Original article

Airway Remodeling in Feline Lungs

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Abstract

Airway remodeling encompass structural changes that occur as the result of chronic injury and lead to persistently altered airway structure and function. Although this process is known in several human respiratory conditions such as asthma and chronic obstructive pulmonary disease (COPD), airway remodeling is poorly characterized in the feline counterpart. In this study, we describe the spontaneous pulmonary changes in three cats paralleling the airway remodeling reported in humans. We observed airway smooth muscle cells (ASMCs) hyperplasia (peribronchial and interstitial), airway subepithelial and interstitial fibrosis, and vascular remodeling by increased number of vessels in the bronchial submucosa. The hyperplastic ASMCs co-expressed α-SMA, vimentin and desmin suggesting that vimentin, which is not normally expressed by ASMCs, may play a role in airway thickening and remodeling. ASMCs had strong cytoplasmic expression of TGFβ-1, which is known to contribute to tissue remodeling in asthma and in various bronchial and interstitial lung diseases, suggesting its involvement in the pathogenesis of ASMCs hyperplasia. Our findings provide histological evidence of airway remodeling in cats. Further studies on larger caseloads are needed to support our conclusions on the value of this feline condition as an animal model for nonspecific airway remodeling in humans.

Keywords: airway remodeling; airway smooth muscle cells hyperplasia; asthma; feline asthma; TGFβ.

Introduction

Airway remodeling is the set of processes encompassing morphological changes in structural cells of airways affected by chronic diseases, such as asthma or chronic obstructive pulmonary disease (COPD) (Prakash et al., 2017).

In human medicine, asthma is defined as a chronic inflammatory disorder characterized by airway hyper-responsiveness and remodeling that leads to thickening of the airway walls and to a variable degree of obstruction (Mims, 2015; Papi et al., 2018).

Analogously, airway obstruction in COPD is due to structural changes in the small airways, although they differ from those of human asthma (Chung, 2005; Sköld, 2010)

Airway remodeling comprises numerous cellular and extracellular alterations including mucous metaplasia of bronchial epithelium, inflammation, basement membrane thickening, subepithelial fibrosis, submucosal angiogenesis, increased number of myofibroblasts and airway smooth muscle cells (ASMCs) hyperplasia and hypertrophy (Fehrenbach et al., 2017; Grigoraş et al., 2016; Harkness et al., 2014; Kim et al., 2007; Pain et al., 2014; Prakash et al., 2017).

Cats and horses are recognized to be spontaneous animal models of asthma-like conditions (Aun et al., 2018), and Norris Reinero et al. (2004) demonstrated that cats develop pathologic changes similar to human patients. However, airway remodeling occurring in feline asthma has not been fully characterized (Masseau et al., 2015).

Different epithelial and mesenchymal cell types, such as bronchial epithelial cells fibroblasts, myofibroblasts and ASMCs, play a role in the pathogenesis of asthma,

producing cytokines and mediators promoting airway remodeling (Davies, 2009; Fixman et al., 2007; Rosethorne and Charlton, 2018). TGFβ is a central factor in epithelial-mesenchymal interactions (Saito et al., 2018) that mediates numerous fibrogenic responses, resulting in modifications of the extracellular matrix (ECM) (Pardali et al., 2017; Rockey et al., 2015). Moreover, TGFβ is involved in the remodeling of asthmatic lung disease, as well in idiopathic interstitial lung diseases and in COPD (Fitch et al., 2011; Michaeloudes et al., 2017) by promoting most of the processes underlying the morphological changes observed (Halwani et al., 2011), including differentiation of fibroblasts to myofibroblasts (Michalik et al., 2009) and proliferation of ASMCs (Chen and Khalil, 2006; Xie et al., 2007).

In this study, we describe the spontaneous pulmonary changes in three cats paralleling the pathological features of airway remodeling in human patients; further, we investigated the immunophenotype of hyperplastic ASMCs and the expression of TGFβ-1 and its receptors TGFβ RI and TGFβ RII.

