Effects of Epigallocatechin Gallate on Diethyldithiocarbamate-Induced Pancreatic Fibrosis in Rats

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Epigallocatechin gallate (EGCG), a major component of green tea extracts, is known to have anti-fibrotic properties in many organs. The aim of present study was to investigate effects of EGCG on rat pancreatic fibrosis induced by diethyldithiocarbamate (DDC). Oral gavages of different dose of EGCG (50, 100 and 200 mg/kg daily for 8 weeks) ameliorated histological changes and significantly suppressed collagen deposition in a dose-dependent manner. Meanwhile, administration of EGCG inhibited overexpression of TGF-β1 and α-smooth muscle actin (a symbol of activation of pancreatic stellate cells). Moreover, EGCG has a potent influence on expression of Smads (downstream transcription factor of TGF-β1). EGCG suppressed the expression of Smad3 and enhanced the expression of Smad7. In conclusion, our results demonstrated that EGCG attenuated rat pancreatic fibrosis induced by DDC and therefore may be an anti-fibrogenic candidate in the pancreatic fibrosis.

Key words epigallocatechin gallate; pancreatic fibrosis; α-smooth muscle actin (SMA); TGF-β1; Smad

Chronic pancreatitis is a progressive and irreversible disease, whose clinical features are intractable pain, loss of pancreatic exocrine and endocrine function. Fibrosis and loss of parenchymal cells in the pancreas are characteristic histopathologic features in chronic pancreatitis. Moreover, pancreatic fibrosis is suggested to contribute to the irreversibility of the disease. Therefore, to search for an available medicine against pancreatic fibrosis is urgently needed.

The pathogenesis of chronic pancreatitis is not clear yet. Recent data demonstrate that pancreatic stellate cells (PSCs) play a major role in pancreatic fibrosis.1—4 PSCs, in their quiescent state, can be identified by the presence of vitamin A containing lipid droplets in their cytoplasm. After pancreas injury, they are activated and transform into myofibroblast-like cells which exhibit positive staining for the cytoskeletal protein α-smooth muscle actin (α-SMA), and synthesise and secrete increased amounts of extracellular matrix (ECM). TGF-β1 is a major mediator of fibrosis in pancreatic fibrosis. Increased evidences demonstrated that the expression of TGF-β1 was up-regulated in the fibrotic areas of the pancreas in human and animal with chronic pancreatitis, whereas with little expression in normal pancreas.5—9 Vogelmann R. et al.10 observed that overexpressed TGF-β1 developed pancreatic fibrosis in transgenic mice. Moreover, inhibition of TGF-β1 by anti-TGF-β1 antibody suppressed ECM production in pancreas.11 Furthermore, TGF-β1 is important to regulate PSCs functions. For instance, TGF-β1 activates PSCs, inhibits PSC growth and enhances ECM production and secretion.1,12

As we know, tea is one of the most frequently consumed beverages in the world, next to water. Epigallocatechin gallate (EGCG) (Fig. 1) is the major constituent found in green tea polyphenols (GTP). EGCG is responsible for the majority of the potential health benefits attributed to green tea consumption. Over the past decade, a vast body of scientific research discovered that EGCG had a wide range of biologic activities, including hypolipidemic,13 anti-microbial,14 anti-tumour activities15 and anti-oxidative stress.16 Recent studies suggested that EGCG also had anti-inflammatory and anti-fibrosis effects15—19 and substantial evidence had shown the role of EGCG in hepatic fibrosis, including inhibiting activation and proliferation of hepatic stellate cells, interrupting TGF-β signaling and suppressing collagen production and collagenase activity.20—23 And as we known, the pathogenesis of pancreas fibrosis is similar to that of hepatic fibrosis,24,25 so scholars have set about to investigate the effect of EGCG in pancreatitis. Recent investigations reported that GTP had a protective effect on the pathogenesis of acute pancreatitis.26,27 Furthermore, EGCG could inhibit activation and impede proliferation and migration of PSCs in vitro.28,29 But the correlative research literature in vivo has not been presented.

