Gastroenterologia Japonica
Copyright © 1986 by The Japanese Society of Gastroenterology

Vol. 21, No. 5
Printed in Japan

—Original Article—

EFFECTS OF ANTIFIBROTIC SUBSTANCES ON PANCREATIC FIBROSIS FOLLOWING ACUTE NECROTIZING PANCREATITIS

Yoshio KINAMI, M.D., Noriaki SAKATA, M.D. and Michiaki TAKATA, M.D.
The Second Department of Surgery, Kanazawa Medical University, Ishikawa 920-02, Japan

Summary

This study was designed to search for a way to inhibit pancreatic fibrosis following acute pancreatitis. Experimental necrotizing pancreatitis was induced by a freezing procedure in the pancreas of male Wistar rats. After the freezing procedure, the rats were divided into 3 groups: nothing addition was done to the control group, while the other 2 groups received daily intraperitoneal administration of antifibrotic substances (colchicine or L-azetidine-2-carboxylic acid (AZC)) for 6 weeks. Pancreatic enzymes in the serum were not markedly influenced by administration of antifibrotic substances, and there were no differences in the ratios of dry to wet weights of the pancreas between groups with and without these drugs. After freezing, the hydroxyproline levels in the pancreas of the control group increased from 1 to 4 weeks and then decreased during the 5th and 6th weeks. All groups receiving colchicine or AZC exhibited a significant decrease in the hydroxyproline levels at 2 to 4 weeks compared with the control group ($P<0.01$). Histological examination also showed the inhibition of pancreatic fibrosis, agreeing with changes in the hydroxyproline levels in groups receiving colchicine or AZC. These results suggest that administration of antifibrotic substances, colchicine and AZC, have the possibility of inhibiting pancreatic fibrosis following acute pancreatitis.

Key Words: *Antifibrotic substances, Hydroxyproline, Necrotizing pancreatitis, Pancreatic fibrosis.*

Introduction

It is generally thought that the characteristic of chronic pancreatitis[1] is an irreversible and progressive fibrous proliferation in the pancreas. This fibrosis causes not only morphologic alteration but also functional impairment in the pancreas. In most patients with acute pancreatitis, the pathological changes in the pancreas normalize together with reduction of inflammation, but in 20% or 30% of cases[2] the necrotized parenchyma is replaced with fibrous tissue. However, at present there is no effective treatment to reduce this pancreatic fibrosis.

Hepatic and pneumonic fibroses have been widely studied[3-5]. In the former, it is thought that as a repair mechanism in tissue after successive necrosis of liver cells, fibrous proliferation occurs, fundamentally similar to wound healing. On the other hand, the latter has been studied from the standpoint of immunology. We investigated experimentally a relationship between pancreatic fibrosis and collagenolytic

Received November 28, 1985. Accepted April 14, 1986.

Address requests for reprints to: Yoshio Kinami, M.D., The Second Department of Surgery, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Kahoku-gun, Ishikawa 920-02, Japan.

activity in pancreatic tissue following necrotizing pancreatitis[6]. Besides such basic studies related to pancreatic fibrosis, there are a few studies concerning prevention of or therapy for fibrous proliferation in the pancreas after acute pancreatitis. In this study, 2 antifibrotic substances were administered to rats with acute pancreatitis, and their effects on pancreatic fibrosis were examined.

Materials and Methods

In this study 432 male Wistar rats weighing 200-250 g and fasting for one day were used. Colchicine (Wako Junyaku Co. Osaka, Japan) an anti-microtubular drug and L-azetidine-2-carboxylic acid (AZC, Sigma Chemical Co. St. Louis, U.S.A.) a proline analogue were examined concerning their effect on pancreatic fibrosis.

Experimental necrotizing pancreatitis was produced by the following procedure. The rats received intraabdominal injections (5 mg/100 g body weight) of pentobarbital sodium and underwent laparotomy. The splenic segment of the pancreas underwent a freezing procedure for 30 seconds at −60°C in a freezing apparatus (Cryos-A) using CO_2 (**Fig. 1**). The rats were given food ad libitum after this procedure. The rats with necrotizing pancreatitis were divided into 3 groups, consisting of a group receiving colchicine, a group receiving AZC, and a control group in which no additional procedures had been carried out. Furthermore, the colchicine group was divided into 3 subgroups. One received 0.04, another 0.2, and the third 0.4 mg/kg body weight of colchicine by injection into the abdominal cavity daily for 6 weeks from the day the pancreatitis was induced. On the other hand, the AZC group was divided into 2 subgroups, one receiving 4 and the other 20 mg/kg body weight of AZC by injection into the abdominal cavity daily for 6 weeks.

