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EXPRESSION OF TRANSFORMING GROWTH FACTOR-β1 AND EPIDERMAL GROWTH FACTOR IN CAERULEIN-INDUCED PANCREATITIS IN RAT

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Growth factors such as TGF-β1 and EGF are known to modulate the deposition of extracellular matrix components and tissue repair and to affect the cellular growth but their expression in the course of pancreatitis has not been studied. In this study we investigated the gene expression of TGF-β1 mRNA and EGF mRNA and other parameters of the pancreas including DNA synthesis, blood flow (PBF), tissue protein content and plasma amylase during the induction of acute pancreatitis. Supramaximal dose of cearulein (10 mg/kg/h s.c.) was infused for 5 h to induce pancreatitis. Animals were killed after 1, 2, 3, 4 and 5 h of infusion. The PBF was measured, blood samples were withdrawn to determine serum amylase concentration, biopsy samples were taken to measure the protein content and DNA synthesis. Expression of TGF-β1 and EGF mRNA was studied by reverse-transcription of polymerase chain reaction (RT-PCR). Caerulein infused caused a time-dependent decrease in DNA synthesis accompanied by gradual decrease of PBF and significant increase in pancreatic weight. The pancreatic protein content and plasma amylase showed progressive rise during 5 h of cearulein infusion. Histology revealed tissue edema, cellular vacuolization and prominent leukocyte infiltration after 3 h of cearulein infusion. TGF-β1 mRNA was strongly expressed at each time interval beginning from the 1 h after the start of cearulein infusion. In contrast, EGF mRNA was detected only at 5 h after induction of pancreatitis. We conclude that 1) the development of caerulein-induced pancreatitis results in the inhibition of pancreatic growth and the reduction in PBF accompanied by enhanced expression of TGF-β1; 2) The expression of EGF that was observed at the end of the induction of pancreatitis may indicate the initiation of pancreatic repair; 3) TGF-β1 seems to lead to subsequent induction of EGF that may stimulate the regeneration of injured pancreas.

Key words: *caerulein; pancreatitis; TGF-β1; EHZ; ΔΞΑ; παξγσεαυιγ βμοοδ ζμοψ*

INTRODUCTION

Acute pancreatitis is a severe disease with significant mortality of which pathophysiology remains obscure and for which treatment is still only supportive. The most useful model for the study of acute pancreatitis includes

the *in vivo* administration of caerulein, a CCK analogue, to rats in doses that are in excess of those required for maximal secretion (1, 2). Although the mechanism of caerulein-induced pancreatitis is not fully explained, recent studies suggest an important role of intraacinar activation of proteases through low affinity binding sites for cholecystokinin, leading to impaired sorting of the zymogen fraction and lysosomal fraction (3). High doses of caerulein induce acute interstitial edematous pancreatitis with depletion of digestive enzymes, decrease of pancreatic blood flow and total DNA and histological evidence of pancreatic damage (4, 5).

The sequence of events leading to the development of the pancreatitis is not clear at present. Several inflammatory mediators have been proposed to play a role in acute pancreatitis, including PAF (6), prostaglandins (7), cytokines (8—10), and free radicals (11, 12). There is also an evidence that polypeptide growth factors, particularly TGF-β, may be involved in this process (13). Although the polypeptide growth factors such as transforming growth factor β1 (TGF-β1) and epidermal growth factor (EGF) regulate various cellular processes, little is known about their involvement in consecutive phases of the evolution of acute experimental pancreatitis.

Transforming growth factor-βS (TGFβS) form a family of structurally related peptides that include at least 3 distinct isoforms, termed TGF-β1, TGF-β2 and TGF-β3. They are synthesized as precursors that undergo proteolytic cleavage to yield biologically active dimers. Mature TGF-β1 and TGF-β2 share 70% aminoacid sequence homology, also TGF-β3 shares 70% homology with two other isoforms (14). TGF-β1, which is predominant form of TGF-β family, is considered as the multifunctional cytokine implicated in the regulation of many cellular processes including cell growth and differentiation on and composition of extracellular matrix (ECM) (15). TGF-β1 plays also an important role in the tissue repair by promoting angiogenesis and stimulating the synthesis of different matrix components such as fibronectin, tenascin, collagens and proteoglycans (16—19). It simultaneously inhibits matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors. All these events may affect locally the tissue injury leading to excessive scarring and fibrosis.