Materials and methods

Necropsy and histology

Pulmonary samples were collected from 3 client-owned cats, referred to the Pathology Service of the Department of Veterinary Medical Sciences for necroscopic examination. Cats were two males (No. 1; No. 2) and one female (No. 3), of 12, 14, and 12 years of age respectively. Two out of three cases (case No. 1 and No. 2) were regularly vaccinated and annually subjected to anti-parasite therapy. In case No. 2, the medical history consisted of sporadic coughing episodes occurring in the summer period. Case No. 3 had an unknown medical history. In cases Nos. 2 and 3 the anamnesis at the time of the

necropsy consisted in sudden death without previous clinical manifestations, whereas in case No. 1 death was preceded by respiratory distress.

The cats underwent necropsy, gross examination and tissue sampling for routine histological examination. All the biological samples used for the research were collected and processed in agreement with informed consent signed by the owners. Two lung samples were collected from each basal lobe (four samples/cat). In addition to H&E, sections of the lungs of each cat underwent Masson's trichrome stain (Bio-Optica, Milan, Italy). Three feline pulmonary samples without morphological alterations were used as control.

Collagen deposition in the alveolar interstitium was quantified on Masson's trichrome stained sections by image analysis using the software ImageJ (version 1.52t). For image analysis, 5 photomicrographs (area of each photomicrograph equal to 1.49 mm^2) from randomly selected 200x fields were used. For each photomicrograph, the ratio of positively stained area to the total parenchymal area (empty spaces were excluded) was used to quantify interstitial fibrosis (Supplementary S1).

The extent of collagen deposition within the bronchial submucosa (subepithelial fibrosis) was assessed in the examined cases and compared to controls.

Immunohistochemistry

Three micrometer-thick sections of lung were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersion in 3% H_2O_2 in methanol for 30' at room temperature. The primary antibodies, dilutions, antigen retrieval methods and tissues used as positive controls are reported in Table 1. Antigen retrieval was followed by cooling

at room temperature for 20'. Blocking of non-specific antigenic sites was achieved by incubating the slides in a solution of 10% goat serum in PBS for 30' at room temperature and afterwards incubated overnight at 4°C with the primary antibodies.

Binding sites were revealed by secondary biotinylated antibody and amplified using a commercial avidin-biotin-peroxidase kit (ABC Kit Elite, Vector, Burlingame, CA). The chromogen 3,3′-diaminobenzidine (0.05%) (Histo-Line Laboratories, Emergo, Europe) was used. Slides were counterstained with Harris hematoxylin and permanently mounted with DPX medium.

Positive internal and external controls were examined, and negative control slides were processed in parallel by replacing the primary antibody with a non-reactive isotype-matched antibody.

A qualitative assessment of the number of small vessels in the bronchial submucosa was performed by comparing the CD31 immunolabeling in the examined cases with the control cases.

Immunofluorescence staining

Three micrometer-thick sections of lung were dewaxed in xylene; the primary antibodies, dilutions, antigen retrieval methods and tissues used as positive controls are reported in Table 1. Antigen retrieval was followed by cooling at room temperature for 20'. Blocking of non-specific antigenic sites was achieved by incubating the slides in a solution of 3% bovine serum albumin, 3% fetal bovine serum and 0.25% Triton X-100 in PBS for 1h at room temperature and afterwards incubated overnight at 4°C with the primary antibodies. Detection of primary antibodies was visualized with Alexa Fluor 488 and 555

(Abcam, Cambridge, UK). Sections were counterstained and mounted with Anti-Fade Fluorescence Mounting Medium with DAPI (Abcam, Cambridge, UK).

Slides were examined using a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes to distinguish the fluorochromes used. Images were recorded using a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands).

Results

Gross findings

In cases No. 2 and 3, bilateral multifocal and patchy grayish-white lesions consistent with chronic interstitial pulmonary disease, localized mainly in caudal lobes, were identified. A mild and bilateral myocardial ventricular hypertrophy was found in case No. 2. Further minor macroscopic findings were found in the liver and kidneys and included mild hepatic congestion and lobular pattern accentuation in case No. 3; and diffuse, chronic, moderate interstitial nephritis in case No.2. In case No. 1 no evident gross alterations were found.