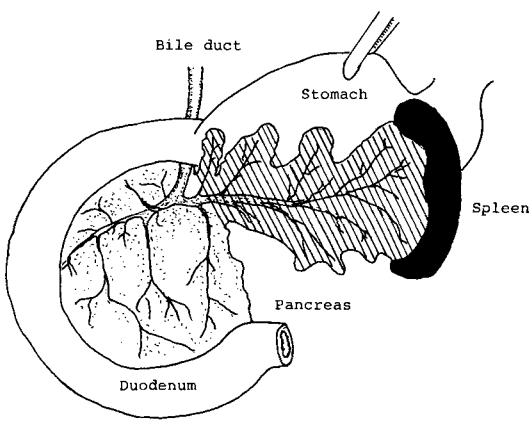

Fig. 1. Frozen area in the pancreas.

Each group was examined as follows at 1-6 weeks after the onset of acute pancreatitis. Besides main examinations, the amylase and lypase levels in the serum were determined at 1, 3 and 6 weeks after administration of colchicine or AZC. In order to determine amylase and lypase, blood was taken from the inferior vena cava. Amylase was determined by the enzymatic method (Amylase test, Wakow), and lypase was done by the British antilewsite tributyrate sodium louryl sulfate-5, 5′-dithiobis (2-nitro-benzoic acid) method (BALB-BTNB, Lypase kit Dainippon Co. Osaka, Japan). In each group, a section of the pancreatic tissue, which was taken from a fixed region of the pancreas, was desiccated by freezing for 20 hours at −40°C after measurement of the wet weight, and the ratio of dry to wet weights of the pancreatic tissue was calculated. Thereafter, hydroxyproline levels in the pancreatic tissue were determined by the KISO method of Inayama et al.[7], a modification of the method of Prochop et al. All values were expressed as μg/mg dry weight of the pancreatic tissue. Furthermore, the pancreas was removed before or 1-6 weeks after the freezing procedure, and the fibrotic changes in the pancreas were evaluated histologically with hematoxylineosin and azan stains.

All data for each examination was presented

as the mean ± SD, and the unpaired Student's t-test was used for comparison.

Results

1. Amylase and lypase levels in serum

The mean (±SD) amylase level in the serum of normal rats was 2858 ± 286 Somogyi U/dl (n=8), and the mean (±SD) lypase level in the serum of normal rats was 124.3 ± 36.5 BALB unit/0.05 ml (n=8). As basic examinations, the influence of administration of colchicine and AZC was examined on the amylase and lypase levels in the serum of 25 rats without the freezing procedure (**Table 1**). However these levels were not affected by administration of any amounts of colchicine or AZC at any week, and there was no significant difference among any of the levels of each enzyme. There were no marked differences in serum amylase levels at any times between rats of the control group and normal rats. The lypase levels in the serum of the control group rose slightly for the period 1 to 6 weeks, but there were no significant differences in serum lypase levels at any time between rats of the control group and normal rats.

On the other hand, there were no marked differences in serum amylase levels at any time between the colchicine groups and the control group, or between the AZC groups and the control group (**Table 2**). Both the colchicine and AZC groups exhibited stable levels of amylase. Furthermore, there were also no significant differences in serum lypase levels throughout the whole course between colchicine or AZC groups and the control group (**Table 3**).

2. Ratio of dry to wet weights of the pancreatic tissue

The mean (±SD) ratio of dry to wet weights of the normal pancreatic tissue was 28.6 ± 2.1% (n=8). The ratio of dry to wet weights in the control group was 39.1 ± 5.9% at 4 weeks after the freezing procedure, and this was sig-

Table 1. *Pancreatic enzymes in serum of normal rats before and after administration of antifibrotic substances*