Increased expression of TGF-β1 was reported in liver cirrhosis, glomerulonephritis and idiopathic pulmonary fibrosis indicating that this growth factor may be an important mediator of these disorders (20—22). However, little is known about the role of TGF-β1 in the development of acute pancreatitis.

EGF is an extensively studied growth factor which was originally isolated from the mouse submandibular salivary glands (23). Substantial amounts of EGF have been demonstrated in different tissues and several biological fluids including saliva, milk and gastric, duodenal and pancreatic juice (24). The

receptors for EGF (EGFR) were found to be expressed in the pancreas suggesting the possible physiological role of EGF in the regulation of pancreatic functions (25). Previous reports showed that EGF stimulates pancreatic acinar enzyme secretion and exerts a trophic influence on the pancreas (25—28). Up to now, the role of EGF in the development of acute pancreatitis has not been examined.

The aim of this work was to assess the gene expression of EGF and TGF-β1 in the course of caerulein-induced acute pancreatitis and to investigate the biochemicals parameters and histological changes in the rat pancreas during the evolution of this pancreatitis.

MATERIALS AND METHODS

Induction of pancreatitis

All studies were performed on male Wistar rats, weighing 150—200g. Animals were housed in cages with wire mesh bottoms at room temperature with a 12 h light dark cycle. Drinking water and food were available *ad libitum*. 18 h before the beginning of the experiments rats were allowed to water but not food.

Pancreatitis was induced by caerulein that was diluted in saline and infused s.c. for 5 h at a dose 10 mg/kg/h. The animals were killed at 1, 2, 3, 4 or 5 h after the beginning of the caerulein-infusion.

Measurement of pancreatic blood flow (PBF)

At the time sequence described before, the rats were anesthetized with ether, weighed and the abdominal cavity was opened. The pancreas was exposed for measurement of the blood flow by hydrogen (H_2) gas clearance used for several years in this laboratory. Blood flow was measured in five different portions of the pancreas and the mean value of these measurements of pancreatic blood flow (PBF) was expressed in ml/100g tissue/min. The results were presented as percent of control value obtained in rats infused with vehicle saline.

Determination of plasma concentration of amylase

Immediately after measurement of pancreatic blood flow, the abdominal aorta was exposed and blood was taken for plasma amylase determination. Plasma amylase was determined by an enzymatic method (Amylase reagent, Dialab Diagnostic GmbH, Wien, Austria). The values were expressed as unit/liter (U/l).

Histological examination

The specimens were excised from the body portion of the pancreas, fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxylin and eosin. The slides were examined histologically by expert without the knowledge of the treatment given. The histologic grading of edema was made using scale ranging from 0 to 3, where 0 = no edema, 1 = interlobular edema, 2 = interlobular edema and moderate intralobular edema and 3 = interlobular edema and

severe intralobular edema. Leukocyte infiltration was also graded from 0 (absent) to 3 (diffuse maximal infiltration in the entire pancreatic gland). Grading of vacuolization was based on the appropriate percentage of cells involved; 0 = absent, 1 = less than 25%, 2 = 25—50%, and 3 = more than 50%.

Determination of DNA synthesis and DNA and RNA and protein contents in pancreatic tissue

After taking the blood, the pancreas was carefully dissected out from its attachment to the stomach, the duodenum and the spleen. Fat and excess of tissue were trimmed away. The pancreas was rinsed with saline, blotted on paper, weighed and minced. The rate of DNA synthesis in the pancreatic tissue was determined by incubating the tissue at 37°C for 45 min in 2 ml medium containing 8 μCi/ml of tritiated thymidine (^{3}H-thymidine, 20—30 Ci/mmol, Research Institute, Production and Application of Radioisotopes, Prague, Bohemia). The reaction was stopped with 0.4 M perchloric acid containing carrier thymidine (5 mM). Tissue samples were centrifuged, precipitated and washed twice in cold 0.2 M perchloric acid and then recentrifuged. RNA was ohydrolyzed in 0.3 M KOH incubater for 90 min. at 37°C. DNA and protein were reprecipitated with 10% perchloric acid. After standing for 10 min on ice, the tubes were centrifuged and supernatant containing RNA was discarded. DNA in residual pellet was solubilized in 10% perchloric acid by heating at 70°C for 20 min. Denatured protein was removed by centrifugation for 20 min. Using calf thymus as a standard, the DNA content of the samples was determined by Giles and Myers procedure (31). The final pellet was solubilized in 1 M NaOH and its protein content was determined by the method of Lowry (32). The incorporation of ^{3}H-thymidine into DNA was determined by counting 0.5 ml DNA containing supernatant in a liquid scintillation system. DNA synthesis was expressed as disintegrations per min (dpm) ^{3}H-thymidine per microgram DNA.

