Expression of Transforming Growth Factor β1, β2, and β3 in Chronic, Cancer-Associated, Obstructive Pancreatitis

Yuki Fukumura, MD, MS; Toshio Kumasaka, MD, PhD; Keiko Mitani, MT; Kanae Karita, MD, PhD; Koichi Suda, MD, PhD

Context.—Myofibroblasts are considered to play central roles in pancreatic fibrosis. The potent fibrogenic capacities of transforming growth factor βs (TGF-βs) have been emphasized in vitro and in animal studies. However, the roles of TGF-βs in human chronic pancreatitis have not been fully clarified.

Objective.—To investigate whether expressions of TGF-βs are related to myofibroblast distribution in chronic, cancer-associated, obstructive pancreatitis (COP).

Design.—Histopathologic studies using hematoxylin-eosin and Elastica-Masson trichrome and immunohistochemical studies using antibodies against α-smooth muscle actin (SMA); CD68; TGF-β1, -β2, and -β3; and TGF-β soluble receptor type II were performed in 19 COP cases and 6 controls. By classifying COP tissues into 3 fibrosis phases by the amount of collagen deposits, immunoreactivities for TGF-βs, histopathologic changes, and myofibroblast distribution were examined for each fibrosis phase.

Results.—Six cases were categorized in the early stage of fibrosis, 8 in the intermediate stage, and 5 in the advanced stage. Immunoreactivities for all 3 isoforms of TGF-β were observed in occasional myofibroblasts. In the early and intermediate stages, TGF-β1-expressing macrophages and neutrophils were distributed in the midst of myofibroblasts. TGF-β2 and TGF-β3 expressions were observed in ductal structures, sometimes even in sites where no or few myofibroblasts were seen. TGF-β soluble receptor type II was immunoreactive for myofibroblasts, endothelium, and ductal structures.

Conclusions.—All 3 isoforms of TGF-βs may contribute to fibrosis in COP. Macrophages and neutrophils may be sources of fibrogenic TGF-β1. Infiltration of these cells appears to play an important role in the progression of COP fibrosis.

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Chronic pancreatitis is characterized by progressive fibrosis that leads to morphologic and functional devastation of the pancreas. Myofibroblasts, a transformed cell type of pancreatic stellate cells, are known to play central roles in collagen deposits in pancreatic fibrosis.1-4 Although the mechanism of pancreatic fibrosis is still far from clear, recent molecular techniques have led to the identification of several growth factors that contribute to fibrogenesis in the pancreas.5

Transforming growth factor βs (TGF-βs) are members of a superfamily of polypeptides. Three isoforms are present in mammals, namely, TGF-β1, -β2, and -β3.6 These 3 TGF-β isoforms bind to common specific transmembrane receptors, TGF-β receptor type I and type II, to target genes via the SMAD family of signal transducing proteins. Both types of receptors are involved in TGF-β signal activation.7 TGF-βs are pluripotent growth factors. They have complex and widely varying effects on cell and tissue differentiation, growth, and response to injury. For example, exogenous TGF-β inhibits epithelial cell proliferation in general1,6,9 and is a potent chemoattractant for monocytes.10 In addition, extracellular matrix production is a relatively well-established role of TGF-βs.11,12

In the pancreas, the potent fibrogenic capacities of TGF-βs have been emphasized in vitro and in animal studies.13-16 In cell culture, exogenous TGF-βs transform pancreatic stellate cells into myofibroblasts and stimulate collagen synthesis by myofibroblasts.15,16 However, the roles of TGF-βs in human chronic pancreatitis have not been fully studied.

In the current study, the immunolocalization of the 3 TGF-β isoforms (TGF-β1, -β2, and -β3) was examined and compared with histopathologic changes and myofibroblast distribution in human chronic, cancer-associated, obstructive pancreatitis (COP) to investigate whether some expressions of TGF-βs are related to pancreatic fibrosis by myofibroblasts. By classifying COP tissues according to the amount of collagen deposits, the expression of each isoform, histopathologic changes, and myofibroblast distribution were examined for each fibrosis phase.

MATERIALS AND METHODS

Specimen Collection and Classification by Fibrosis Phase

Nineteen cases with a confirmed diagnosis of COP were studied. Pancreatic tissues 5 to 10 mm grossly distal to the carcinoma
invasion of ampullary carcinoma, carcinoma of the pancreas head, or carcinoma of the pancreas body were selected from the surgical pathology files of Juntendo University Hospital, Tokyo, Japan, and Yamanashi University Hospital, Yamanashi, Japan.