Antifibrotic Substances	Amounts mg/kg	Serum Amylase (somogyi U/dl, Mean±SD)				Serum Lypase (BALB U/0.05 ml, Mean±SD)			
		Before Administration*	After Start of Administration (weeks)**			Before Administration*	After Start of Administration (weeks)**		
			1	3	6		1	3	6
Colchicine	0.04	2858±286	2870±303	2790±412	2620±442	124.3±36.5	132.4±30.2	146.1±32.2	144.3±28.4
	0.20	2858±286	2690±412	2882±372	2530±386	124.3±36.5	143.2±40.4	122.8±40.6	130.6±33.4
	0.40	2858±286	2850±652	2768±574	2682±652	124.3±36.5	118.7±38.2	154.2±30.4	136.8±40.2
AZC	4	2858±286	2546±442	2554±324	2563±402	124.3±36.5	142.8±28.6	148.9±40.1	136.5±25.6
	20	2858±286	2610±532	2910±420	2748±526	124.3±36.5	118.6±41.5	155.6±34.8	140.8±26.8

*: n=8 **: n=5

Table 2. Changes in serum amylase levels of each group after the freezing procedure

Groups	Amounts mg/kg	Serum Amylase (somogyi U/dl, Mean±SD)						
		Before Administration*	After Freezing Procedure (weeks)*					
			1	2	3	4	5	6
Control	—	2858±286	2652±584	2826±576	3412±894	3106±553	3094±980	2772±608
Colchicine	0.04	2858±286	2485±598	2684±504	2372±574	2495±526	2240±386	2114±398
	0.20	2858±286	1963±314	3214±1202	3776±645	2702±680	2818±738	2510±549
	0.40	2858±286	3325±473	2094±498	2889±796	3152±787	2715±514	2698±738
AZC	4	2858±286	1694±384	2009±504	2372±398	2198±401	2516±490	2394±412
	20	2858±286	2189±398	2573±610	3941±604	2846±843	2838±621	2385±404

*: n=8

Table 3. Changes in serum lypase levels of each group after the freezing procedure

Groups	Amounts mg/kg	Serum Lypase (BALB U/0.05 ml, Mean±SD)						
		Before Administration*	After Freezing Procedure (weeks)*					
			1	2	3	4	5	6
Control	—	124.3±36.5	121.6±40.4	204.5±35.3	188.7±30.5	157.5±27.8	208.3±46.2	189.5±35.4
Colchicine	0.04	124.3±36.5	160.3±36.4	215.6±37.0	156.9±39.1	171.4±46.2	186.0±47.0	198.0±33.7
	0.20	124.3±36.5	187.5±34.3	212.7±46.7	235.2±31.5	178.1±39.2	191.5±36.2	208.4±31.7
	0.40	124.3±36.5	217.6±62.7	220.3±65.5	224.1±31.2	237.3±57.3	240.5±56.9	223.2±68.7
AZC	4	124.3±36.5	168.5±27.6	217.3±19.7	219.2±22.5	224.7±30.1	231.2±26.7	228.7±26.4
	20	124.3±36.5	198.7±22.5	221.5±31.7	241.3±34.9	237.0±35.8	244.9±35.4	206.3±24.7

*: n=8

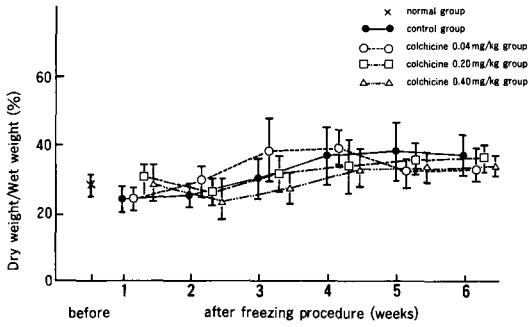

Fig. 2. Changes in the ratio of dry to wet weights of the pancreas after the freezing procedure. Each point represents the mean ± S.D. in 8 rats. (comparison between colchicine and control group)