Application of RT-PCR for detection of TGF-β1 and EGF gene expression

After killing of the rats, the pancreas samples were removed, immediately snap frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from pancreas using a rapid guanidinium isothiocyanate/phenol chloroform single step extraction kit from Stratagene^R. Following precipitation, the RNA was resuspended in RNase-free TE buffer and the concentration was estimated by absorbance at 260 nm wavelength. Samples were stored frozen at −80°C until analysis. First-strand cDNA was synthesized from total cellular RNA (5 μg) using 200 U StrataScript™ reverse transcriptase (Stratagene, La Jolla, USA) and oligo (dT) primers (Stratagene, La Jolla, USA). Following the reaction, the transcriptase activity was destroyed by heating and the products were then stored at −20 °C until PCR.

Primers were synthesized by Biometra^R (Göttingen, Germany). The primers pairs for TGF-β1 and EGF were synthesized based on the published cDNA sequences; EGF sense primer:

5'-GACAACTCCCCTAAGGCTTA-3',

the EGF antisense primer;

5'-CATGCACACGCCACCATTGAGGCAGTACCCATCGTACGA-3',

defining a 566 bp product and TGP sense primer;

5'-CTTCAGCTCCACAGAGAAGAACTGC-3',

TGF antisense primer;

5'-CACGATCATGTTG GACAACTGCTCC-3',

defining a 298 bp product (32, 33). Concomitantly, amplification of GAPDH was performed on the same samples to assess RNA integrity. Reaction mixtures for PCR contained cDNA templates, 50 pmol of each primer, and 2.5 U of Taq DNA polymerase (Promega) in 10 mM Tris-HCl (ph 8.8), 50 mM KCL, 1.5MM $mgCl_2$, 0.5 mM dNTPs in a volume of 50 µl. 2RT blanks (no RNA included) and PCR blanks (no cDNA products included) were included in each analysis. To maximize amplification specificity, hot start PCR was performed in a Perkin Elmer Cetus DNA thermal Cycler for 33 cycles (94 °C for 1 min, 60 °C for 45 sec, 72 °C for 2 min) using AmpliWax^R PCR Gem 50 beads.

Briefly, after adding primers, buffer and dNTPs, a AmpliWax PCR Gem was oadded and heated to 80° C for 10 min. Then, the DNA Tag Polymerase, cDNA sample and buffer was pipetted into the mixture. Semiquantitative evaluation of amplified PCR products on 1.5% agarose gels, staining with ethidium bromide, and visualization under UV light was performed. In addition to size analysis by agarose gel electrophoresis, specificity of the primer pair for preproEGF was assessed by sequencing of PCR products.

Statistical analysis

Results are expressed as means ±SEM. Statistical comparisons were made by analysis of variance and, where appropriate, by unpaired Student t-test with p value <0.05 considered significant. For statistical analysis the nonparametric Mann-Whitney U and Kruskal-Wallis tests for unpaired comparisons were also applied where appropriate.

RESULTS

Morphologic features (histology findings)

Infusion of caerulein at a dose of 10 µg/kg/h s.c. for 5 h, consistently produced pancreatitis in all tested rats. The pancreas appeared grossly swollen and enlarged with visible collections of edematous fluid. Peritoneal fluid was present in all animals. Edema vacuolization and infiltration increased progressively reaching their highest score at the end of 5 h infusion of caerulein (for edema 2.33 ± 0.23 vs 0.25 ± 0.16 in the control group; $p<0.05$, for vacuolisation 2.22 ± 0.36 vs 0 in the control group; $p<0.05$ and for infiltration 1.77 ± 0.27 vs 0.12 ± 0.12 in the control group; $p<0.05$) *(Table 1)*.

Pancreatic blood flow (PBF)

As shown of *Fig 1.* caerulein induced a progressive decrease of pancreatic blood flow beginning in the first of infusion ($\sim 71\% \pm 5{,}9\%$ of control value, $p<0.05$) and reaching its maximum after 5 h of caerulein infusion ($\sim 46{,}1\% \pm 3{,}9\%$ of control value, $p<0.05$).

