FULL PAPER

Pathology

Immunophenotypical analysis of pancreatic interstitial cells in the developing rat pancreas and myofibroblasts in the fibrotic pancreas in dogs and cats

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ABSTRACT. Pancreatic fibrosis develops as the results of the activity of myofibroblasts capable of producing collagens. The myofibroblasts derive from pancreatic interstitial cells, including pancreatic stellate cells (PSCs), which can express glial fibrillary acidic protein (GFAP). First, we investigated the expression patterns of vimentin, desmin, α-smooth muscle actin (α-SMA), Thy-1 and GFAP in the developing rat pancreas (in fetuses at 18 and 20 days, neonates from 1 to 21 days, and adults). Interstitial cells in the developing pancreas expressed vimentin, desmin, GFAP and Thy-1 at varying degrees; interestingly, the reactivity for desmin and vimentin was the highest in fetuses. GFAP expression was consistent between fetuses and neonates, and Thy-1 reactivity transiently increased after birth; however, α-SMA-positive interstitial cells were rarely seen. Next, we analyzed the immunophenotypical characteristics of myofibroblasts appearing in pancreatic fibrosis in dogs and cats. With increasing fibrotic grade, myofibroblasts showed increased expression of vimentin, desmin and α-SMA, in addition to increased GFAP expression. Collectively, pancreatic interstitial cells and myofibroblasts may have similar immunophenotypes, and myofibroblasts might originate partly from GFAP-expressing PSCs.

KEY WORDS: fibrosis, GFAP, immunohistochemistry, myofibroblasts, pancreas

Fibrosis occurs after a tissue injury as a healing process. However, chronically progressive fibrosis may result in organ failure, as seen in cases of liver cirrhosis [8, 9]. Pancreatic fibrosis may develop after acute inflammation and subsequent repeated injury [13, 16]. Generally, the fibrotic lesions are characterized by myofibroblast development and abundant collagen deposition produced mainly by myofibroblasts. The myofibroblasts derive from interstitial fibroblasts; in addition, the cells may also be generated from hepatic stellate cells (HSCs) in hepatic fibrosis [11, 17] and be induced via the epithelial-mesenchymal transition (EMT) in liver fibrosis or injured renal tubules in renal fibrosis [4, 14]. Myofibroblasts express α-smooth muscle actin (α-SMA), and during development, the cells may express other cytoskeleton components such as vimentin and desmin at various degrees [5, 15]. The properties of myofibroblasts in hepatic and renal fibrosis have been well described, whereas those in pancreatic fibrosis remain to be analyzed.

Interestingly, HSCs express GFAP, a type III intermediate filament protein that was originally reported as an immunohistochemical marker for astrocytes [3, 15]; the nature of myofibroblasts appearing in hepatic fibrosis can be assessed using GFAP immunohistochemistry because HSCs are thought to be the major contributor to the development of myofibroblasts capable of producing collagens in liver cirrhosis [6]. It has been reported that pancreatic stellate cells (PSCs), which are located around the exocrine gland as the interstitial cells, exhibit GFAP immuno-expression as seen in HSCs [1, 12]. Therefore, in this study, using immunohistochemistry with antibodies against vimentin, desmin and α-SMA, as well as GFAP, we investigated the characteristics of interstitial cells, including PSCs, during the development of the rat pancreas to establish baseline data and then analyzed the cytoskeletal expression pattern of developing myofibroblasts in pancreatic fibrosis in dogs and cats. The purpose of this study was to determine the properties of interstitial cells in the developing rat pancreas and myofibroblasts appearing in the fibrotic pancreas in dogs and cats.

