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# Transforming growth factor-β-induced upregulation of transforming growth factor-β receptor expression in pancreatic regeneration

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**Abstract**

The transforming growth factor-β (TGFβ) signaling pathway is one important player in the regulation of extracellular matrix turnover and cell proliferation in epithelial regeneration. We used cerulein-induced pancreatitis in rats as a model to investigate the regulation of TGFβ receptor type I and type II expression on protein and messenger RNA level during regeneration. In the regenerating pancreas, mRNA levels of TGFβ receptor I and II were significantly increased with a maximum after 2 days. On protein level, expression of TGFβ receptor II was significantly increased after 3–5 days. This elevated expression could be inhibited by neutralizing the endogenous biological activity of $TGF\beta_1$ with a specific antibody. In cultured pancreatic epithelial cells, $TGF\beta_1$ reduced cell proliferation as measured by [$^3$H]thymidine incorporation. Furthermore the transcript levels of $TGF\beta_1$ as well as mRNA and protein concentrations of type I and type II receptor increased during TGFβ stimulation in vitro. These results indicate that epithelial pancreatic cells contribute to the enhanced $TGF\beta_1$ synthesis during pancreatic regeneration by an autocrine mechanism. $TGF\beta_1$, furthermore, upregulates the expression of its own receptors during the regenerative process, thereby contributing to the increase of the TGFβ-induced cellular responses. 

*Keywords:* Transforming growth factor-β; Transforming growth factor-β receptor; Gene expression; Epithelial regeneration

## 1. Introduction

The members of the transforming growth factor-β family (TGFβ) are important regulatory components in cell differentiation and proliferation as well as in tissue morphogenesis from flies to mammals [1,2]. Among the growing number of members of the TGFβ superfamily, which includes bone morphogenetic proteins (BMP) and activins, $TGF\beta_1$ is considered to represent the prototype factor for characterizing receptor signaling mechanisms and various cellular responses. The three mammalian isoforms $TGF\beta_{1-3}$ are highly conserved and functional inter-

Abbreviations: PBS, calcium and magnesium free phosphate buffered saline; ECM, extracellular matrix; TGFβ, transforming growth factor-β; TβRI, TGFβ receptor type I; TβRII, TGFβ receptor type II; $\alpha TGF\beta_1$, neutralizing antibody against $TGF\beta_1$; MMP, matrix metalloprotease; EGFR, epidermal growth factor receptor

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changeable in most biological assays [3,4]. The growth inhibitory effect of TGFβ in a variety of cell types, in particular in epithelial cells, as well as its ability to induce gene expression leading to increased extracellular matrix deposition, were characterized by many groups [3,5].

TGFβ exerts its activity by interacting with two types of cell surface serine/threonine kinase receptors, namely type I and type II (TβRI and TβRII) [6–8]. Both receptors represent transmembrane glycoproteins of 55 and 75 kDa, respectively. After ligand binding to TβRII, the receptor forms a dimer with TβRI and as a consequence TβRI is phosphorylated in the GS domain. Concomitantly, the activated serine/threonine kinase of the receptor type I phosphorylates downstream acting elements, such as Smad proteins [9,10], which transduce the signal into the nucleus.

Recent studies revealed the functional importance of TGFβ in various regenerative events [11–13]. In different intestinal organs, TGFβ is involved in regulating cell proliferation, differentiation, production and deposition of extracellular matrix after injury or inflammation [14–16]. In contrast to low constitutive expression of $TGF\beta_{1-3}$ in a healthy pancreas, these growth factors are overexpressed in acute and chronic pancreatitis. Furthermore, this overexpression correlates with increased deposition of collagen and other ECM components [15,17,18]. Recently we have shown that the inhibition of the biological activity of $TGF\beta_1$ caused a reduction of the collagen content by 30% in the pancreatitis model used in this study [19].