Table 1. Histological changes such as edema, leukocyte infiltration and acinar cell vacuolization in the course of caerulein induced pancreatitis after 1, 2, 3, 4 and 5 h of caerulein infusion. Mean ± 8 tests on 8 rats on 8 rats. Asterisk indicates significant ($p < 0.05$) increase above the value obtained with saline (vehicle) infusion

	HISTOLOGY		
	EDEMA (0—3)	INFILTRAFION (0—3)	VACUOLIZATION (0—3)
CONTROL (saline infusion)	0.25±0.1	0.12±0.04	0
CAERULEIN (infusion)			
After 1 h	0.70±0.26	0.40±0.16	0.05±0.22
After 2 h	1.50±0.18*	0.75±0.36	0.87±0.29*
After 3 h	1.90±0.26*	1.00±0.21*	1.00±0.21*
After 4 h	2.55±0.24*	1.55±0.29*	1.89±0.26*
After 5 h	2.33±0.25*	1.77±0.27*	2.22±0.38*

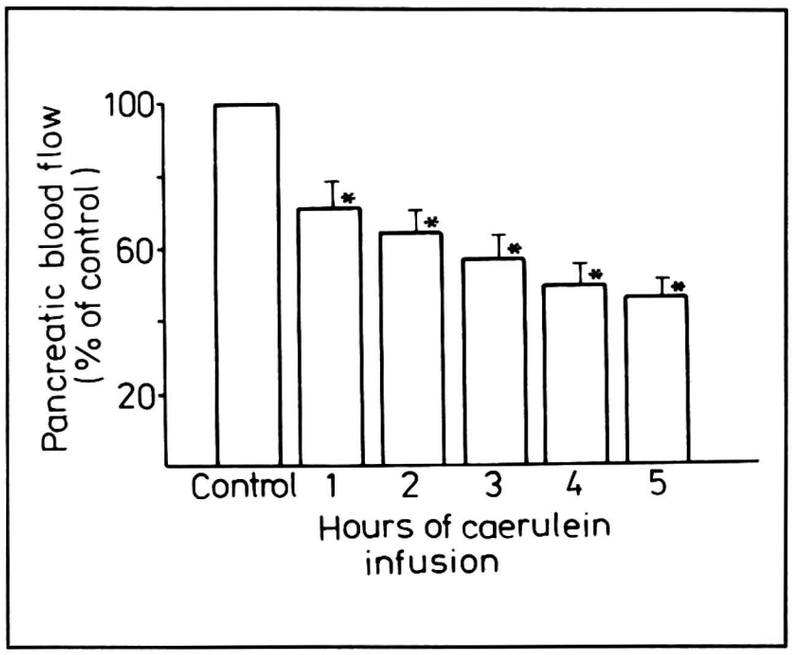

Fig. 1. Pancreatic blood flow in intact rats and in caerulein-induced pancreatitis after each hour of caerulein infusion. Means±SEM of 8—10 tests on 8—10 rats. Asterisk indicates significant decrease as compared to the control values in the intact rats infused with vehicle (saline).

Biochemical parameters

Infusion of caerulein for 5 h resulted in progressive increase of pancreatic protein content *(Fig 2.)*, total pancreatic weight *(Fig. 3)* and plasma amylase level *(Fig. 4)* with the peak achieved for all these parameters after 5 h. All these parameters were significantly raised ($p<0.05$) as compared to the values obtained in the control group infused with saline (protein content: 281.9±12.6 mg vs 211.8±9.4 mg in control group; pancreatic weight 1709±105 mg vs 986±37 mg in control group; plasma amylase: 8330±621 vs 1207±63). Parallel to the above changes, a time-dependent decrease in DNA synthesis was observed, reaching its peak after 5 h of caerulein infusion (22.5±2 DPM/μg DNA vs. 49.3±1.9 DPM/μg DNA in control group, $p<0.05$) *(Fig 5)*.