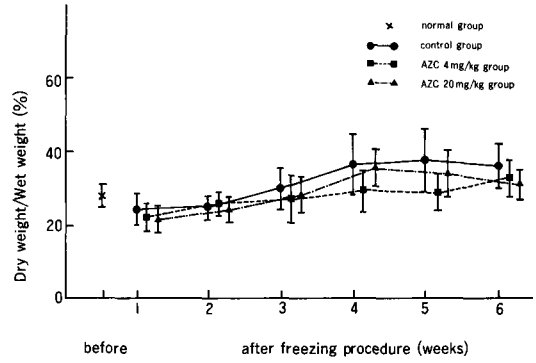

Fig. 3. Changes in the ratio of dry to wet weights of the pancreas after the freezing procedure. Each point represents the mean ± S.D. in rats. (comparison between AZC and control group)

nificantly higher than that of normal rats ($P<0.01$). The ratios at 5 and 6 weeks were similar to that at 4 weeks. The changes in the ratios of dry to wet weights of the pancreatic tissue in the colchicine groups were similar to those changes in the control group, and there were no significant differences between the colchicine groups and the control group (**Fig. 2**). On the other hand, the ratios of dry to wet weights of the pancreatic tissue in the AZC groups revealed the same changes as those in the control group, and there were no significant differences among these groups (**Fig. 3**).

3. Hydroxyproline levels in the pancreatic tissue

The mean (±SD) level of hydroxyproline in normal pancreatic tissue was 3.42 ± 0.76 μg/mg dry weight of pancreas (n=8). After the freezing procedure, the hydroxyproline level in the pancreatic tissue of the control group was 5.14 ± 1.26 μg/mg (n=8) at 1 week, and significantly higher than the level in normal pancreatic tissue ($P<0.01$). Thereafter, the levels increased to 7.56 ± 1.14 μg/mg at 2 weeks, 7.58 ± 1.26 μg/mg at 3 weeks and 7.88 ± 1.48 μg/mg at 4 weeks, but declined to 5.04 ± 0.69 μg/mg at 5 weeks and 5.14 ± 1.12 μg/mg at 6 weeks.

On the other hand, the hydroxyproline levels in the pancreatic tissue for the subgroup receiving 0.04 mg/kg colchicine were 3.89 ± 0.49 μg/mg at 1 week, 5.41 ± 0.40 μg/mg at 2 weeks, 5.59 ± 0.36 μg/mg at 3 weeks and 5.71 ± 0.47 μg/mg at 4 weeks, being significantly lower than those of the control group, except at 1 week ($P<0.01$). But the levels at 5 and 6 weeks were 4.29 ± 0.37 μg/mg and 4.72 ± 0.29 μg/mg respectively, and there were no significant differences between this subgroup and the control group (**Fig. 4**). Levels in the subgroup employing 0.2 mg/kg colchicine were 4.42 ± 0.36 μg/mg at 1 week, 5.03 ± 0.68 μg/mg at 2 weeks, 5.77 ± 0.78 μg/mg at 3 weeks and 5.20 ± 0.66 μg/mg at 4 weeks, being significantly lower than those of the control group, except at 1 week ($P<0.01$). However levels at 5 and 6 weeks were 4.73 ± 0.64 μg/mg and 4.66 ± 0.37 μg/mg respectively, and there were no significant differences between this subgroup and the control group. Levels in the subgroup employing 0.4 mg/kg colchicine were 4.61 ± 0.62 μg/mg at 1 week, 3.78 ± 0.27 μg/mg at 2 weeks, 4.63 ± 0.43 μg/mg at 3 weeks and 4.59 ± 0.57 μg/mg at 4 weeks, being significantly lower than those of the control group, except at 1 week ($P<0.01$). But the levels at 5 and 6 weeks were 4.36 ± 0.32 μg/mg and 4.16 ± 0.42

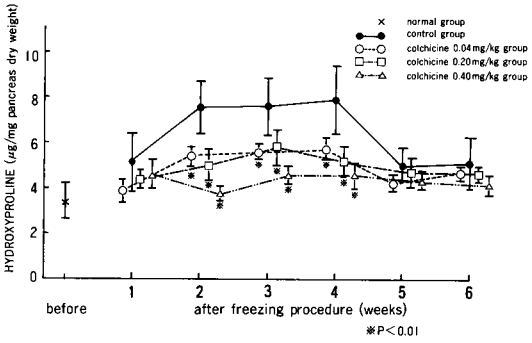

Fig. 4. Comparison of hydroxyproline levels in the pancreatic tissue after the freezing procedure between colchicine groups and the control group. Each point represents the mean ± S.D.