Control specimens were obtained from 6 patients (2 men and 4 women; mean age, 66.1 years; age range, 56–76 years); among them, 3 were 5–10 mm proximal portions of carcinoma of the pancreas body from the COP cases, and the remaining 3 were collected from the autopsy files of Juntendo University Hospital. None of the patients who underwent autopsy had histories of pancreatic diseases. The times between the deaths of the 3 patients who underwent autopsy and pancreas removal were 5, 6, and 6 hours.

Thirteen serial sections (4 μm thick) were cut from each for hematoxylin–eosin, Elastica–Masson trichrome, and other immunohistochemical stainings. With the hematoxylin–eosin–stained sections and with routine diagnostic procedures, each block was confirmed to be COP tissue, that is, each block was more than 5 mm away from any microscopic focus of cancer invasion, including pancreatic intraepithelial neoplasms, and showed clearly different fibrosis and inflammatory patterns from cancer-associated desmoplasia.

With Elastica–Masson trichrome–stained slides, COP cases were classified into fibrosis phase 1 (early stage) when less than one third of the normal tissue was replaced by fibrosis, phase 2 (intermediate stage) when fibrotic tissue constituted between one third and two thirds of the field, and phase 3 (advanced stage) when more than two thirds of acinar and ductal cells were replaced by fibrotic tissue.

All of the experiments conducted in this study were in compliance with the current laws of Japan.

**Immunohistochemical Analysis**

To study the distribution of myofibroblasts and macrophages, immunohistochemical stainings were performed using monoclonal antibodies against α-smooth muscle actin (α-SMA) and CD68, respectively. The expression of TGF-β1 and TGF-β2 soluble receptor type II (TGF-βRII) were investigated with polyclonal antibodies against TGF-β1, β2, and β3 and against TGF-βRII. An EnVision kit (DakoCytomation, Carpinteria, Calif) was used for the immunostaining of α-SMA and TGF-β1, β2, and β3 and against TGF-βRII. An EnVision kit (DakoCytomation, Carpinteria, Calif) was used for the immunostaining of α-SMA and TGF-β1, β2, and β3 and against TGF-βRII. Antibody Dilution Source Pretreatment

<table>
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<th>Antibody</th>
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<td>TGF-βRII (goat polyclonal)</td>
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* α-SMA indicates α-smooth muscle actin; TGF-β, transforming growth factor β; TGF-βRII, transforming growth factor β soluble receptor type II.

RESULTS

**Fibrosis Phases, Histopathologic Changes, and Myofibroblast Distribution of COP**

Six cases (32%), 8 cases (42%), and 5 cases (26%) were categorized into phase 1 (early stage), phase 2 (interme-

diate stage), and phase 3 (advanced stage) fibrosis, respectively (Figure 1, A through C, and Table 2). In all control cases (6 cases), no or scant autolytic changes, almost no fibrosis, and no or mild acinar atrophy were noted. Scattered CD68-positive macrophages were seen, but almost no inflammatory cells were distributed. Scant collagen fibers were seen mainly at interlobular areas and around the walls of large pancreatic ducts, and α-SMA-positive myofibroblasts were seen only around the walls of pancreatic ducts.

In all COP cases (19 cases), dilatations of the main pancreatic duct were consistently observed. Interlobular fibrosis was mainly observed in phase 1 (Figure 1, A), whereas intralobular fibrosis was also marked in phases 2 and 3 (Figure 1, B and C). Inflammatory cell infiltration was seen mildly and interlobularly in phase 1 (Figure 1, D), markedly and both interlobularly and intralobularly in phase 2 (Figure 1, E), and mildly and diffusely in phase 3 (Figure 1, F) and included macrophages, neutrophils, and lymphocytes. Myofibroblasts highlighted by α-SMA staining were seen mainly interlobularly and focally intralobularly in mild to moderate numbers in phase 1 (Figure 1, G) and massively both interlobularly and intralobularly in phase 2 (Figure 1, H). In phases 1 and 2, the distribution of myofibroblasts corresponded to the sites of collagen deposits. In phase 3, despite the massive replacement by collagen, myofibroblasts were much looser than in phase 2 (Figure 1, I).