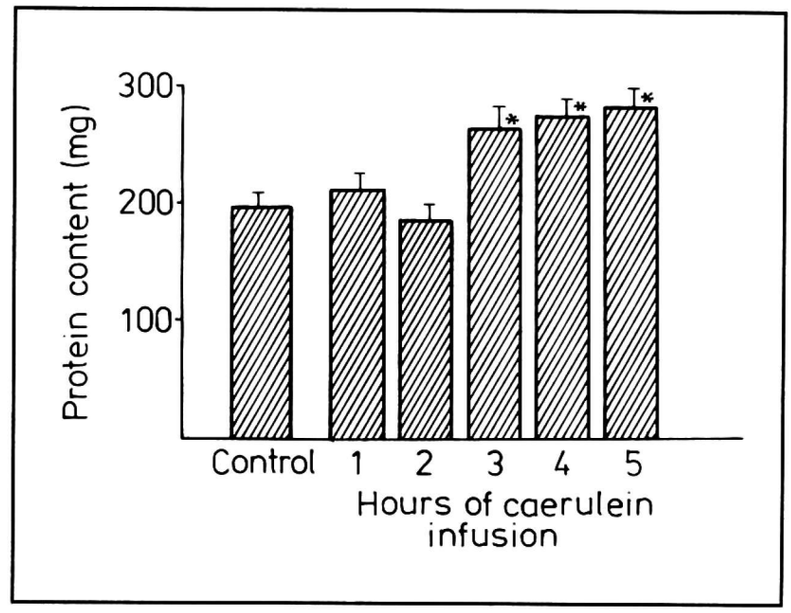

Fig. 2. Pancreatic protein contents in intact rats and in caerulein-induced pancreatitis after each hour of caerulein infusion. Means ± SEM of 8—10 tests on 8—10 rats. Asterisk indicates significant increase as compared to the control values in the intact rats infused with vehicle (saline).

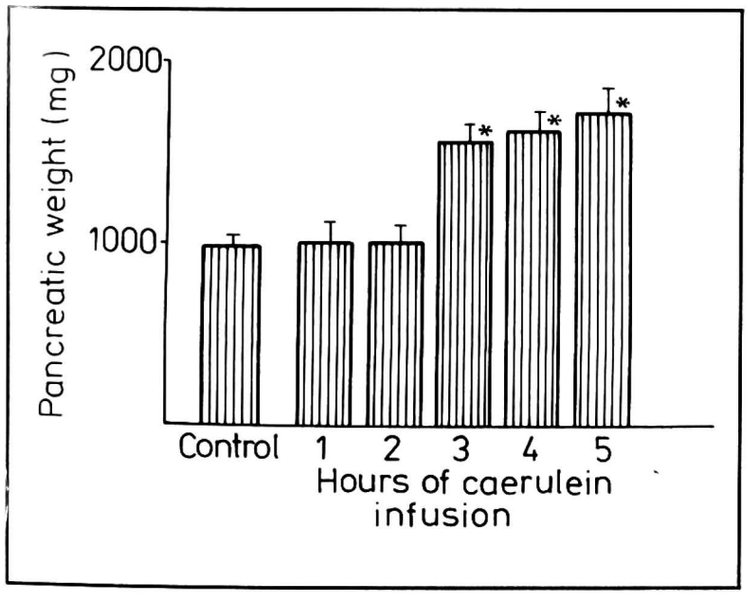

Fig. 3. Weight of the pancreas in intact rats and in caerulein-induced pancreatitis after each hour of caerulein infusion. Means ± SEM of 8—10 tests on 8—10 rats. Asterisk indicates significant increase as compared to the control values in the intact rats infused with vehicle (saline).

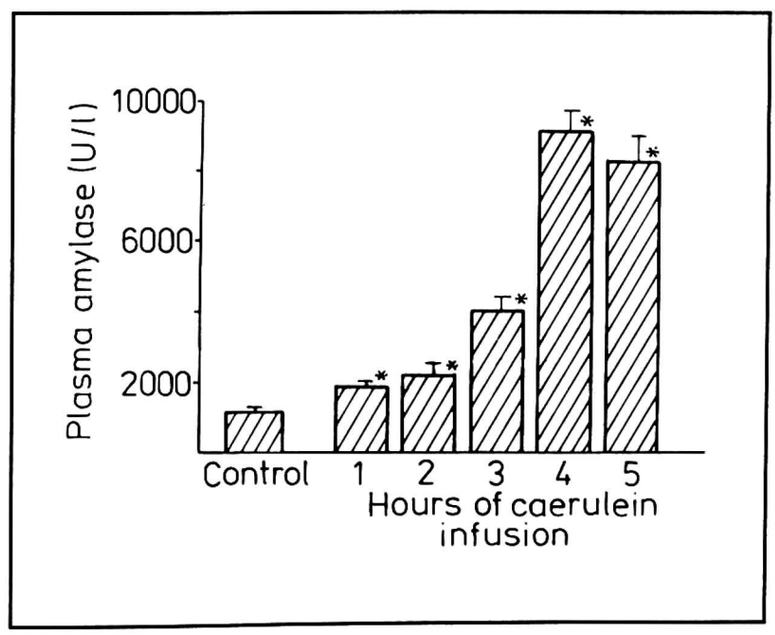

Fig. 4. Plasma amylase concentration in intact rats and in caerulein-induced pancreatitis after each hour of caerulein infusion. Means ± SEM of 8—10 tests on 8—10 rats. Asterisk indicates significant increase as compared to the control values in the intact rats infused with vehicle (saline).