The detailed mechanisms by which TGFβ is involved in the regulation of ECM turnover are hardly understood. However, there are supporting data that TGFβ stimulates the expression of various collagen genes and reduces the expression of extracellular matrix degrading proteases [3,20,21].

In this study, we investigated the influence of $TGF\beta_1$ on the regulation of its own expression and on the concentration of the TGFβ receptors during the regenerative process after cerulein-induced pancreatitis in vivo as well as in cell culture experiments. We could demonstrate that $TGF\beta_1$ stimulated its own expression and the production of its receptor proteins type I and type II in the regeneration of the pancreas as well as in cultured pancreatic cells.

## 2. Materials and methods

### 2.1. Materials

Male Wistar rats (150–200 g) were obtained from the Charles River Laboratories (Sulzbach, Germany). The cDNA probes for rat TGFβ receptor type I (ALK-5) and type II were kindly provided by Dr. M.G. Brattain (Medical College of Ohio, Toledo, USA [22,23]). The $TGF\beta_1$ cDNA probe was provided by Dr. R. Dernyck (University of California, San Francisco, USA [24]). The antibody against TGFβ receptors was purchased from UBI (Lake Placid, USA; [7]). EGFR antiserum was commercially available from Santa Cruz (Santa Cruz, USA; sc1005). The polyclonal neutralizing anti-$TGF\beta_1$ antibody AB-101 from R&D Systems (Minneapolis, USA) was used to inhibit the biological activity of $TGF\beta_1$. Normal chicken Ig from R&D Systems served as control serum.

### 2.2. Induction of experimental pancreatitis

Experimental acute pancreatitis was induced by intravenous infusion of supramaximal doses of cerulein (Farmitalia, Freiburg, Germany) (10 μg/kg/h) for 12 h. Control rats were infused with saline. The protocol was approved by the institutional animal use and care committee. Serum amylase was measured daily in order to control the development of the pancreatitis as described earlier [25].

To neutralize the activity of $TGF\beta_1$, the rats received three injections (i.v.) of 150 μg neutralizing $TGF\beta_1$ antibody or control serum in PBS. The first injection was given 30 min before, the second 24 h and the third 48 h after start of infusion. Three to five animals were examined at 0 h (start of infusion), 1, 2, 3, 5 and 7 days after the start of cerulein infusion. The pancreata were quickly removed, weighed, and shock frozen in liquid nitrogen for further studies. All investigations were done in three unrelated experimental setups.

### 2.3. RNA studies

The RNA was extracted according to the method described by Chomczynski and Sacchi [26]. $Poly(A)^+$ RNA was isolated with a Qiagen kit in accordance to

the manufacturer's instructions. For Northern blots 30 μg total RNA or 6 μg of poly(A)$^+$ RNA were transferred on Hybond-N membranes by capillary blotting. Equal loading of the slot blots was verified by 18S rRNA hybridization. The blots were hybridized with [$^{32}$P]dCTP labeled purified cDNA probes as described earlier [19]. The membranes were exposed to X-ray films (Kodak XAR, Rochester, USA) at −70°C for 7–10 days.

Optical densities of hybridization signals were measured by using the 1D gel analysis software from Phoretix (Newcastle upon Tyne, UK). The different cDNA signals were corrected by the optical densities of the 18S rRNA hybridization of each blot.

### 2.4. Isolation and culture of pancreatic epithelial cells

Dispersed rat pancreatic cells were prepared by sequential enzymatic and mechanical dissociation as described previously [27]. The cells were incubated in Waymouth medium containing 10% fetal calf serum (FCS), 1.6 nM EGF, 0.8 nM insulin and 10 nM choleratoxin. The epithelial origin of the cultured cells was confirmed by immunohistochemical demonstration of E-cadherin using an antibody (C20820) from Transduction Laboratories (Lexington, USA). After 2 days, the medium was replaced by Waymouth medium containing 0.1% FCS for 24 h and afterwards the cells were stimulated for 18 h with 10 ng/ml TGFβ$_1$ (PeproTech, London, UK) in Waymouth medium with 0.1% FCS. These cultures were used for RNA extraction or proliferation assays.