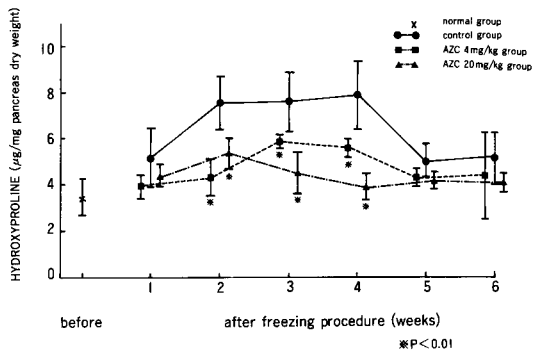

Fig. 5. Comparison of hydroxyproline levels in the pancreatic tissue after the freezing procedure between AZC groups and the control group. Each point represents the mean ± S.D. in 8 rats.

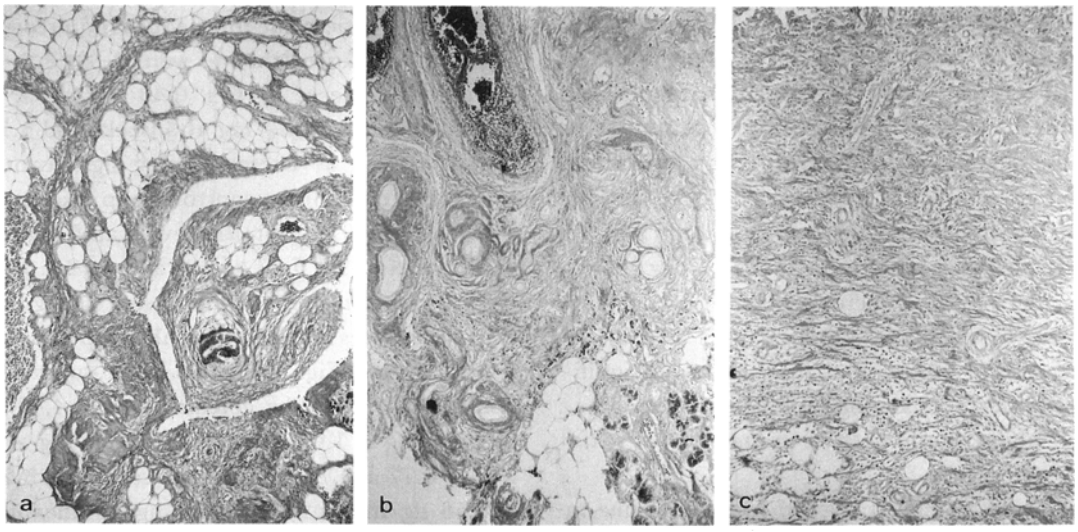

Fig. 6. Histological findings of the pancreas 4 weeks after the freezing procedure (azan stain, ×100). A: The pancreas of the control group revealed a strong fibrosis and a mild adipose tissue. B: The pancreas of a colchicine group at receiving 0.2 mg/kg/day exhibited a mild fibrosis and adipose tissue. C: The pancreas of AZC group, receiving 4 mg/kg/day of it, also indicated a mild fibrosis and adipose tissue.

μg/mg respectively, and there were no significant differences between this subgroup and the control group.

The hydroxyproline levels in the pancreatic tissue of the subgroup employing 4 mg/kg AZC were 3.91 ± 0.49 μg/mg at 1 week, 4.29 ± 0.82 μg/mg at 2 weeks, 5.85 ± 0.30 μg/mg at 3 weeks and 5.58 ± 0.40 μg/mg at 4 weeks, being significantly lower than those of the control group, except at 1 week ($P<0.01$). But the levels at 5 and 6 weeks were 4.32 ± 0.36 μg/mg and 4.39 ± 1.87 μg/mg respectively, and there were no significant differences between this subgroup and the control group (**Fig. 5**). The levels in the subgroup employing 20 mg/kg AZC were 4.33 ± 0.36 μg/mg at 1 week, 5.36 ±

0.64 μg/mg at 2 weeks, 4.48 ± 0.90 μg/mg at 3 weeks and 3.93 ± 0.58 μg/mg at 4 weeks, being significantly lower than those of the control group, except at 1 week ($P<0.01$). However the levels at 5 and 6 weeks were 4.16 ± 0.37 μg/mg and 4.11 ± 0.42 μg/mg respectively, and there were no significant differences between this subgroup and the control group.