Table 1. The species, sex, age and grades of pancreatic fibrosis in dogs and cats

Species	Sex	Age	Histopathology (HE stain)	Fibrosis grade
Dog; Miniature Schnauzer	Male	6 year 2 month	No change	-
Dog; Border Collie	Female	4 year 9 month	No change	-
Dog; Shiba Inu	Spayed female	4 year 10 month	No change	-
Dog; Papillon	Male	4 year	No change	-
Dog; Labrador Retriever	Male	10 year 5 month	Multiple focal necrosis, Hemorrhage, No inflammation, Acute necrotizing, Fibrosis	±
Dog; Miniature Schnauzer	Castrated male	8 year 8 month	Slight fibrosis	±
Dog; Beagle	Male	12 year	Congestion, Thrombus	+
Dog; Shih Tzu	Male	12 year	Hemorrhage, Early stage of Islet of Langerhans neoplasm, Fibrosis	+
Dog; Pembroke Welsh Corgi	Male	9 year 2 month	Focal fibrosis	+
Dog; Kishu Inu	Female	11 year 5 month	Nodular hyperplasia	+
Dog; Maltese	Male	7 year	Disappearance of Langerhans Islet, Vacuolation	++
Dog; Bernese Mountain Dog	Female	5 year	Metastatic malignant histiocytosis, Fibrosis	++
Dog; Giant Schnauzer	Female	10 year 8 month	Pancreas nodular hyperplasia	++
Cat; Mix	Female	Unclear	Fibrosis	++
Cat; Mix	Male	Unclear	Diffuse fibrosis	+++

Grades of pancreatic fibrosis are as follows: -, normal without fibrosis; ±, very slight fibrosis; +, slight fibrosis or focal fibrosis; ++, moderate fibrosis; and +++, severe fibrosis with diffuse lesion.

Table 2. Primary antibodies used for immunohistochemistry and immunofluorescence

Antibody	Clone	Type	Dilution	Pre-treatment	Source	Specificity
Vimentin	V9	Mouse monoclonal	1:500	Microwave in citrate buffer, 20 min	Dako Corp., Glostrup, Denmark	Cells of mesenchymal origin
Desmin	D33	Mouse monoclonal	1:200	No	Dako Corp., Glostrup, Denmark	Smooth muscle cells, Ito cells (rat)
α-SMA	1A4	Mouse monoclonal	1:1,000	Microwave in citrate buffer, 20 min	Dako Corp., Glostrup, Denmark	Smooth muscle cells, myofibroblasts
GFAP	-	Rabbit polyclonal	1:500	10 μg/ml proteinase K, 10 min at 37°C	Dako Corp., Glostrup, Denmark	Astroglial cells
CK19	B170	Mouse monoclonal	1:100	0.1% trypsin, 20 min at 37°C	Novocastra Laboratories Ltd., Newcastle, U.K.	Pancreatic ducts
Thy-1	CD90	Mouse monoclonal	1:500	Microwave in citrate buffer, 20 min	Cedarlane Laboratories Ltd., Ontario, Canada	Immature mesenchymal cells

MATERIALS AND METHODS

Animals and experimental procedures

For the analysis of PSCs in the developing rat pancreas, 4-week-old male F344 rats and pregnant F344 rats were purchased from Charles River Japan (CRJ, Hino, Japan). These animals were maintained in a room at 21 ± 3°C with a 12 hr light-dark cycle, and they were fed a standard diet for rats (DC-8, CLEA Japan, Tokyo, Japan) and supplied with tap water *ad libitum*. Pancreases collected from 10-week-old rats were used as adult pancreases. Fetal pancreases were taken from pregnant rats on gestational (fetus) days 18 and 20; after delivery, pancreatic tissues were also obtained from neonates on days 1, 8, 15 and 21. At each examination point, three rats were euthanized by exsanguinations under deep isoflurane anesthesia. Animal housing and sampling conformed to the institutional guidelines approved by the ethics committee of Osaka Prefecture University for the Care and Use of Experimental Animals.