Oxidative stress was reported to be involved in pancreatic fibrosis.29—33 Our previous report also confirmed this viewpoint.30 From this viewpoint, Matsumura N. et al.31 succeeded in inducing pancreatic fibrosis in rat using a superoxide dismutase inhibitor, diethyldithiocarbamate (DDC). This model is easy to handle, and it needn’t require long-term duration for the development of pancreatic fibrosis. The aim of the present study was to evaluate effects of EGCG on DDC-induced pancreatic fibrosis in rats.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 170—190 g, were purchased from the Laboratory Animal Center of Shandong University and kept in a temperature-controlled room under
dual light cycle. They were allowed free access to water and standard laboratory food. Experiments were performed in accordance with the Laboratory Animal Care and Use Regulations of Shandong University.

Groups of Animals and Treatment The rats were randomly divided into control group, model group, low-, medium- and high-dose EGCG groups, and each group included 8 animals. Except for rats in control group, all rats were received intraperitoneal injections with 1 ml DDC (75 mg/ml; diluted in distilled water) (Sigma, U.S.A.) per 100 g body weight twice a week for 10 weeks. From the third week, rats in EGCG administration groups were given oral gavages of 1 ml EGCG (5 mg/ml, 10 mg/ml and 20 mg/ml; diluted in 0.5% sodium carboxymethyl cellulose) (Sigma, U.S.A.) per 100 g body weight once a day for 8 weeks.

At the end of the experimental period, all rats were sacrificed under anesthesia, and pancreases were immediately excised. Some pancreas samples were processed for morphological examination, the remainder was immediately frozen and stored at −80 °C for molecular analysis.

Hematoxylin–Eosin (H&E) Staining Pancreas samples were embedded in 10% formalin, routinely dehydrated and paraffin-embedded. Serial sections (5 μm thick) were stained with H&E for routine histopathologic examination.

Pancreatic Collagen Staining To evaluate the pancreatic fibrosis, pancreas sections were analyzed by Sirius red staining. Slides were de-paraffined and immersed for 25 min in saturated aqueous picric acid containing 0.5% Sirius red. All the Sirius red-stained sections were observed and photographed under a polarization microscope. Under polarization microscope, collagen appears bright orange-red and/or bright green. The images were digitized using Image-Pro Plus (IPP) software (Media Cybernetics, U.S.A.). Areas of pixels for pancreatic collagen were determined. Pancreas collagen deposition was presented by a fibrosis index (%), which indicates the ratio of the mean Sirius red stained area to the mean whole area of the section.

Immunohistochemical Analysis Pancreas sections were deparaffinized, exposed to 3% H2O2 (v/v) to quench endogenous peroxidase activity. After heat-induced epitope retrieval (microwave treatment for 10—15 min in 10 mM sodium citrate buffer [pH 6.0]), the slides were rinsed in phosphate-buffered saline (pH 7.4). Then the sections were exposed to 10% normal goat serum for 15 min at room temperature to block nonspecific reactions, subsequently incubated overnight at 4 °C with the primary antibodies (anti-α-SMA, anti-TGF-β1, anti-Smad3, anti-Smad7; all 1 : 150 dilution) (Santa Cruz, U.S.A.). Bound antibody was detected by using the avidin-biotin immunolabeling procedures and diaminobenzidine as a visualizing agent. Nuclei were counterstained with Harris-Hematoxylin. Negative control preparations involved omitting the primary antibodies and replacing them with antibody diluent. Positively stained optical density were quantified by densitometric analysis (IPP). The immunostaining results were expressed as mean optical density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from frozen pancreatic tissues with Trizol reagent (Invitrogen, U.S.A.), and was reverse transcribed into cDNA using an Oligo (dT)12—18 primer (Invitrogen, U.S.A.), M-MLV reverse transcriptase (Promega, U.S.A.).