Histopathology

On microscopic examination, in all three cases, the most evident pulmonary lesion was a multifocal to coalescing interstitial thickening, consisting of bundles of spindle cells and a variable amount of collagen. Cells had abundant eosinophilic cytoplasm and small, oval to cigar-shaped and central basophilic nuclei and were interpreted as hyperplastic ASMCs. All cases had small- to medium-sized multifocal foci of ASMCs hyperplasia in terminal bronchioles, involving the whole parenchyma (Figs. 1A, 1B). Additionally, in case No. 3, the coalescence of hyperplastic ASMCs led to the formation of a focally extensive

lesion entrapping scattered bronchioles (Fig. 1C). Associated with extensive ASMCs hyperplasia, alveolar walls were multifocally lined by hyperplastic type II pneumocytes. Moderate peribronchial ASMCs hyperplasia were detected in each case. Multifocally, peribronchial and intraluminal bronchial mild inflammatory infiltrate of lymphocytes, plasma cells and numerous eosinophils was evident (Fig. 1D). Diffusely, the tunica media of pulmonary arteries was markedly hyperplastic.

Compared with controls, the alveolar interstitium of all three cats was thickened by fibrosis, based on image analysis assessment of Masson's trichrome stained sections (Fig. 2) and by ASMCs hyperplasia.

The extent of fibrosis was mild (case No. 1) to moderate (cases Nos. 2 and 3) and the distribution was multifocal (cases Nos. 1 and 2) and multifocal to coalescing (case No. 3), predominantly centered on the airways in all cases. In case No. 3, interstitial fibrous tissue was associated with the local extensive lesion characterized by ASMC hyperplasia (Supplementary S2).

Bronchial submucosa had hyperplastic glands admixed with an increased number of small vessels, while Masson's trichrome stain revealed an increased amount of collagen bundles beneath epithelium (subepithelial fibrosis) (Fig. 3).

Based on the evaluated criteria, the histopathological findings of each case are summarized in Table 2.

In all cases, histological examination of the liver revealed a diffuse and moderate congestion, interpreted as a peri-mortem finding. In case No. 3, a multifocal and moderate

hepatic lipidosis was observed, corresponding to the gross finding of accentuated lobular pattern. Examination of the myocardium revealed a mild, multifocal, interstitial fibrosis in all cases. Furthermore, in case No. 1, the myocardium was multifocally replaced by proliferation of fibroblasts and new thin-walled, delicate capillaries (angiogenesis) immersed in a loose extracellular matrix (granulation tissue). Tubulointerstitial lymphoplasmacytic nephritis was confirmed in case No. 2.

Immunohistochemistry

Strong cytoplasmic expression of α-SMA and TGFβ-1 was detected in hyperplastic ASMCs in all cases (Fig. 4B). TGFβ-1 was also expressed, but discontinuous and less intense, in normal ASMCs and less consistently in arterial smooth muscle cells (Fig. 4A). Cytoplasmic expression of TGFβ RI and RII was detected in bronchial epithelial cells and bronchial glands, and rarely within the cytoplasm of hyperplastic type II pneumocytes and in scattered alveolar macrophages (Fig. 4C, 4D). CD31 immunolabeling was identified within the endothelial cells lining of small vessels, showing an increased vascular density in the bronchial submucosa compared with control cases (Fig. 5).

Immunofluorescence staining

Immunofluorescence staining revealed the cytoplasmic co-expression of α-SMA, vimentin, and desmin in hyperplastic smooth muscle cells. Submucosal ASMCs always co-expressed desmin and α-SMA, while vimentin immunolabeling was not detected in normal ASMCs (Fig. 6). A mildly positive vimentin stain was present in a few vascular smooth muscle cells of the arteries.

Discussion

This report describes the histological features suggestive of airway remodeling in the lungs of three cats which parallels the changes reported in human chronic airway diseases.

In the lungs of all three cases, interstitial bundles of hyperplastic ASMCs were detected, involving the whole parenchyma. Lesions were more severe in small non-cartilaginous airways (e.g. case No. 3), similarly to changes observed in horses with pasture asthma (Ferrari et al., 2018). In humans, asthma-associated ASMCs remodeling involves both large and small airways (Elliot et al., 2015). On the contrary, feline asthma is classified as a bronchial disease, whit bronchiolar involvement considered secondary change extending from bronchi (Reinero et al., 2019).