For the analysis of spontaneous pancreatic fibrosis, along with four cases of dogs with a normal pancreas, a total of 15 cases (13 dogs and 2 cats) were examined; the age, sex, species and grades of pancreatic fibrosis are shown in Table 1. These cases were necropsied in our laboratory, revealing various causes of the death, but it was not clear whether the pancreatic fibrosis was the direct cause of their death.
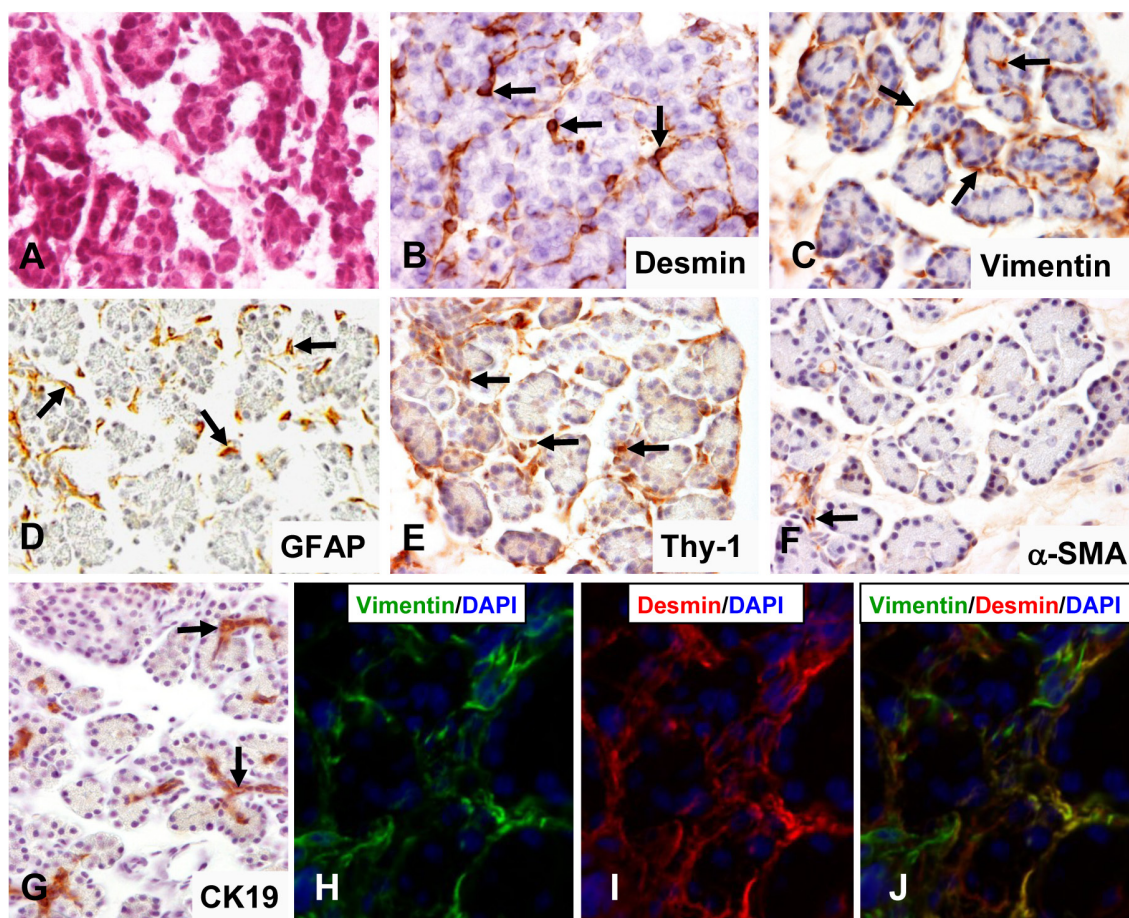
Histopathology and immunohistochemistry

Pancreatic tissues were fixed in 10% neutral buffered formalin; in addition, rat pancreases were fixed in periodate-lysine-paraformaldehyde (PLP) solutions [7]. Deparaffinized sections that were 4 μm in thickness were stained with hematoxylin and eosin (HE) for morphological observations and with azan-Mallory stain for detecting collagen deposition. Deparaffinized sections were also immunostained with (monoclonal or polyclonal) primary antibodies except for desmin. Fresh frozen sections from

Table 3. Semiquantitative evaluation of desmin, vimentin, GFAP-, α-SMA-, Thy-1- and CK19-positive interstitial cells or myofibroblasts in the developing (fetus and neonate) and adult rat pancreas

Antibody	Fetus		Neonate					Adult
	18 day	20 day	1 day	8 day	12 day	15 day	21 day	
Desmin	+++	++~+++	++~+++	+++	++	++	++	+
Vimentin	+++	++	+++	+++	++~+++	++	+ ~++	+
GFAP	+	+	+	+	+	+	+	+
α-SMA	-	-	-	±	±	±	±	±
Thy-1	+	+	++	+~++	+~++	++	+	+
CK19	+	+	+	+~++	++	++	++	++

The semiquantitative grading of immunopositive cells, which were diffuse or scattered, was evaluated as follows: -: no immunopositive cells; ±: a few immunopositive cells; +: a small number of immunopositive cells; ++: a moderate number of immunopositive cells; and +++: a large number of immunopositive cells.