PCR was performed with reaction mixtures containing 2.5 mM dNTP, 10 mM sense and antisense primers, and 5 units/ml TaqDNA polymerase (Takara, Japan). The primers and protocols used for RT-PCR are listed in Table 1.

The RT-PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and quantified by densitometric analysis (IPP). It was calculated according to the following formula: (α-SMA or TGF-β1 or Smad3 or Smad7 in sample)/(β-actin in sample).

Statistical Analysis Data were expressed as the mean± S.D., the significance of these differences among five groups was analyzed by means of One-Way ANOVA using SPSS for windows. p-values less than 0.05 were considered statistically significant.

RESULTS

Results of H&E Staining The effects of EGCG on DDC-induced pancreatic fibrosis were evaluated by histopathologic examination of the pancreas sections by H&E staining. Sections from rats of control group displayed normal structure and no pathologic changes were detected (Fig. 2A). In model group, DDC succeeded in inducing pancreatic fibrosis (Fig. 2B). H&E stained sections of the pancreas showed inflammatory cells and lymphocytes infiltration, interstitial edema, and acinar cells atrophy. Microvesicles were observed in acinar and islet cells and in some local areas the acinar vacuolization was extensive. In addition, interlobular space was enlarged and the structure of some lobules was destroyed in pancreatic specimens. Sparse interlobular and intralobular fibrosis was observed. Administration of EGCG resulted in conspicuous attenuation of these pathologic changes. Results showed the vacuolization in pancreas...
tissues of low-dose EGCG group was decreased compared to that of model group (Fig. 2C). More interestingly, the histopathology of pancreas in high-dose EGCG group was close to that of control group (Fig. 2D).

Effects of EGCG on Pancreatic Collagen Deposition
Pancreatic collagen accumulation was determined by Sirius red staining. With polarized microscopy, collagens were red-orange and/or green with the black background, whereas the cells appear black because of a lack of birefringence. In rats of control group, no or only slight collagen was present (Fig. 3A). In contrast, the pancreas of model rats exhibited abundant collagen accumulation, and displayed bundles of collagen fibers surrounding the lobules (Fig. 3B). EGCG noticeably suppressed collagen deposition (Figs. 3C, D). Moreover, pancreatic fibrosis was semiquantitatively analyzed as described above, Fig. 3E showed the fibrosis index of pancreas in five groups. Control rats showed much lower fibrosis index compared to that of model rats ($p<0.001$). EGCG treated groups showed higher fibrosis index than that of control group, but much lower than that of model rats ($p<0.001$). Besides, the index in different dose EGCG groups had also significant difference between every two groups ($p<0.001$).

Effects of EGCG on PSCs Activation
Activated PSCs were characterized by the expression of $\alpha$-SMA, thus $\alpha$-SMA was determined as a symbol to evaluate PSCs activation in pancreas. By immunohistochemistry, $\alpha$-SMA-positive cells were found very rare in rats of control group (Fig. 4A), whereas they were observed easily in model group (Fig. 4B). $\alpha$-SMA-positive cells were localized in the periacinar fibrotic areas and vascular walls in rats of model group. However, EGCG treatment was associated with down-regulation of $\alpha$-SMA expression in the pancreas (Figs. 4C, D). $\alpha$-SMA-positive cells were observed only in the vascular walls in the high-dose EGCG group. The results analyzed by IPP indicated that the mean optical density values of $\alpha$-SMA in three different doses of EGCG groups decreased significantly in comparison with that of model group ($p<0.01$; Table 2). Besides, there was also significant difference between low-dose EGCG group and high-dose EGCG group ($p<0.01$; Table 2).
served the changes of expression of TGF-

Likewise, using immunohistochemistry and RT-PCR, we ob-
tained in control group, the expression of Smad7 mRNA in

Table 2. Effects of EGCG on Expressions of α-SMA, TGF-β1, Smad3 and Smad7 in Pancreas of Five Groups