**Immunolocalization of TGF-β1, -β2, and -β3**

The immunoreactivities for TGF-β1, -β2, and -β3 are summarized in Table 3. All 3 TGF-β isoforms were immunoreactive in occasional myofibroblasts in all COP cases (Figure 2). TGF-β1 was also positive in inflammatory cells, mostly macrophages and neutrophils (Figure 3, A). Immunoreactivity for TGF-β2 was also observed in most ductal structures, that is, cells of interlobular and intralobular ducts, intercalated ducts, centroacinar cells, and atrophic acinar cells, the former 2 structures with more intensity and the latter 3 with less intensity (Figure 3, B). As for TGF-β3, some endothelial cells, most ductal structures, and some inflammatory cells showed immunoreactivities for this isoform. In contrast to TGF-β2, intercalated ducts, centroacinar cells, and atrophic acinar cells showed more intense reactivity for TGF-β3 than interlobular and intralobular ducts (Figure 3, C). Normal acinar cells were negative for TGF-β1, -β2, or -β3. TGF-β1-positive inflammatory cells were seen mainly interlobularly in phase 1 and both interlobularly and intralobularly in phase 2, thus corresponding to the sites of myofibroblast distribution in these phases. TGF-β2- and TGF-β3-positive ductal structures were often surrounded by myofibroblasts; however, TGF-β2 and TGF-β3 immunoreactivities

Arch Pathol Lab Med—Vol 130, March 2006

TGF-βs in Obstructive Pancreatitis—Fukumura et al 357
in intercalated ductules and centroacinar cells in the controls and those in the remaining ductal structures in phase 3 cases were sometimes seen with no or few myofibroblasts in the proximity. Multiple areas of the pancreatic specimens from each fibrosis phase showed the same immunoreactivity for TGF-β1, -β2, and -β3, confirming the reproducibility of the immunohistochemical data.

**Immunolocalization of TGF-βRII**

Immunoreactivity for TGF-βRII was seen in the endothelium and centroacinar cells in the controls, endothelium, mainly small ductal structures, and myofibroblasts in phase 1 and the endothelium, all types of ductal structures, and myofibroblasts in phases 2 and 3 (Figure 4).

**COMMENT**

Chronic obstructive pancreatitis is a form of chronic pancreatitis in which pancreatic duct obstructions are the main origins, causing fibrosis in the pancreatic tissue distal to the obstruction. Tissues from patients with COP were used to study pancreatic fibrosis.

In our COP cases, myofibroblast distributions corresponded to the sites of collagen deposits in the early to intermediate stages of fibrosis. It is common knowledge that myofibroblasts play an important role in not only wound healing but also collagenization, which is followed by fibrosis in many organs. Hence, it is suggested that collagen deposits and fibrosis in COP are also formed in a similar way.

The purpose of our study was to investigate the isoform of TGF-βs that contribute to COP fibrogenesis and its expressing cells. Since fibrogenic capacities of TGF-βs range not only in pancreatitis but also in cancer-associated desmoplasia, it was necessary to differentiate our materials from cancer desmoplasia. There were clear fibrosis patterns...
differences between them. Our COP fibrosis distributed in a lobule and ductule oriented way. In our cases, interlobular fibrosis distributed surrounding lobules, and most intralobular fibrosis distributed dividing lobules or surrounding ductules. On the other hand, cancer desmoplasia was rather chaotic, with fibrosis distributed more homogeneously, irrespective of interlobular or intralobular. In addition, the amount of neutrophil infiltration was more prominent in cancer desmoplastia. At the clinical level, in frozen section diagnosis, the distinction might be helpful in differentiating cancer invasion from COP.

This study showed the immunolocalization of each isoform of TGF-β in common sites and differential sites as follows: (1) immunoreactivities for all 3 isoforms of TGF-β (TGF-β1, -β2, and -β3) were found in myofibroblasts, and (2) differential immunoreactivities were seen in macrophages and neutrophils for TGF-β1, in ductal epithelia for TGF-β2, and in ductal epithelia, endothelial cells, and macrophages and neutrophils for TGF-β3. Although several in vitro studies have reported similar activities for each isoform, some in vivo studies reported differential expression patterns of these isoforms, suggesting a distinct in vivo function for each isoform. Our study suggests that each has both different and similar functions in COP.

TGF-βs are pluripotent growth factors. Since the receptors for TGF-βs are expressed by cells almost universally, local concentrations of the growth factors are important in the regulation of their effects. To investigate whether TGF-βs play any roles in human COP fibrosis, this study examined whether some expressions of TGF-βs were spatially related to myofibroblasts.

In this investigation, all 3 isoforms and TGF-βRII were immunoreactive for occasional myofibroblasts. The ex-
pression of TGF-βs for myofibroblasts has also been reported in other types of inflammatory organ fibrosis, including chronic alcoholic pancreatitis (CAP),24,25 supporting the role of TGF-βs in organ fibrosis. Our study suggests that all 3 isomers may play roles in COP fibrogenesis.