Fig. 1. A: Histology of the developing pancreas in the neonatal rat on day 12. HE stain. B-F: Immunohistochemistry results showing the expression of desmin (B), vimentin (C), GFAP (D), Thy-1 (E) and α-SMA (F) in interstitial cells of the developing pancreas in rats (neonatal day 12). Arrows indicate immune-positive cells. G: Pancreatic ducts reacting to CK19 are clearly observed on neonatal day 12 (arrows), indicating the formation of exocrine glands with ducts. H-J: Double immunostaining in the pancreas on neonate day 12. Vimentin (green) and desmin (red) are co-expressed interstitial cells (yellow) in the developing pancreas.

developing rat pancreases were immunolabeled with anti-desmin antibody. Details on the antibodies are listed in Table 2. After primary antibody treatment, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (Histofine simplestain MAX-PO, Nichirei, Tokyo, Japan) for 30 min. Positive reactions were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB substrate kit, Vector Laboratories, Burlingame, CA, U.S.A.), and the sections were lightly counter-stained with hematoxylin. As negative controls, tissue sections were treated with mouse or rabbit non-immunized serum instead of the primary antibody.
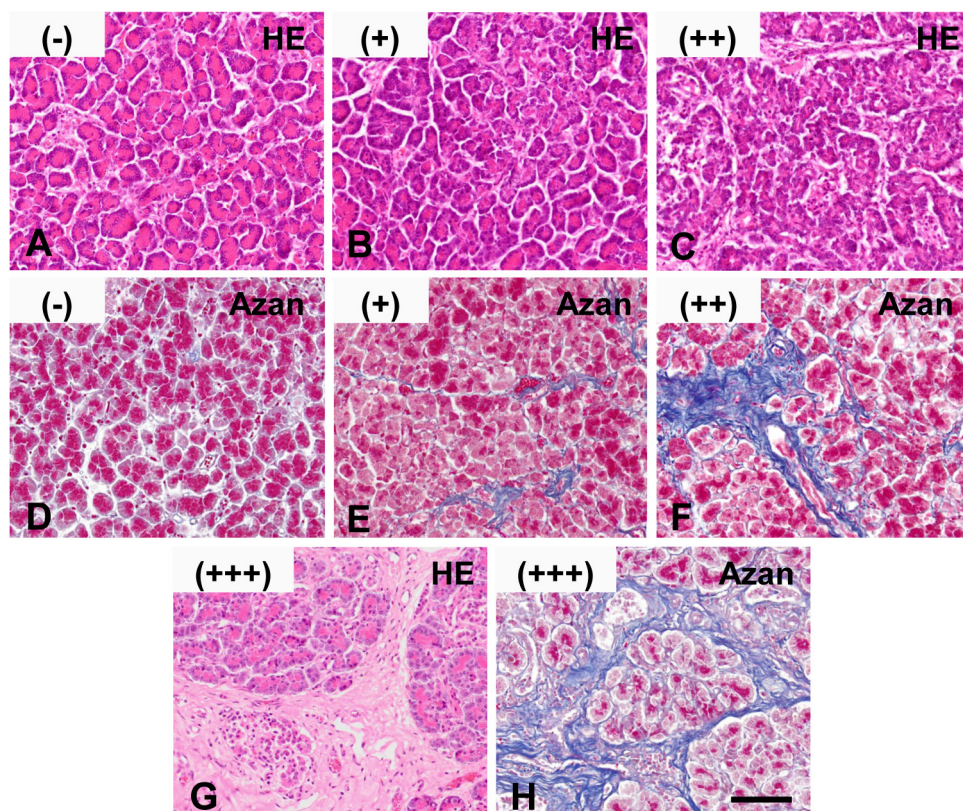
Fig. 2. Histopathology of the normal pancreas without fibrosis (−) (A, D) and fibrotic pancreases of different grades (+ to +++) (B, C, E–H) from dogs and cats. Normal pancreas showing a normal architecture. Fibrous bridges appear in the fibrotic pancreas of grades ++ to +++. A–C, G, HE stain; D–F, H, Azan-Mallory stain. Bar, 100 μm.