### 2.5. Measurement of DNA synthesis

Cell cultures were stimulated with different TGFβ$_1$ concentrations or control buffer for 24 h. [$^3$H]thymidine incorporation was determined by adding [$^3$H]thymidine (0.5 μCi/ml) for a period of 6 h during the end of the stimulation period. Subsequently, the cells were rinsed by ice-cold PBS, precipitated with 10% trichloroacetic acid, unprecipitated activity was removed by washing with ice-cold ethanol (70%). The DNA-bound [$^3$H]thymidine was solubilized by incubation with 0.5 M NaOH plus 1% SDS and determined in a scintillation counter (Beckman, Palo Alto, USA). Proliferation in control medium containing 0.1% FCS was set as 100%, the cell proliferation data obtained with TGFβ$_1$ supplementation were expressed in relation to the controls. The experiments were measured in quadruplicates with $n = 3$.

### 2.6. Protein determination of TGFβ receptors

For protein analysis, the samples were homogenized in RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 7.2, 20 mM EDTA) containing 5 μM aprotinin (Bayer-Leverkusen, Leverkusen, Germany), 1 mM pefabloc, 10 mM leupeptin, 5 mM vanadate and 5 μM soybean trypsin inhibitor (all Boehringer-Mannheim, Mannheim, Germany). SDS-polyacrylamide gel electrophoresis was done according to standard procedures [28]. Thirty micrograms of protein were separated on each lane. The fractionated proteins were blotted by semidry technique onto nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany).

For immunodetection, blots were incubated for 1 h at 37°C with the first antibody and incubated 30 min with a secondary peroxidase-coupled antiserum (Pierce, Rochester, USA). The antibody detection was carried out using the enhanced chemiluminescence reaction system (Amersham-Buchler, Braunschweig, Germany).

## 3. Results

The development of acute pancreatitis induced by cerulein infusion was followed up by monitoring serum amylase concentration and morphological examination of parts of the pancreata as described [25].

As shown earlier [19], quantification of mRNA levels of the transforming growth factors revealed an elevated transcription of TGFβ$_1$ peaking at day 2, with a 17-fold expression compared to control pancreatic tissue (Fig. 1A). This increased mRNA concentration during regeneration was nearly diminished by injections of antibody against TGFβ$_1$ before and during induction of pancreatitis by cerulein infusion (Fig. 1A). TGFβ receptor type I (TβRI, ALK-5) and type II (TβRII) mRNAs were expressed at low levels in the normal pancreas, but 2 days after