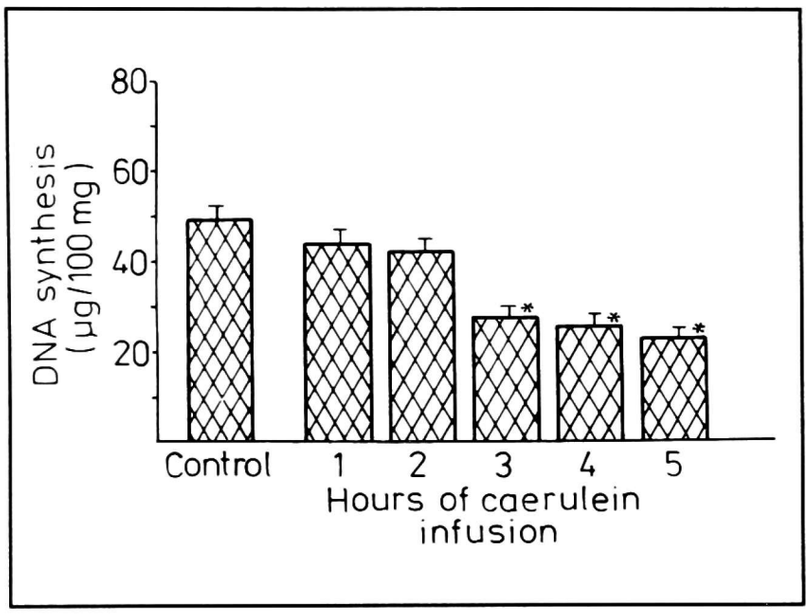

Fig. 5. DNA synthesis in intact rats and in caerulein-induced pancreatitis after each hour of caerulein infusion. Means ± SEM of 8—10 tests on 8—10 rats. Asterisk indicates significant decrease as compared to the control values in the intact rats infused with vehicle (saline).

RT-PCR detection of TGF-β1 and EGF transcripts

TGF-β1 mRNA (298 bp) was detected already after 1, 2, 3, 4 and 5 h of caerulein infusion. In the control group (infused with saline), TGF-β1 mRNA was not detected *(Fig. 6)*. EGF mRNA (566 bp) was not detected in control group and within first 4 h of caerulein infusion. Only after 5 h of caerulein infusion, a strong signal for EGF mRNA was detected *(Fig. 7)*. In all samples a strong signal for GAPDH mRNA (983 bp) was detected indicating the integrity of analyzed RNA *(Fig. 8)*.

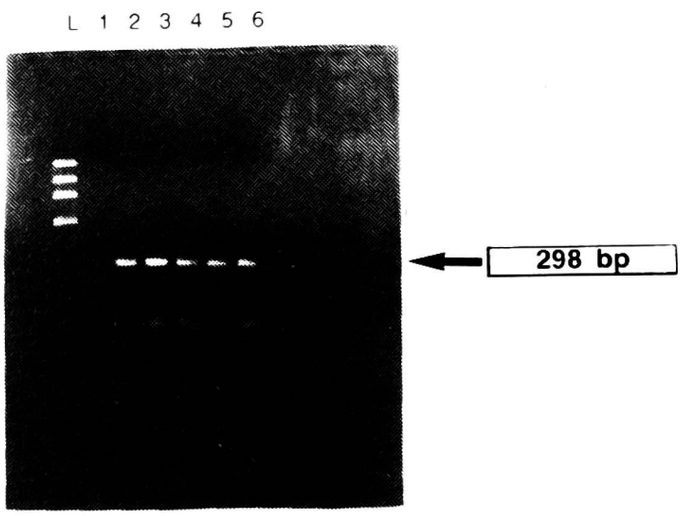

Fig. 6. Typical record of RT-PCR analysis of TGF-β1 mRNA in the pancreatic tissue of intact control rat infused with saline (*lane 1*) and in the pancreatic tissue after 1, 2, 3, 4 and 5 h of caerulein infusion to develop pancreatitis (*lane 2—6*). The size of the predicted amplified product TGF-β1 (298 bp) is indicated on the right. Similar data were recorded in other vehicle- and cearulein-infused rats.