Double immunofluorescence

Fresh frozen sections (10 μm in thickness) from developing rat pancreases on neonate day 12 were used. Double immunofluorescence was carried out using antibodies against vimentin and desmin. Briefly, after fixation in cold acetone:methanol (1:1) for 10 min at 4°C, the sections were incubated with 10% normal goat serum for 30 min. The sections were reacted with the primary antibodies against vimentin labeled with HyLyte flour 555 (Dojindo Laboratories, Kumamoto, Japan) and desmin overnight at 4°C. After rinsing with PBS, the sections were incubated for 45 min with the secondary anti-mouse IgG-conjugated with Alexa 568 (Invitrogen, Carlsbad, CA, U.S.A.). The sections were visualized in Vectashield mounting medium containing 40, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc.) for nuclear staining and analyzed using a virtual slide scanner (VS-120, Olympus, Tokyo, Japan).

Histological evaluation

In HE-stained sections, pancreatic fibrosis in dogs and cats was evaluated by grading as shown in Table 1: -, normal without fibrosis; ±, very slight fibrosis; +, slight fibrosis or focal fibrosis; ++, moderate fibrosis; and +++, severe fibrosis with diffuse lesion. There were no canine cases with severe fibrosis. The immunopositive cells in the developing rat pancreas and pancreases of dogs/cats were evaluated semi-quantitatively as follows: -, no immunopositive cells; ± , a few immunopositive cells; +, a small number of immunopositive cells; ++, a moderate number of immunopositive cells; and +++, a large number of immunopositive cells (Tables 3 and 4).
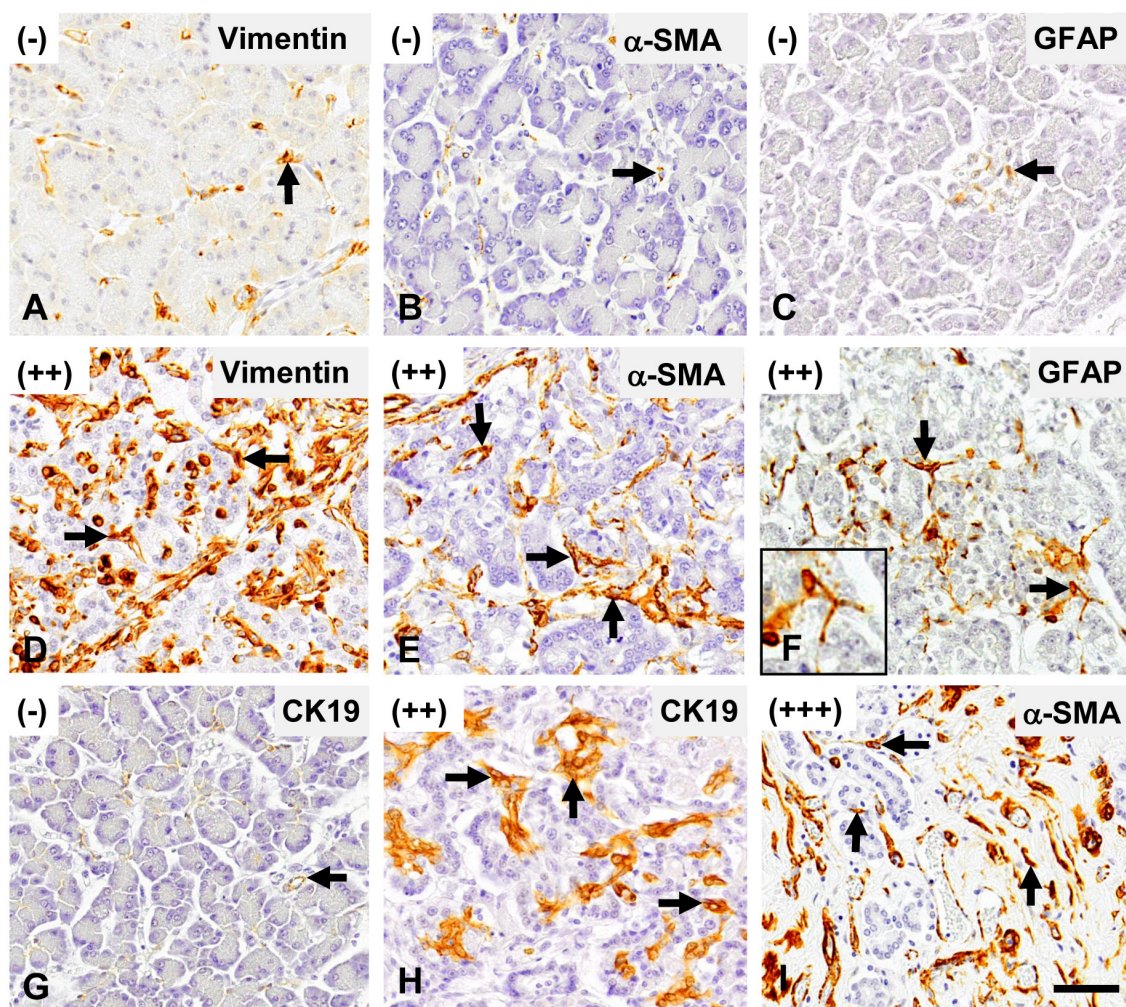
RESULTS