In all the pulmonary samples, the Masson's trichrome stain allowed to assessment of the increased amount of collagen in bronchial submucosa. This finding corresponds to subepithelial fibrosis, one of the histological changes of airway remodeling, that is mediated by resident fibroblasts, myofibroblasts (Brewster et al., 1990) and bone marrow derived-precursors (Nihlberg et al., 2006).

Among the structural changes found in our cases, we identified an increased number of small vessels in the bronchial submucosa, demonstrated by CD31 immunolabeling. Angiogenesis and microvascular changes are common features of chronic airway disease and are referred to as vascular remodeling changes (Alagappan et al., 2013; Harkness et al., 2014; Keglowich and Borger, 2015; Saito et al., 2018). Morphological changes typical of this process include an increased number of bronchial vessels as well as an increased size of pulmonary vessels due to hyperplasia of tunica intima and media. In the cases described in this report, both lesions were identified.

Hyperplasia and hypertrophy of the arteries is a common histologic finding in cats, recognized over time as a possible effect of lungworm infestation such as *Aelurostrongylus abstrusus* (Hamilton, 1970; Vezzosi et al., 2020). Furthermore, smooth muscle hyperplasia in the pulmonary arteries can be observed in severe equine asthma (Ceriotti et al., 2020). It remains to be clarified whether the thickening of the arterial walls observed in our cases may be linked to a vascular remodeling mechanism associated with airway remodeling in feline asthma or represents a sequela of lungworm infestation.

Pulmonary artery remodeling is known to be associated with increased pulmonary vascular resistance, which can lead to cardiac fibrosis (Siamwala et al., 2020); this could explain the cardiac interstitial fibrosis detected in the heart of the cases examined.

In all the cases included in the study, immunofluorescent staining revealed simultaneous expression of α-SMA, vimentin and desmin in the hyperplastic interstitial ASMCs, whereas vimentin was not normally expressed in ASMCs.

Actin, desmin and vimentin are involved in smooth muscle cells contraction as well as in cell migration, and vimentin expression has been associated with an increased cellular motility (Tang and Gerlach, 2017). Since smooth muscle cells migration and motility is putatively related to thickening of smooth muscle in the asthmatic airways (Cleary et al., 2014; Tang and Gerlach, 2017), the increased vimentin expression found in the foci of ASMC hyperplasia may indicate an increased cellular motility in airway remodeling.

In healthy lung ,TGFβ-1 expression is confined to the airway epithelium, alveolar macrophages and fibroblasts (Coker et al., 1996; Kelley et al., 1991; Magnan et al., 1994), while in the three cases here described cytoplasmic expression of TGFβ-1 was detected in hyperplastic smooth muscle cells, mainly ASMCs and less consistently in vascular smooth muscle cells.

ASMCs are known to be synthetically active, producing and/or expressing several cytokines, including TGFβ-1, secondary to extracellular stimuli as occurs in chronic asthma (Howarth et al., 2004; Tliba and Panettieri, 2009). In asthmatic patients, increased immunoreactivity of TGFβ-1 and TGFβ-1 localization in submucosal smooth muscle cells is reported (Vignola et al., 1997), although eosinophils and fibroblasts are the main source of this growth factors. More recently, Xie et al., (2007) identified and increased TGFβ-1 mRNA and protein expression in ASMCs of human asthmatic patients compared with non-asthmatics patients. The high expression of TGFβ-1 in hyperplastic ASMCs (Xie et al., 2007) and in structural ASMCs in the cases here described compared to controls, supports its role in the morphologic changes associated to these feline cases.

TGFβ-1 is a pleiotropic factor involved in different biological processes such as immune response, wound healing, tissue repair, and proliferation of fibroblasts (Xiao et al., 2012). In asthma, TGFβ-1 is responsible for the differentiation of fibroblasts, epithelial cells and also ASMCs into cells with higher contractile phenotype, thus contributing to increased airway hyper-responsiveness (Gawaziuk et al., 2007). Ojiaku et al. (2018) demonstrated that TGFβ-1 directly modulates cell shortening and increases contractility of human ASMCs; additionally, TGFβ-1 directly induces proliferation of smooth muscle cells (Chen and Khalil, 2006), with greater action in severe asthma (Perry et al., 2014).