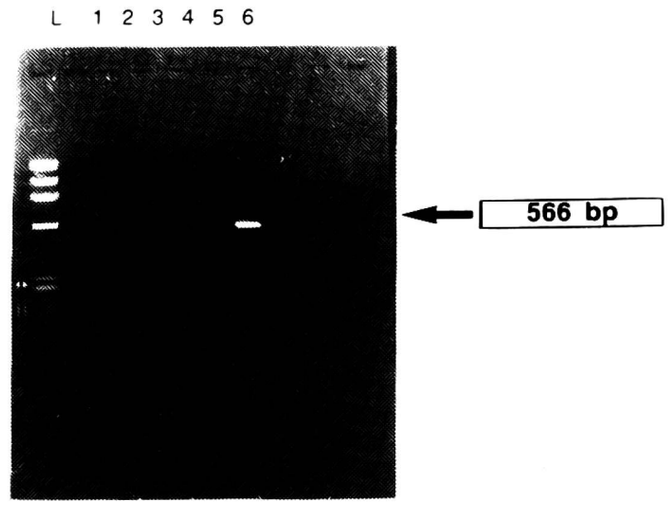

Fig. 7. Typical record of RT-PCR analysis of EGF mRNA in the pancreatic tissue of intact control rat infused with saline (*lane 1*) and in the pancreatic tissue after 1, 2, 3, 4 and 5 h of caerulein infusion to develop pancreatitis (*lane 2-6*). The size of the predicted amplified product TGF-β1 (566 bp) is indicated on the right. Similar results were obtained in vehicle- or caerulein-treated rats.

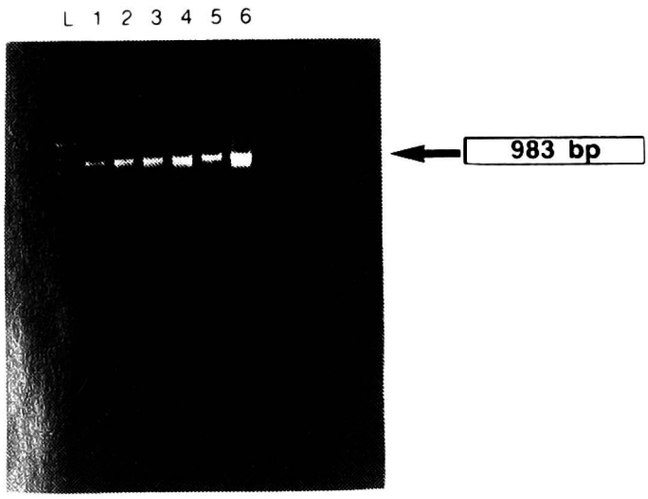

Fig. 8. Typical record of RT-PCR analysis of GAPDH mRNA in the pancreatic tissue of intact control rat infused with saline (*lane 1*) and in the pancreatic tissue after 1, 2, 3, 4 and 5 h of caerulein infusion to develop pancreatitis (*lane 2-6*). The size of the predicted amplified product TGF-β1 (983 bp) is indicated on the right. Similar results were obtained from other vehicle- or caerulein-treated rats.

DISCUSSION

The purpose of our study was to evaluate the gene expression of TGF-β1 and EGF as well as the changes of biochemical and morphological parameters during the early phase of evolution of acute caerulein-induced pancreatitis. Our results show that acute pancreatitis provoked by infusion of caerulein (10 μg/kg/h) results in immediate induction of gene expression for both TGF-β1 and this is followed 5 h later by the expression of EGF.

The demonstration of expression of TGF-β1 mRNA with subsequent appearance of EGF mRNA suggests that both growth factors are implicated in the induction of acute pancreatitis although at different time period of this induction.

The precise mechanism responsible for the gene activation of TGF-β1 and the role of this cytokine in the development of acute pancreatitis have not been fully elucidated. Recent reports indicate that TGF-β1 may play an important role at the site of tissue injury by chemoattracting inflammatory cells, stimulating the production and release of different inflammatory mediators, and promoting the deposition of extracellular matrix (13, 18, 34—36). The first two properties may increase inflammatory response and have an aggravating effect on the course of acute pancreatitis, the later one seems to be important in subsequent events of acute pancreatitis such as remodeling and regeneration of pancreatic tissue (13, 36). Van Laethem *et al.* (36) have recently reported that the course of acute caerulein-induced pancreatitis in mice was not influenced by exogenous recombinant TGF-β1, administered 2 h before the first injection of caerulein. One possible reason for these negative results was that the systemically administered TGF-β1 could not induce significant changes in pancreas because of its short half life in circulation and failure to reach the pancreas in sufficient concentration to affect this organ. On the other hand,