Immunoreactivity for vimentin, desmin, GFAP, a-SMA and Thy-1 in interstitial cells of the developing pancreas in rats

As early as fetal days 18 and 20, the exocrine glands were clearly formed (Fig. 1A), including sporadically distributed islets of Langerhans; the glandular lobules were surrounded by interstitial mesenchymal cells and collagens. The interstitial mesenchymal cells were distributed diffusely or were scattered and labeled mainly for desmin and vimentin; the reactivity was the greatest (++~+++) on fetal days 18 and 20, as well as on neonatal days 1 and 8, and then, it decreased gradually with age until adulthood (Fig. 1B and 1C; Table 3). Consistent with the immunohistochemistry results, double immunofluorescence revealed similar expression patterns for desmin or vimentin in interstitial mesenchymal cells; interestingly, almost all vimentin-positive cells also

Table 4. Semiquantitative evaluation of vimentin, GFAP-, α-SMA- and CK19-positive mesenchymal cells/myofibro-
blasts in the normal pancreas without fibrosis and fibrotic pancreases of different grades in dogs and cats

Fibrosis grade antibody	Normal without fibrosis (-)	Very slight fibrosis (±)	Slight fibrosis or focal fibrosis (+)	Moderate fibrosis (++)	Severe fibrosis with diffuse lesion (+++)
Vimentin	+	++	++	++	+++
GFAP	±	± ~+	± ~+	+~++	++
α-SMA	- ~±	+	+~++	++	++
CK19	- ~±	- ~+	+~++	+~++	+++

The semiquantitative grading of immunopositive cells, which were diffuse or scattered, was evaluated as follows: -: no immunopositive
cells; ± : a few immunopositive cells; +: a small number of immunopositive cells; ++: a moderate number of immunopositive cells; and
+++: a large number of immunopositive cells.

Fig. 3. Distribution of mesenchymal cells/myofibroblasts in a normal pancreas (−) without fibrosis (A–C, G); moderate (++) fibrotic pan-
creases (D-F, H) from dogs; and a severe (+++) fibrotic pancreas (I) from a cat, showing immunoreactivity for vimentin (A, D), α-SMA
(B, E, I), GFAP (C, F) and CK19 (G, H). Arrow indicates immune-positive cells. Inset (F) showing GFAP-positive fusiform-shaped mesen-
chymal cells with long cytoplasmic projections at a higher magnification. Immunohistochemistry. Bar, 50 μm.

expressed desmin (Fig. 1H–J). On the other hand, interstitial cells that reacted to GFAP were consistently seen in the developing
pancreas of fetuses and neonates as well as in adult samples in small numbers (Fig. 1D, Table 3). Thy-1 is a marker for immature
mesenchymal cells; interstitial cells that reacted to Thy-1 tended to increase on neonatal days 1–15 (Fig. 1E; Table 3). α-SMA-
expressing interstitial cells were rarely observed in the developing pancreas (- or ±) (Fig. 1F).