The TGFβ signaling pathways occurs when one ligand of TGF family (e.g., TGFβ-1) binds to TGFβ RII, an intramembranous serine/threonine kinase receptor which then phosphorylates and activates TGFβ RI. The binding to the receptor complexes may activates both canonical SMAD-mediated and non SMAD-mediated cascade. Thus, the cellular response to TGFβ is regulated by the availability of receptors on the cell surface, which can be modified under certain conditions (Budi et al., 2017). So, we tested the expression of TGFβ receptors (TGFβ RI and TGFβ RII), hypothesizing a possible autocrine mechanism leading to ASMCs hyperplasia. Unexpectedly, hyperplastic ASMCs did not express TGFβ RI and TGFβ RII, which were nevertheless expressed in the cytoplasm of bronchial and glandular epithelium, scattered alveolar macrophages and type II pneumocytes. We speculate that, as previously reported (Gressner, 2011), in hyperplastic ASMCs the expression of TGFβ-1 might exert its function by an intracrine signaling involving the interaction of TGFβ with an unknown binding site in the intracellular domain of the Alk5 receptor with consequent activation of the non SMAD-mediated signaling pathway.

Considering the interstitial increase of collagen bundles, idiopathic pulmonary fibrosis (IPF) should be included among the differential diagnoses. A spontaneous, idiopathic pulmonary fibrosis-like condition has been described in cats with or without obvious respiratory clinical signs (Cohn et al., 2004; Williams et al., 2004). This chronic respiratory condition in domestic cats shows morphologic features similar to interstitial pneumonia typical of human IPF (Cohn et al., 2004; Evola et al., 2014; Williams et al., 2004): interstitial fibrosis, fibroblasts/myofibroblasts proliferation and enlarged alveolar spaces lined by bronchiolar epithelium (honeycombing), affecting mainly the subpleural

parenchyma (Travis et al., 2002). Moreover, ASMCs hyperplasia is commonly seen in IPF (Kanematsu et al., 1994). At least in one case (No. 3), the morphological changes resembled those described as “probable unusual interstitial (UIP) pattern” by Le Boedec et al., (2014) following human criteria (Raghu et al., 2011).

Conclusions

In cats, airway remodeling associated with feline asthma and other bronchial and interstitial disease remains a poorly characterized process. Considering that the cat represents a potential spontaneous animal model of asthma or interstitial fibrosis, it may be useful to know and characterize the lesions and pathogenesis of airway remodeling in the lungs of cats. We report the histological findings of airway remodeling in cats, and insights on the role of TGFβ-1 in their pathogenesis. Nevertheless, future studies on larger caseloads are needed to confirm our conclusions and to support the value of airway remodeling in cats as an animal model for nonspecific airway remodeling in humans.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: …

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581 **Tables**

582 **Table 1**. Immunohistochemistry and immunofluorescence materials and methods

583 information. INT, internal; EXT, external; CTR, control; MW, microwave; ON, overnight.

Marker	Type, Clone	Supplier	Dilution/ incubation	Ag retrieval	Positive INT and EXT CTR
Immunohistochemistry					
TGFβ-1	Mouse monoclonal anti TGFβ -1 (3C11)	Santa Cruz Biotechnology, California, USA	1:100/ON 4°C	10' Citrate pH6 MW:750W	Smooth muscle cells (INT)
CD31	Mouse monoclonal anti-PECAM-1 (JC70)	Santa Cruz Biotechnology, California, USA	1:30/ON 4°C	10' EDTA pH8 MW:750W followed by 30' in Pepsin 0.05% at 37°	Endothelial cells (INT)
α-SMA	Mouse monoclonal anti- α-SMA (1A4)	Santa Cruz Biotechnology, California, USA	1:500/ON 4°C	10' Citrate pH6 MW:750W	Smooth muscle cells (INT)
TGFβ RI	Rabbit polyclonal IgG (T-19)	Santa Cruz Biotechnology, California, USA	1:200/ON 4°C	10' Citrate pH6 MW:750W	Granulation tissue (cat, skin) (EXT)
TGFβ RII	Rabbit polyclonal IgG (C-16)	Santa Cruz Biotechnology, California, USA	1:600/ON 4°C	10' Citrate pH6 MW:750W	Granulation tissue (cat, skin) (EXT)
Immunofluorescence					
Desmin	Rabbit polyclonal anti-Desmin (H76)	Santa Cruz Biotechnology, California, USA	1:20/ON 4°C	20' EDTA buffer pH9 pressure cooker (110-120°C, high pressure)	Smooth muscle cells (INT)
Vimentin	Mouse monoclonal anti-Vimentin (V9)	Dako, Glostrup, Denmark	1:100/ON 4°C	20' EDTA buffer pH9 pressure cooker (110-120°C, high pressure)	Mesenchymal cells (INT)
α-SMA	Mouse monoclonal anti- α-SMA (1A4)	Santa Cruz Biotechnology, California, USA	1:100/ON 4°C	20' EDTA buffer pH9 pressure cooker (110-	Smooth muscle cells (INT)