4. *Histological changes in the pancreatic tissue*

To carry out histological examinations, 111 rats were sacrificed. In the control group, necrosis of acinar cells, infiltration of the inflammatory cells, increased pancreatic ductules and fibrous proliferation were obviously observed in frozen sections obtained 1 week after freezing. On the other hand, the colchicine group also exhibited fibrous proliferation at 1 week, but its grade was mild compared with the control group. The AZC group also revealed the same findings as the colchicine group. In the control group, these fibrous proliferations advanced markedly in the period from 2 to 4 weeks, while there were reductions of the necrotic area, inflammatory cells and pancreatic ductules. On the contrary, colchicine and AZC groups revealed further inhibition of fibrous proliferation in the same period (**Fig. 6**). However, in both the colchicine and AZC groups the intensity of inhibition of fibrosis was not affected by administered amounts of these substances. Thereafter, in all groups the fibrous tissue and pancreatic ductules began to decrease, and fatty tissue increased gradually. There was no difference in the histological findings among any of the groups at 6 weeks.

Discussion

Acute pancreatitis is one of the causes of irreversible chronic pancreatitis, and there are many reports[1,2] about this subject. In general, it is mentioned that irreversible and progressive pancreatic fibrosis occurs as a result of inflammation in the pancreas, being due to various causes, and is accompanied by morphologic and functional changes in the pancreas. Pancreatic fibrosis observed after pancreatitis is a serious clinical problem, but there have been few studies concerning the etiology and inhibition of fibrosis.

Although it is not easy to experimentally induce pancreatic fibrosis for clinical investigation, models of pancreatitis have been developed by various methods. We also developed a rat model of necrotizing pancreatitis by contact freezing and observed changes in pancreatic fibrosis[6]. In this study, the contact freezing method was used because it is certain that fibrous proliferation in the pancreatic tissue will follow within a relatively short term.

Hydroxyproline, one of the characteristic amino acids of which collagen protein is composed, exhibits a constant ratio in collagen. Therefore, this level could indicate the amount of collagen. In order to determine the hydroxyproline level in pancreatic tissue as an index of pancreatic fibrosis after experimental necrotizing pancreatitis, Prockop-Udenfreind's method[8] and Nauman's method[9] are used in Japan. However, we employed the KISO method of Inayama et al.[7] because it is a simpler method to obtain reliable data. After the freezing procedure, the hydroxyproline levels increased for 4 weeks, but decreased thereafter. The change in these levels was similar to their changes in pancreatic tissue reported previously[6], and agreed with histological changes.

To observe the development of pancreatic fibrosis and to search for a possible way to inhibit it, we administered antifibrotic substances that have an influence upon each step in collagen synthesis, to rats with necrotizing pancreatitis. Colchicine was used as one of these substances. It is known that colchicine[10-12], an anti-microtubular drug, inhibits collagen biosynthesis and interfers with the transport of col-

lagen precursors to extracellular space. Furthermore, this drug is also considered to act on the existing collagen fiber and reduce its amount[4,13]. On the other hand, AZC[14-16], a proline analogue that competes with proline for transport and acylation of proline-specific transfer-RNA, was also examined.

It is important to examine any influences caused by administration of antifibrotic substances on exocrine pancreas, especially those that accompany acute pancreatitis. At first, the influences of colchicine or AZC were studied on the exocrine enzymes in the serum of normal rats. As a result, no influence caused by these substances were observed. After the freezing procedure in rat pancreas, examination of the levels of serum exocrine enzymes showed that their levels in the control, colchicine or AZC group were similar to those in normal rats, and there were no significant differences in changes occurring among these groups. Furthermore, the changes in exocrine enzymes were not affected by the amounts of these substances administered. Accordingly, these results indicate that the antifibrotic substances have no marked influence on the exocrine pancreas.

The amount of water included in the tissue is almost constant in each organ. The histological changes in the pancreas were inferred according to examination of the amount of water in the pancreatic tissue at all time periods. The ratio of dry to wet weights of the pancreas was 30% in normal tissue, but exhibited a mild reduction at 1 and 2 weeks after the freezing procedure. Therefore, edema in tissue was assumed to be present at these times. The ratio reached approximately 40% after 4 weeks. From histological findings observed previously by us[6] or recognized similarly in this study, it was presumed that this result was due to a decrease of parenchyma cells, fibrous proliferation and increase in fatty tissue appearing after 3 weeks. The colchicine and AZC groups also revealed the same changes in ratios as those observed in the control group.