<table>
<thead>
<tr>
<th></th>
<th>α-SMA</th>
<th>TGF-β1</th>
<th>Smad3</th>
<th>Smad7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model group</td>
<td>0.35±0.08</td>
<td>0.36±0.08</td>
<td>0.31±0.05</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>Low-dose EGCG group</td>
<td>0.25±0.01**</td>
<td>0.23±0.04**</td>
<td>0.21±0.02**</td>
<td>0.16±0.01*</td>
</tr>
<tr>
<td>Medium-dose EGCG group</td>
<td>0.22±0.01***</td>
<td>0.18±0.01***</td>
<td>0.16±0.02****</td>
<td>0.17±0.01**</td>
</tr>
<tr>
<td>High-dose EGCG group</td>
<td>0.19±0.01****</td>
<td>0.14±0.02****</td>
<td>0.15±0.01****</td>
<td>0.19±0.01****</td>
</tr>
<tr>
<td>Control group</td>
<td>0.14±0.02**</td>
<td>0.10±0.02**</td>
<td>0.12±0.01**</td>
<td>0.26±0.03**</td>
</tr>
</tbody>
</table>

Values are mean±S.D. (n=7). Statistical significance: ∗p<0.05, ∗∗p<0.001 vs. model group; ∗∗p<0.01, ∗∗∗p<0.001 vs. low-dose EGCG group; ∗∗∗∗p<0.05 vs. medium-dose EGCG group.

α-SMA mRNA levels were also detected by RT-PCR (Fig. 5). Similarly to the results of immunohistochemistry staining, α-SMA mRNA expression increased significantly in rats of model group compared with that of control group (p<0.001). EGCG suppressed the overexpression of α-SMA mRNA (Fig. 5), and there was also significant difference between every two groups of three EGCG groups (p<0.001 vs. low-dose EGCG group; p<0.05 vs. medium-dose EGCG group).

Effects of EGCG on Expressions of TGF-β1/Smads

Likewise, using immunohistochemistry and RT-PCR, we observed the changes of expression of TGF-β1, Smad3 and Smad7 in pancreas to evaluate the effects of EGCG on TGF-β1/Smads signaling pathway. TGF-β1-positive and Smad3-positive cells were observed easily in rats of model group (p<0.001 vs. low-dose EGCG group; p<0.05 vs. medium-dose EGCG group).

In present study, we observed the effect of different doses of EGCG on rat pancreatic fibrosis induced by DDC in vivo.

The results of H&E staining indicated that administration of EGCG could effectively ameliorate pancreatic pathologic changes induced by DDC. Meanwhile, we assessed the degree of fibrosis by means of Sirius red staining. The fibrosis index of different doses of EGCG groups decreased about 28—66% compared with that of model group. These results

DISCUSSION

In present study, we observed the effect of different doses of EGCG on rat pancreatic fibrosis induced by DDC in vivo. The results of H&E staining indicated that administration of EGCG could effectively ameliorate pancreatic pathologic changes induced by DDC. Meanwhile, we assessed the degree of fibrosis by means of Sirius red staining. The fibrosis index of different doses of EGCG groups decreased about 28—66% compared with that of model group. These results
showed that EGCG could attenuate experimental pancreatic fibrosis.

Activated PSCs are important effector cells and the primary source of ECM in pancreatic fibrosis. In our study, we determined the expression of α-SMA (the symbol of activated PSCs) in experimental animals administered with and without EGCG by immunohistochemistry and semiquantitative RT-PCR. Our present data showed that DDC increased the α-SMA-positive cells and the expression of α-SMA mRNA in pancreas and that these increases were suppressed by different doses of EGCG. At the same time, EGCG significantly suppressed the increased collagen deposition in rats. These findings indicate that EGCG attenuates rat pancreatic fibrosis due to, at least, suppressing activation of PSCs.