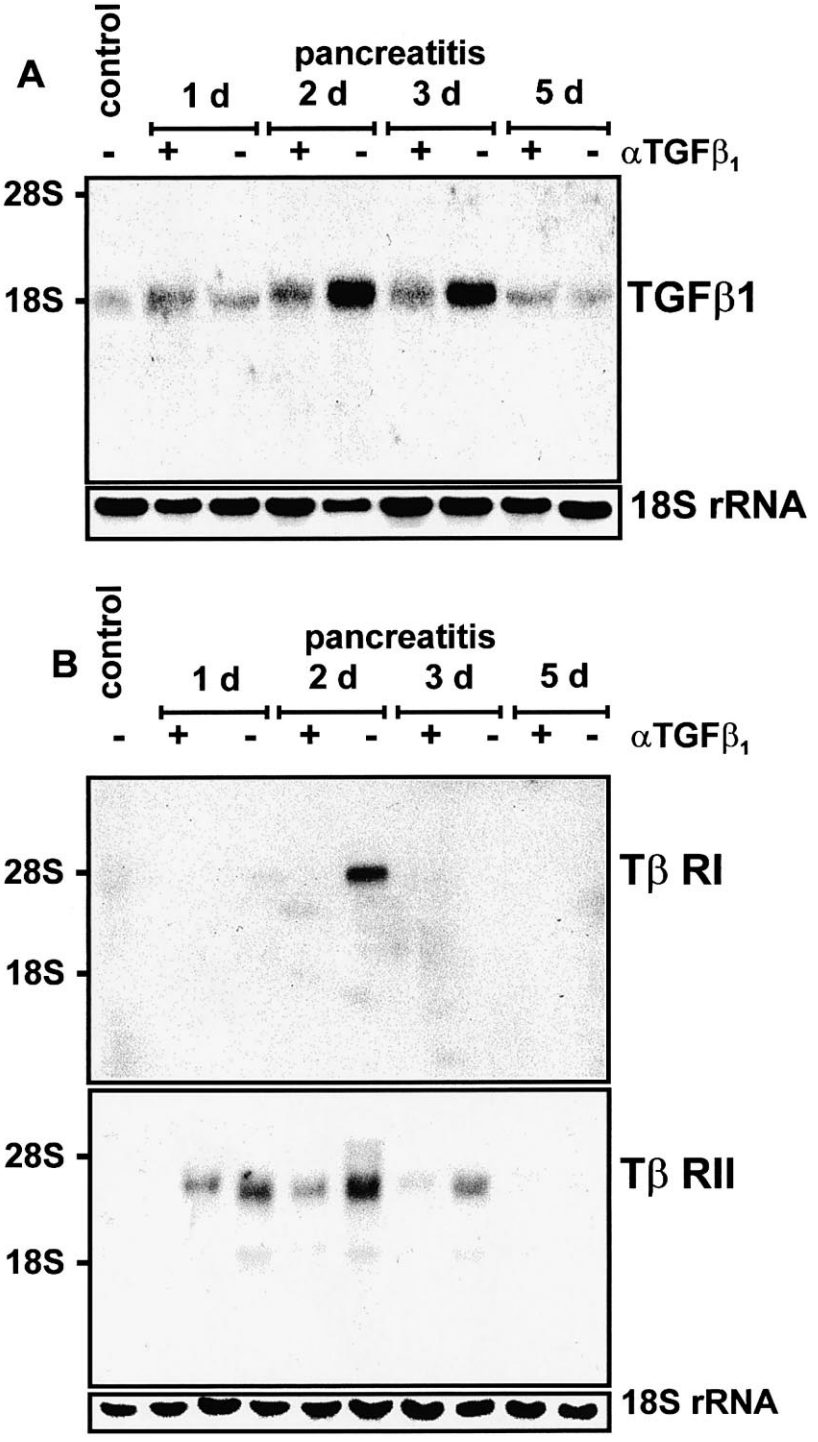


Fig. 1. Expression of $TGF\beta_1$, TGFβ receptor type I and type II during regeneration from cerulein-induced pancreatitis in rats. Neutralization of the biological activity of $TGF\beta_1$ results in decreased mRNA concentrations as demonstrated by these representative hybridizations. (A) The increased mRNA concentration of $TGF\beta_1$, 1–5 days after induction of pancreatitis, was diminished by three injections of neutralizing antibody against $TGF\beta_1$. 18S rRNA is shown as control for equal loading of the gel. (B) Both receptor types were transcribed at low levels in normal pancreas. After induction of pancreatitis, clearly elevated mRNA levels were detected ($-\alpha TGF\beta_1$). This elevated expression was suppressed by $TGF\beta_1$ antibody injections ($+ \alpha TGF\beta_1$). The 18S rRNA control hybridization is shown in the lower part.