The hydroxyproline levels in pancreatic tissue of all groups were determined for 6 weeks after the freezing procedure. As a result, the levels in colchicine or AZC groups were significantly lower for 4 weeks than those in the control group. On the other hand, a histological examination was performed on all groups during the same period. The grades of fibrous proliferation were histologically slight in groups treated with antifibrotic substances, which was in accord with the changes of hydroxyproline levels in these groups. Rojking et al.[13] administered colchicine to rats with injured livers, which had been chronically treated with carbon tetrachloride, and recognized a reduction in collagen biosynthesis and an improvement in some liver-function tests. Furthermore, they mentioned that an antifibrotic effect was obtained in cirrhotic patients by administration of this drug. On the other hand, Kershenobich et al.[17] administered AZC to rats with livers injured by carbon tetrachloride, and reported an inhibition of hepatic fibrosis and an improvement in liver-function tests. In our experiments, an antifibrotic effect due to the administration of these substances was also observed in pancreatic tissue after the freezing procedure. However, there were no obvious differences in the reduced grades of hydroxyproline levels or in the inhibited grades of fibrous proliferation when comparing the administered amounts of both drugs. From these results obtained in our study, it is suggested that there is a possibility for the clinical application of both colchicine and AZC, because these drugs produce an inhibition of pancreatic fibrosis and no apparent injury to the pancreatic function.

References

1) Baggenstoss AH: Pathology of pancreatitis, in "Pan-

creatitis" edited by Gambill EE, CB Mosby Co, Saint Louis, 1973, p 202
2) Sato T, et al: Chronic pancreatitis, in "Pancreatic Disease" edited by Yamagata K, Kanahara Co, Tokyo, 1976, p 209 (Jpn)
3) Rojkind M, et al: Colchicine and the treatment of liver cirrhosis. Lancet 6: 38, 1973
4) Kershenobich D, et al: Treatment of cirrhosis with colchicine, a double-blind randomized trial. Gastroenterology 77: 532, 1979
5) Snider GL: Interstitial pulmonary fibrosis which cell is the culprit? Am Rev Resp Dis 127: 535, 1983
6) Kinami Y, et al: Pancreatic fibrosis and collagenolytic activity following acute pancreatitis in the rat. J Kanazawa Med Univ 9: 187, 1984
7) Inayama S, et al: A new microanalytical method for determination of hydroxyproline in connective tissue. Keio J Med 27: 43, 1978
8) Prockop DJ, et al: A specific method for the analysis of hydroxyproline in tissue and urine. Anal Biochem 1: 228, 1960
9) Neuman R, et al: The determination of hydroxyproline. J Biol Chem 184: 299, 1950
10) Diegelman R, et al: Inhibition of collagen secretion from bone and cultured fibroblasts by microtubular disruptive drugs. Proc Natl Acad Sci USA 69: 892, 1972
11) Ehrlich HP, et al: Microtubules in transcellular movement of procollagen. Nature New Biol 238: 257, 1972
12) Ehrlich HP, et al: Effects of antimicrotubular agents in the secretion of collagen. A biochemical and morphological study. J Cell Biol 62: 390, 1974
13) Rojkind M, et al: Effect of colchicine on collagen, albumine and transferrin synthesis by cirrhotic rat liver slices. Biochim Biophys Acta 378: 415, 1975
14) Takeuchi T, et al: Biosynthesis of abnormal collagens with amino acid analogues. I. Incorporation of L-azetidine-2-carboxylic acid and cis-4-fluoro-L-proline into protocollagen and collagen. Biochim Biophys Acta 175: 142, 1969
15) Takeuchi T, et al: Biosynthesis of abnormal collagens with amino acid analogus. II. Inability of cartilage cells to extrude collagen polypeptides containing L-azetidine-2-carboxylic acid or cis-4-fluoro-L-proline. Biochim Biophys Acta 175: 156, 1969
16) Uitto J, et al: Incorporation of proline analogues into polypeptides. Effects on the production of extracellular procollagen and on the stability of the triplehelical structure of the molecule. Biochim Biophys Acta 336: 234, 1974
17) Kershenobich D, et al: Effect of the administration of L-azetidine-2-carboxylic acid on albumin and transferrin biosynthesis by liver slices of rats treated with carbon tetrachloride. Biochim Biophys Acta 319: 216, 1973