immunohistochemistry for type IV collagen (Fig. 2h). Thus, VEGF+ and CD31+ mononuclear polygonal cells, which were not positive for CD34, vWF, and type IV collagen, were thought to be endothelial progenitor cells. In the area where such mononuclear cells were densely packed, blood vascular channels were scarcely formed. Most of the VEGF+ and CD31+ mononuclear cells showed round or polygonal shapes and occasionally spindle shapes, which were partially aligned in rows suggesting endothelial arrangements for tubule formations (Fig. 2e, g). Some of the VEGF+ and CD31+ spindle-shaped cells, which were located next to or in the vicinity of newly formed capillaries, were also considered to be endothelial progenitor cells. However, it was difficult to distinguish other VEGF+ and CD31+ round-shaped or polygonal cells from foamy macrophages floating in the mucous pool, because some of the macrophages also showed immunopositivities for VEGF and CD31.

To further characterize the VEGF+ or CD31+ round-shaped or polygonal cells in the mucous pools, double immunostainings were performed. Simultaneous VEGF- and CD68-immunopositivities (Fig. 3a) as well as CD31- and CD68-immunoreacitivities (Fig. 3b) were obtained in these round-shaped or polygonal mononuclear cells. Figure 3c shows that most of them were immunopositive for CD68. Most of these double-immunolabeled cells were floating in singular but sometimes in aggregated forms in the mucous background, while a small number of them tended to aligning in a row. Apparent endothelial cells forming vascular channels were consistently VEGF+ and CD31+ but not CD68+. Thus, the CD68+ round-shaped or polygonal cells were thought to lose the immunoreactivity when they were differentiated to spindle-shaped endothelial cells.

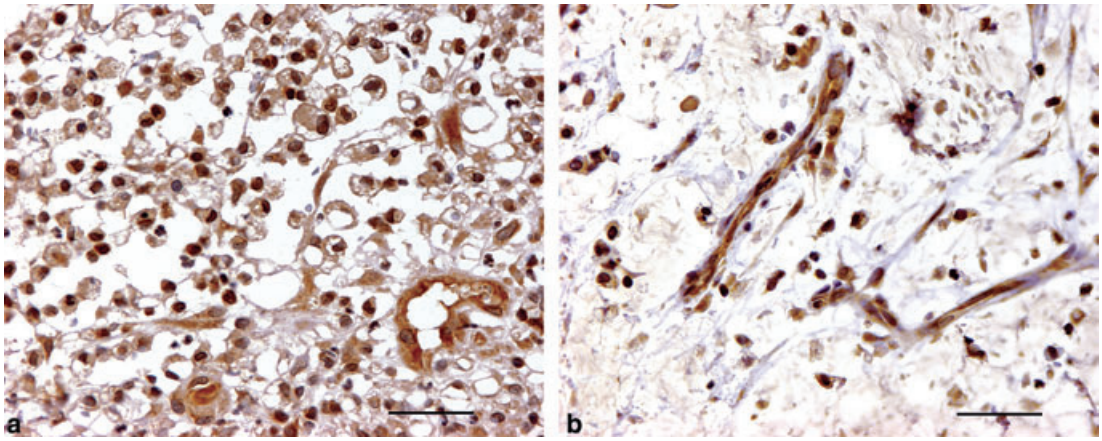

Figure 4 Immunohistochemical demonstration of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) in endothelial progenitor cells in mucous retention cyst. Immunoperoxidase stain for Flk-1. Hematoxylin counterstain. Bars = 50 μm. Polygonal-shaped endothelial progenitor cells were strongly immunopositive for Flk-1 (a), as well as mature endothelial cells in vascular structures (b).

Flk-1, one of the VEGF receptors, was also labeled in the round-shaped or polygonal mononuclear cells, whose staining intensities were varied from cell to cell. Strong Flk-1 signals were seen in capillary endothelial cells and the clustered mononuclear cells (Fig. 4a). In addition, short or interrupted vascular channels in the looser stroma were well visualized by the immunoreactivity for Flk-1 (Fig. 4b). These results also suggested that the aggregated mononuclear cells were endothelial progenitor cells.