TGF-β1 is the key mediator in the development of pancreatic fibrosis and can promote the PSCs activation and ECM secretion by paracrine and autocrine. Using the immunohistochemistry and semiquantitative RT-PCR methods, we examined the expression of TGF-β1 protein and mRNA in rat pancreas. Expressions of TGF-β1 both at protein and mRNA level were higher in rats of model group. However, after 8 weeks of EGCG administration, the up-regulated levels of expression of them in EGCG groups were significantly decreased. We presume it could be relevant with inhibition the phosphorylation of R-Smads thereby preventing the activation of PSCs. In the rats of pancreatic fibrosis, however, expression of Smad7 mRNA and protein were down-regulated. We presume it could be relevant with that activated PSCs lose the sensitivity to the up-regulated expression of Smad7 induced by TGF-β1. Interestingly, we found that administration of EGCG suppressed the expression of Smad3 and meanwhile enhanced the expression of Smad7 both at mRNA and protein level in a dose-dependent manner. Taken together, these results signify that EGCG has a potent influence on expression of Smads. It can regulate the

Upon TGF-β1 binding to TGF-β type II receptor, the type II receptor kinase phosphorylates the GS domain of TGF-β type I receptor, leading to activation of type I receptor. The activated type I receptor triggers the downstream Smad signal transduction pathway. Smads can be categorized into three different subgroups: the receptor-regulated Smad2 and Smad3 (R-Smads); the common-mediator Smad4 (co-Smads), and the antagonistic or inhibitory Smad6 and Smad7 (I-Smads). The activated type I receptor kinase phosphorylates Smad2 and Smad3 at two serine residues in the SSXS motif at their extreme C termini. Phosphorylated Smad2 and Smad3 form oligomeric complexes with Smad4 (Co-Smad); the complexes then translocate into the nucleus. These complexes subsequently mediate the transcriptional regulation of target genes. Thus, Smads transmit signals directly from the receptor kinase into the nucleus. On the other hand, Smad7 antagonizes the signaling transduction by inhibiting the phosphorylation of R-Smads thereby preventing their association with Co-Smad, and participating in a negative feedback loop to control TGF-β1 responses.

Therefore, in order to further elucidate the mechanism of protective effects of EGCG on pancreatic fibrosis, we also observed effects of EGCG on downstream transcription factors of TGF-β1, Smad3 and Smad7.

This study showed that expressions of Smad3 mRNA and protein were up-regulated in model rats. Usui T. et al. deemed that overexpression of Smad2, Smad3, and/or Smad4 could accelerate the ECM production stimulated by TGF-β. Our results are consistent with that report and we speculate that Smad3 participates in a positive feedback loop to facilitate TGF-β1 signal transduction. TGF-β1 can rapidly induce expression of Smad7 mRNA, demonstrating its participation in a negative feedback loop to control TGF-β1 responses and prevention the activation of PSCs. In the rats of pancreatic fibrosis, however, expression of Smad7 mRNA and protein were down-regulated. We presume it could be relevant with that activated PSCs lose the sensitivity to the up-regulated expression of Smad7 induced by TGF-β1. Interestingly, we found that administration of EGCG suppressed the expression of Smad3 and meanwhile enhanced the expression of Smad7 both at mRNA and protein level in a dose-dependent manner. Taken together, these results signify that EGCG has a potent influence on expression of Smads. It can regulate the
balance between Smad3 and Smad7, thereby impede signal transduction of TGF-β1, and further suppress activation of PSCs induced by TGF-β1.

In conclusion, our results demonstrate that EGCG attenuated rat pancreatic fibrosis induced by DDC. The mechanism may be that EGCG not only inhibits activation of PSCs but also suppresses expression of TGF-β1 directly. Besides, EGCG also influences downstream transcription factors of TGF-β1: it could down-regulate expression of Smad3 and up-regulate expression of Smad7. Therefore, it impedes TGF-β1 signals to transmit into the nucleus. Our results provide the novel insights into the mechanisms of EGCG as anti-fibrogenic candidate in the pancreatic fibrosis and long-term studies in animal models and cells are the next step to establish its safety and clinical potentials.

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REFERENCES