CK19 is expressed in pancreatic ducts and hepatic bile ducts [2, 7]. Pancreatic ducts that reacted to CK19 were clearly visible
on neonatal day 8 to onward and in adulthood (Fig. 1G), indicating the formation of exocrine glands and ducts. These results are
summarized in Table 3.

Immunoreactivity for vimentin, GFAP and α-SMA in myofibroblasts in the fibrotic pancreas in dogs and cats

Collagen deposition, which stained blue with the azan-Mallory stain, increased with the grade of fibrosis (+, ++), although the highest grade of pancreatic fibrosis (+++) was seen in only one cat (Fig. 2A–H). Acinar structures were markedly lost as the grade increased (Fig. 2C, 2F–H). When we evaluated canine and feline fibrotic pancreases, we confirmed that the immunoreactivity for cells positive for vimentin, GFAP, and α-SMA was similar to that in the other dogs and cats. In the normal pancreas, a very small number of interstitial cells that reacted to vimentin, α-SMA and GFAP were present (± or +) (Fig. 3A–C). Vimentin- and α-SMA-expressing myofibroblasts increased with the fibrotic grade (Fig. 3A, 3B, 3D, 3E and 3I) and were distributed diffusely or focally, although the expression degree for vimentin was greater than that for α-SMA at each fibrotic grade (Table 4). Interestingly, the expression pattern for GFAP was almost the same as that for α-SMA. GFAP-positive mesenchymal cells were morphologically fusiform-shaped with long cytoplasmic projections, and their numbers increased with the grade of pancreatic fibrosis (Fig. 3C and 3F). CK19-reacting pancreatic ducts also increased with the grade of pancreatic fibrosis (Fig. 3G and 3H). These results are summarized in Table 4.

DISCUSSION

Hepatic fibrosis is characterized by the abnormal deposition of collagens produced mainly by myofibroblasts [5]. The myofibroblasts derive from interstitial fibroblasts and HSCs expressing GFAP [15]. First, we analyzed the immune-expression of the cytoskeletons of interstitial cells in the developing rat pancreas to determine the basic features of these cells. It was found that interstitial cells in the pancreas expressed vimentin, desmin, GFAP and Thy-1 at varying degrees; the reactivities for desmin and vimentin were the highest in the fetuses; GFAP expression was consistent between fetuses and neonates; and Thy-1 reactivity transiently increased after birth; on the other hand, α-SMA-positive interstitial cells were rarely seen in the normal developing pancreas [10]. Based on these findings, immature interstitial cells may express vimentin, desmin and Thy-1, whereas although their number is small, GFAP-positive interstitial cells are regarded as PSCs whose cell features may be similar to those of HSCs. Because α-SMA expression was observed in myofibroblasts, the absence of its expression in the pancreatic interstitial cells was expected.

Next, we analyzed pancreatic fibrosis in samples obtained from dogs and cats, focusing on developing myofibroblasts. It is well known that myofibroblasts seen in hepatic and renal fibrosis express vimentin, desmin and α-SMA at varying degrees [5]. As expected, in the fibrotic pancreas, the number of cells that reacted to vimentin and α-SMA increased with the grade, although acceptable results were not obtained for desmin immunoreactivity because the fixative method was not suitable for desmin immunostaining [15]. In addition, GFAP-positive cells were observed in the pancreatic fibrotic lesions of dogs and cats, and their number increased with the grade. The cells that reacted to vimentin, α-SMA and GFAP were regarded as pancreatic myofibroblasts; these findings suggest that GFAP-positive myofibroblasts might derive partly from PSCs and contribute to pancreatic fibrosis. CK19-reacting pancreatic ducts increased with the grade in the fibrotic pancreas, indicating the possible proliferation of pancreatic ducts that might be reactive and increase fibrosis. This was the first analysis of pancreatic fibrosis in dogs and cats.

In conclusion, using immunohistochemical analyses of vimentin, desmin, α-SMA and GFAP, we determined the characteristics of interstitial cells in the developing rat pancreas and myofibroblasts in dogs and cats with pancreatic fibrosis. The findings obtained in this study will provide the basic information needed to understand lesion development in the fibrotic pancreas, which is an intractable disease.

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