Logsdon *et al.* (37) showed that TGF-β1 inhibits the growth of pancreatic acinar cells. Additionally, the report of Lee *et al.* (37) provided an evidence for the inhibited proliferation of acinar calls in the transgenic mice overexpressing TGF-β1 in the pancreatic β-islet cells. These results as well as our detection of TGF-β1 transcripts in early phase of pancreatitis when acute edema and neutrophil infiltration occur suggest that this cytokine may be an important factor responsible for the increase in vascular permeability and the accumulation of protein in edematous pancreatic tissue as well as the decrease of the DNA synthesis, observed in our study during the development of acute pancreatitis.

The major finding of this study is the demonstration of EGF transcripts during the later phase of the evolution of acute caerulein-induced pancreatitis. In contrast to TGF-β1, EGF mRNA was detected only in later phase of the development of pancreatitis that is after 5 h of caerulein infusion. EGF displays many kinds of biological effects, including stimulation of proliferation and differentiation of epithelial and nonepithelial tissues, stimulation of DNA synthesis, protection of gastroduodenal mucosa against various irritants and healing of gastroduodenal ulcerations (39). The presence of EGF in pancreatic juice (24, 25) and receptors for EGF in pancreatic tissue (25) as well as the stimulation by this peptide of pancreatic growth (26) and protein enzyme secretion (27, 28) indicate that this peptide may play an important role in the trophic and secretory functions of pancreas. So far, no study reported on gene expression for EGF in pancreas during the development of acute pancreatitis. Our detection of strong EGF mRNA signal at the end of caerulein-infusion may indicate the initiation of pancreatic repair by this growth factor. There is no satisfactory explanation for this finding, but we hypothesize that TGF may lead to subsequent upregulation of EGF mRNA expression at the late period of the evolution of caerulein pancreatitis in rat.

Our study describes also histological and biochemical changes occurring in the pancreas in response to caerulein infusion. Infusion of supramaximally stimulating dose of caerulein caused pancreatic edema as well as acinar vacuolisation and inflammatory infiltration in the pancreas. Some morphological changes such as vacuolisation and edema were significant already after 3 h of caerulein-infusion. The vacuoles, which appear in the acinar cells, contain activated lysosomal enzymes, which in turn may activate the digestive enzymes leading to cell destruction. The edema reflects the increased capillary leakage of plasma proteins and collection of interstitial fluid. The increased leukocyte infiltration is induced by inflammatory cytokines including TGF. Parallel

to the histological changes, an increase of pancreas weight, protein content in pancreas and plasma amylase concentration were observed. The initial increase of pancreatic weight during the development of caerulein-induced pancreatitis was probably due to the edema of pancreatic tissue due to the leakage of plasma proteins from blood vessels to pancreatic interstitial tissue. The increased amylase level is closely related to the pancreatic cell damage.

Infusion of caerulein resulted also in a significant fall in pancreatic blood flow, which was significant already after 2 h of caerulein infusion and which further progressed to reach after 5 h, about half of the normal flow. These changes have been previously reported by our as well as other laboratories (5, 6, 38, 39). The hemodynamic changes during acute pancreatitis have been investigated previously and most reports proposed that the reduction in pancreatic blood flow and resulting ischemia play a major role in the pathogenesis and the course of acute pancreatitis (38—40).

In our studies, DNA synthesis an index of cell proliferation, decreased time-dependently after caerulein infusion, reaching its lowest level 5 h after infusion. The mechanism responsible for this decrease is unknown but the fact, that TGF-β1 is a powerful inhibitor of growth of several epithelial cells (41), leads us to speculation, that this peptide may be, at least in part, responsible for the decrease in DNA synthesis in pancreatic acinar cells.

In summary: 1) during the development of caerulein-induced pancreatitis, the inhibition of pancreatic growth and the reduction in pancreatic blood flow accompanied by enhanced expression of TGF-β1 have been observed; 2) the increased TGF-β1 expression in the early course of pancreatitis may be responsible for the observed gradual fall in DNA synthesis; and 3) initial expression of TGF-β1 may lead to subsequent activation of EGF gene and increased formation in the pancreatic tissue to stimulate the regeneration of injured pancreas.

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