Although type IV collagen was not immunolocalized in the extracellular space other than in the vascular basement membranes (Fig. 2d, h), tenascin, another ECM molecule which is closely related to the basement membrane, was immunolocalized in an interrupted fashion along CD68+ spindle-shaped or polygonal cells scattered haphazardly in the mucous pool (Fig. 5a). In a similar cell density, isolated or aggregated cells showed strong mRNA signals for tenascin in ISH (Fig. 5b). When CD68+ endothelial progenitor cells were aligned in a row, they showed strong intracellular signals for tenascin, in addition to its linear extracellular positivities, which indicated that the cells were regularly arranged on basement membrane-like cords which would predict their maturation to vascular channels (Fig. 5d). ISH for tenascin mRNA showed that these aligned cells contained definite messages for tenascin mRNA (Fig. 5e). In the periphery of the mucous pool, dilated venules were often observed in myxoid stromata, which were diffusely and strongly immunopositive for tenascin, although a small number of strongly tenascin+ dots were localized around venules and no cellular staining for tenascin was observed (Fig. 5g). ISH showed strong mRNA signals for tenascin in sporadic round-shaped or polygonal cells in the stromal space between venules, but only faint signals in endothelial cells of the fully developed venules were observed (Fig. 5h). In control experiments in which antisense probes were replaced with sense probes, no hybridization signals were obtained (Fig. 5c, f, i).

Discussion

The present results indicated that vascular endothelial cells are recruited from monocytic populations which are mobilized as a usual inflammatory reaction to extravasated mucous materials. Since mucous retention in the interstitial space of the oral mucosa is thought to be coagulated and circumscribed by granulation tissues, the mucous substance casted into gels in the presence of tissue fluids could be utilized similar to agar culture plates by any kind of cells mobilized into it, as matrigel was used for this purpose in an *in vitro* experiment (16). Therefore, vasculogenetic or angiogenetic processes are easily observed in this particular gel milieu, in which endothelial cells are differentiated to form vascular channels without complex interactions with such interstitial cells as fibroblasts which are thought to mainly participate in a usual process of granulation tissue formation, because extracellular matrices produced by them have mainly been expected to serve for endothelial differentiation (17). Only one model which is similar to this may be neovascularization in the cornea, which is purely collagenous (18). However, no attention had been paid to this ideal *in vivo*-like model in the oral mucosa prior to our trial.

The most striking observation in the present study is that monocytic lineage cells were the most responsible sources for endothelial differentiation in mucous retention cysts of the oral mucosa. As many of the CD68+ monocytic cells in the mucous pool were at the same time immunopositive for endothelial markers, such as CD31, VEGF, and Flk-1, in the very initial step of the organization process of the mucous pool, some of the monocytes in this particular milieu were differentiated to endothelial cells as well as to macrophages which should have scavenged gelled mucous substances as foreign bodies. There have been two major hypotheses for vasculogenesis in pathologic conditions: one is that endothelial cells sprout directly from injured microvasculatures and form new blood vessel blanches (19), and the other is that monocytes in the blood stream are