				120°C, high pressure)	

584

585 **Table 2.** Pathological findings of each case according to histological criteria evaluated.

	Case No. 1	Case No. 2	Case No. 3
ASMCs hyperplasia	small- to medium foci in the peribronchial interstitium	small- to medium foci in the peribronchial interstitium	medium sized coalescent foci entrapping scattered bronchioles
Subepithelial fibrosis	Moderate, beneath bronchial epithelium	Moderate, beneath bronchial epithelium	Moderate, beneath bronchial epithelium
Interstitial fibrosis	Mild, multifocal	Moderate, multifocal	Moderate, multifocal to coalescent
Airway inflammation	peribronchial and mural mild inflammatory infiltrate*	peribronchial and mural mild inflammatory infiltrate*	peribronchial and mural mild inflammatory infiltrate*
Microvascular changes	Mild increased number of small vessels	Mild increased number of small vessels	Mild increased number of small vessels

586 *The inflammatory infiltrate was characterized by lymphocytes, plasma cells and numerous eosinophils.

587

Figures legend

Fig. 1 Multifocal interstitial bundles of hyperplastic smooth muscle cells spread within pulmonary parenchyma in case No. 1 (A) and in case No. 2 (B); (C) Coalescent bundles of hyperplastic smooth muscle cells in case No. 3. H-E stain, Magnification 100x. (D) Mild airways inflammation characterized by bronchial infiltrations of eosinophils; H-E, Magnification 200x.

Fig. 2 Lung alveolo-septal interstitial collagen deposition in case No. 3 (B) compared with control case (A); Magnification 400x, Masson's trichrome stain. (C) quantitation by Image analysis show a higher content (fibrosis) in the 3 lungs compared to controls.

Fig. 3 (A) Normal lower airway in control case (Magnification 40x); insert: detail (Magnification 400x). (B) diffuse subepithelial fibrosis in lower airways of case No. 2 (Magnification 40x); insert: detail of increase in collagen bundles (Magnification 400x). Masson's trichrome stain.

Fig. 4 (A) Immunohistochemical TGFβ-1 expression by airway smooth muscle cells and vascular smooth muscle cells of arteries detected in control cases; Magnification 40x. (B) Cytoplasmic immunohistochemical TGFβ-1 expression by hyperplastic smooth muscle cells in case No. 3; Magnification 40x. (C) Multifocal TGFβ RI cytoplasmatic expression by scattered pneumocyte type II and alveolar macrophages in case No. 2; Magnification 400x. (D) TGFβ RII cytoplasmatic expression by pneumocyte type II, alveolar macrophages and bronchial epithelial cells in case No. 2; Magnification 400x.

Fig. 5 Immunohistochemical CD31 expression by endothelial cells of proliferated submucosal vessels (arrowheads) in case No. 2 (B, D) compared with control cases (A, C); Magnification 100x (A, B) and 400x (C, D).

Fig. 6 Co-expression of desmin, alpha-smooth muscle actin (α-SMA), and vimentin. Double immunofluorescence of desmin and α-SMA (A, B) or vimentin (C, D) indicates co-expression of all three mesenchymal markers in the bundles of hyperplastic cells (B, D), suggesting an increased in motile capacity. However, normal ASMCs (internal control, C) did not express vimentin. Case No. 3, magnification 100x (A, C) and 400x (B, D).