A close spatial relationship between TGF-β1 immunopositivities and myofibroblast distribution was seen in the early to middle phases in our study. The contribution of inflammatory cell infiltration, including macrophages and polymorphonuclear cells, in pancreatic fibrosis has been reported in chronic pancreatitis.26–28 The present results also support the contributing roles of macrophages and neutrophils in COP fibrosis.

The expressions of TGF-β2 and -β3 for ductal structures in COP cases and controls were another finding of this study, with some expressions spatially related to myofibroblasts and others not. To clarify the latter observation, 11 serial sections (4 μm thick) were made, with the first to fifth and seventh to 11th sections stained with α-SMA, and the sixth sections were stained with TGF-β2 and -β3, resulting in the confirmation that TGF-β2 and -β3 expressions in intercalated ductules and centroacinar cells in the controls and those expressions in the remaining ducts in advanced phases were often observed, even in sites where no or few myofibroblasts were seen within 20 μm. Several studies on human chronic pancreatitis have reported TGF-β expression for ductal structures adjacent to fibrotic areas, suggesting them to be sources of fibrogenic TGF-β.29,30 Our study suggested that in COP, TGF-β expressed in some ductal structures play a fibrogenic role, whereas those factors in other ductal structures may have other functions. Immunoreactivities for TGF-β receptors were also observed in ductal structures in COP cases. Thus, our study suggests that the TGF-β2 and -β3 expressed by the ductal structures, particularly in the advanced stage of fibrosis, may have targeted themselves, inhibiting their own proliferation, possibly leading to exocrine atrophy.

Figure 2. Double immunostaining for transforming growth factor β3 (TGF-β3) and α-smooth muscle actin (SMA) in chronic, cancer-associated, obstructive pancreatitis (COP). Arrows demonstrate spindle-shaped cells positive for both TGF-β3 (red) and α-SMA (brown), indicating TGF-β3 expression in myofibroblasts. Epithelial cells of the dilated duct, upper right, were positive only for TGF-β3. Case 11, phase 2 COP (double immunostain for TGF-β3 and α-SMA, original magnification ×150).

Figure 3. Representative immunolocalizations of transforming growth factor βs (TGF-βs) in phase 2 chronic, cancer-associated, obstructive pancreatitis (COP). A, TGF-β1 was intensely positive for inflammatory cells. Arrows (inset) demonstrate some that were positive for both TGF-β1 (brown) and CD68 (red), indicating TGF-β1 expression in macrophages. B, TGF-β2 was intensely positive in ductal cells. C, TGF-β3 was intensely positive in endothelial cells (arrowhead). Ductal structures and some inflammatory cells were also positive for TGF-β3 with mild to moderate intensities. A through C are from a portion similar to B (case 10) (immunostain for TGF-β1, -β2, and -β3 [A through C, respectively] and double immunostain for TGF-β1 and CD68 [inset], original magnifications ×80 [A through C] and ×150 [inset]).
Intense staining was also seen for TGF-β3 in endothelial cells. However, we could not find a spatial relationship between TGF-β3-expressing endothelium and myofibroblast proliferation, because only scattered endothelial cells showed immunoreactivities for the isoform. Further studies are needed on the role of the endothelium in COP.

In conclusion, all 3 isoforms were suggested to contribute to fibrogenesis in COP. Our study showed macrophages and neutrophils to be possible candidate sources of fibrogenic TGF-β1, and the infiltration of macrophages and neutrophils appeared to play an important role in the progression of COP fibrosis.

Finally, CAP is also a fibrosing disease. To elucidate the fibrosing mechanism of CAP is clinically more important than that of COP. It has been reported that the pattern of development of fibrosis in COP is different from that in CAP. In COP, both intralobular and interlobular fibrosis are seen homogeneously, as are the findings of our cases of middle stages. On the other hand, in typical CAP, fibrosis is mainly formed interlobularly in early stage, and even in advanced stage, there are some lobules surrounded with interlobularly distributed fibrosis, leading to unevenly distributed fibrosis. Hence, the fibrosing mechanism mediated with TGF-βs suggested in this study may not be applicable to CAP. Studies of TGF-βs using human CAP tissues are needed.

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References

4. Powell DW, Mifflin RC, Valentich JD,Crowe SE, Saada JI, West AB. Myo-

15. Muller-Pillacs F, Menke A, Yamaguchi H, et al. TGF-beta and the extra-
19. Lörh M, Schmidt C, Ringel J, et al. Transforming growth factor-β induces morpho-
20. Miller DA, Pelton RW, Derynck R, Moses HL. Transforming growth factor-
21. Cheisietz S, Weatherbee JA, Tsang ML, et al. The transforming growth factor-
erlands; 1984:77–85.
32. Suda K, Takase M, Takei K, et al. Histopathologic and immunohisto-