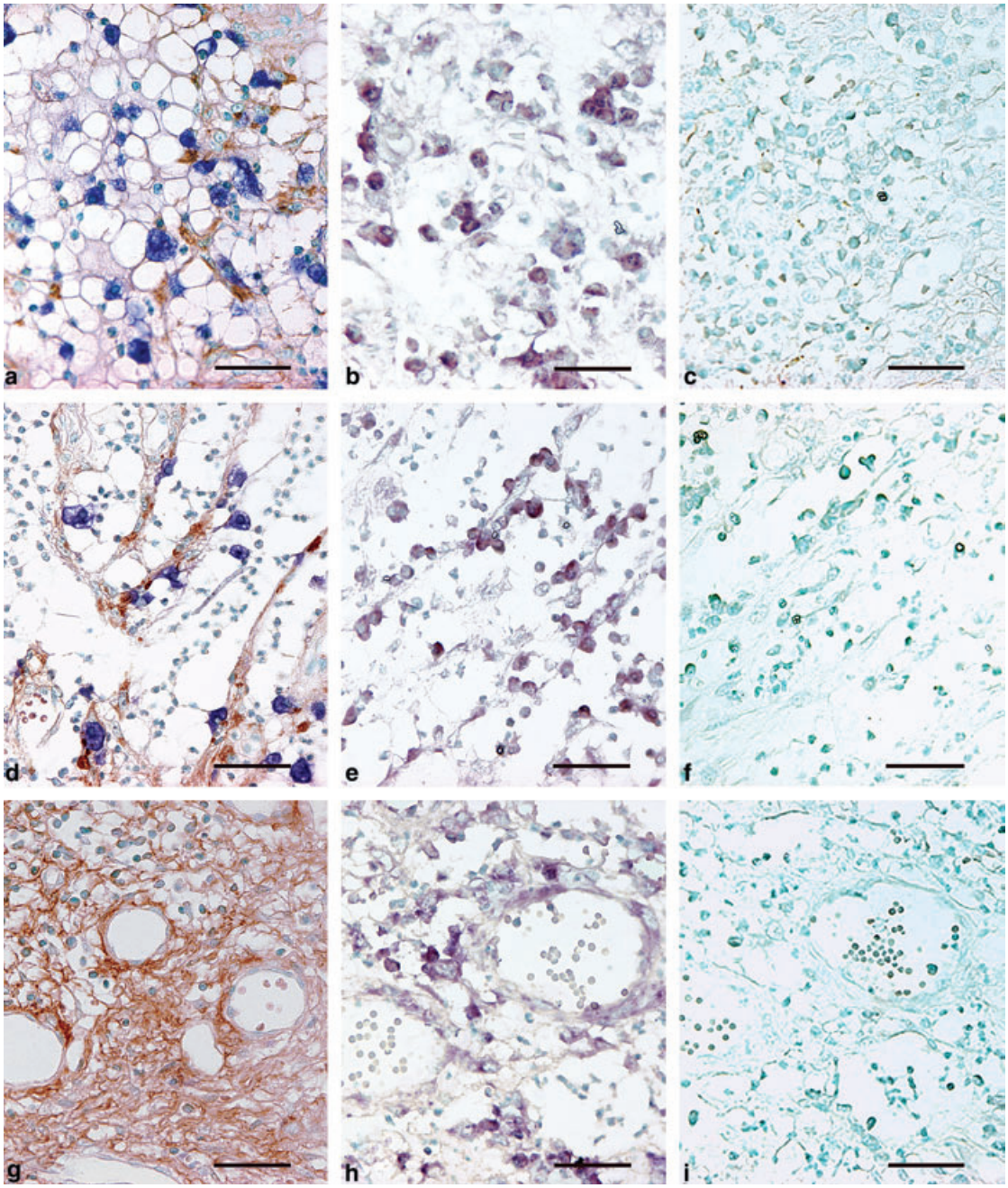

Figure 5 Differential expression of tenascin in steps of endothelial cell maturation steps at mRNA and protein levels in endothelial progenitor cells in mucous retention cyst. Double immunoperoxidase-alkaline phosphatase stains for (a, d and g) tenascin [brown with DAB] vs. CD68 (blue with NBT/BCIP), methyl green counterstain. (b, e and h) *In situ* hybridization for tenascin mRNA with methyl green counterstain. (c, f and i) *In situ* hybridization with sense probes for tenascin mRNA as negative controls, methyl green counterstain. (a–c) Polygonal-shaped endothelial progenitor cells, (d–f) immature endothelial cells forming vascular channels, (g–i) definitely mature endothelial cells in venules. Bars = 50 μm. CD68-immunopositive polygonal-shaped endothelial progenitor cells were simultaneously immunopositive for tenascin (a) and contained mRNA signals for tenascin (b). In slightly matured endothelial cells just starting to form vascular channels, there were simultaneous immunopositivities for tenascin and CD68 (d), and mRNA signals for tenascin were demonstrated (e). In the interstitial space around fully developed venules, tenascin was strongly and diffusely distributed (g), but tenascin mRNA signals were not so obvious in endothelial cells of venules but still definite in polygonal-shaped endothelial progenitor cells around venules (h). No positive signals were obtained when antisense probes were replaced with sense probes (c, f and i).

extravasated into the site of inflammation and are differentiated into endothelial cells to form new vascular vessels (16). The former idea has been developed by Folkman and Haudenschild in a sophisticated manner since the 1980s (20). Although the sprouting of endothelial cells is highly possible in various pathophysiologic conditions, the latter theory has also attracted more attention in recent years because *in vitro* studies have shown how angiogenic factors function in migration, proliferation, tubule formation of angioblasts or

endothelial progenitor cells (20). As Asahara et al. have demonstrated, such progenitor cells from the blood stream could be differentiated in experimentally induced ischemic sites (4), where endothelial cell-specific tyrosin kinase receptors, such as Flk-1 or Flt-1, as well as their ligand VEGF have been identified and their functions in each step of vasculogenesis not only in postnatal vasculogenesis but also in embryonic angiogenesis have been characterized (9, 21, 22).

CD34 is known to be expressed by endothelial progenitor cells derived from the peripheral blood (4, 23). CD34+ endothelial progenitor cells have been shown to be differentiated by VEGF via its receptors, Flk-1 or Flt-1, and subsequently to express CD31 (8) or vWF (24) in their more differentiated status. However, recent studies have demonstrated that the immunoreactivity for CD34 is not necessarily restricted to the vascular endothelium but to a wide spectrum of tissue stem cells such as those in the bone marrow (4), muscles (8), or in any other sources (23). Our present result that not CD34, but CD31 was expressed in the initial differentiation stage of endothelial progenitor cells is not consistent with these previous data. Since ours were only immunohistochemical data, this discrepancy may be due to the use of the monoclonal antibody, which recognizes only a limited epitope on CD34 glycoprotein (25). However, Engelhardt et al. have claimed to determine which is the more primitive between CD34+ and CD34− from their experiment by using murine and human models (26). As VEGF has been demonstrated to be produced in the human salivary gland and to be secreted into the saliva (27), mucous pools in mucous retention cysts are considered to contain VEGF which must serve for endothelial progenitor cells, which may explain one of the functions of saliva in wound healing (28). In regard to the role of VEGF in vasculogenesis, at least two mechanisms have been suggested: one is that VEGF functions as a direct mitogen on endothelial cells as well as a potent mediator of microvascular hyperpermeability resulting in extravasations of plasma proteins into the surrounding stroma, which would lead to pro-angiogenic alterations in the extracellular milieu (17). The other is that it enhances collagenase activities as an indirect effect by stimulating plasminogen activators (29). It is also known that several vascular endothelial cell growth factors other than VEGF, such as fibroblast growth factor (FGF) (30) or platelet-derived growth factor (PDGF) (31) are in the salivary contents. These cytokines may also function in the differentiation process of endothelial cells in the particular circumstance of mucous retention cysts, which should be elucidated in the next step.

During the differentiation process of endothelial progenitor cells, not only VEGF but also ECM molecules, such as tenascin (32, 33), collagen type IV (34), or fibronectin (35), are shown to play important roles. In the present study, we were successful in the demonstration of tenascin mRNA expression in CD68+/VEGF+ endothelial progenitor cells, which were about to form tubules in mucous retention cysts. As tenascin disappeared in fully developed capillaries whose endothelial cells resided on collagen type IV-immunopositive basement membranes, it is suggested that tenascin functions in the very early stages of endothelial differentiation. Similar results for the role of tenascin in cellular differentiation have been demonstrated in neoplastic liver cells (36) or in activation of lymphocytes (37).

In conclusion, it was evident from the present observation that vasculogenesis in mucous retention cysts preceding the organization process of pooled mucous substance is mainly driven by monocytic lineage cells which are recruited from the periphery blood via the granulation tissue wall of the retention cyst. These periphery blood-derived cells may be activated by the saliva-derived or endogenous growth factors including VEGF or by some specific composition of ECM including tenascin within the mucous pool.

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