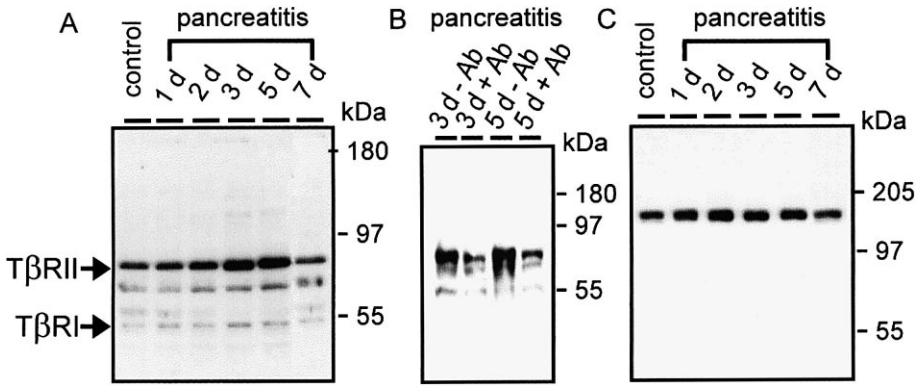


Fig. 2. Western blots showing TGFβ receptors in the regeneration of the pancreas. (A) As documented for the mRNA, TGFβ receptor types I and II concentrations were elevated 3–5 days after induction of pancreatitis. (B) Injection of neutralizing TGFβ-antibody (+ Ab) abolished the increase of TGFβ receptors. (C) In contrast to the TGFβ receptor, the EGF receptor showed only a slight increase in concentration.

induction of pancreatitis, the concentrations were significantly increased for both receptors as shown in Fig. 1B by representative Northern blot hybridization. To demonstrate the influence of $TGF\beta_1$ on the expression of its own receptor, we injected neutralizing antibody against $TGF\beta_1$ during the induction of pancreatitis. As a consequence, the increased expression of the TGFβ receptors was completely reduced by application of a neutralizing antibody against $TGF\beta_1$ (Fig. 1B). Additionally to mRNA quantification, we examined changes of the protein concentrations of the receptors during regeneration by Western blot analyses. We could show that type I and type II TGFβ receptor proteins were expressed in the normal rat pancreas (Fig. 2A). During the regeneration period after cerulein-induced pancreatitis the concentration of type II receptor was significantly increased within the first 5 days, whereas the elevation of type I receptor was less pronounced. After 3 days, the TβRII concentration reached a maximum and decreased continuously to control values at day 7. The same was true in the case of TβRI, with the maximum at day 5. In contrast to these findings, no alteration in the EGF receptor content could be demonstrated. The immunoneutralization of $TGF\beta_1$ by a neutralizing antibody also caused a reduction of TGFβ receptor protein expression, but was less pronounced than the decrease on mRNA level (Fig. 2B).

In further experiments, the possibility was proved that TGFβ stimulates the expression of its own receptor by examining the TGFβ responsiveness of isolated epithelial cells from the pancreas. The functional importance of $TGF\beta_1$ for these cultured epithelial cells was shown by its influence on cell proliferation measured by [$^3$H]thymidine incorporation in the presence or absence of TGFβ. $TGF\beta_1$ reduced the DNA synthesis in a dose-dependent manner: 10 ng $TGF\beta_1$/ml inhibited proliferation of isolated pancreatic epithelial cells to about 66% of controls grown in pure culture medium (Fig. 3A). In parallel samples, we estimated mRNA concentrations of $TGF\beta_1$ and its receptor type I and type II after stimulation with $TGF\beta_1$ (10 ng/ml culture medium). As shown in Fig. 3B, the stimulation of cultured pancreatic epithelial cells resulted in enhanced expression of $TGF\beta_1$ (13-fold) and also in elevated mRNA levels of TGFβ receptor type II. Type I receptor again was less overexpressed in the presence of $TGF\beta_1$ compared to TβRII. Examination of the protein concentrations revealed similar changes. Expression of TβRII was significantly elevated after TGFβ stimulation in cultured primary epithelial cells, whereas TβRI amounts showed only a minor increase after stimulation (Fig. 3C).

## 4. Discussion

The recovery of the pancreas after acute inflammation, as it is shown for other epithelial organs as well, is characterized by an increased production of extracellular matrix proteins [12,29]. It has been shown previously that 1 day after induction of an acute

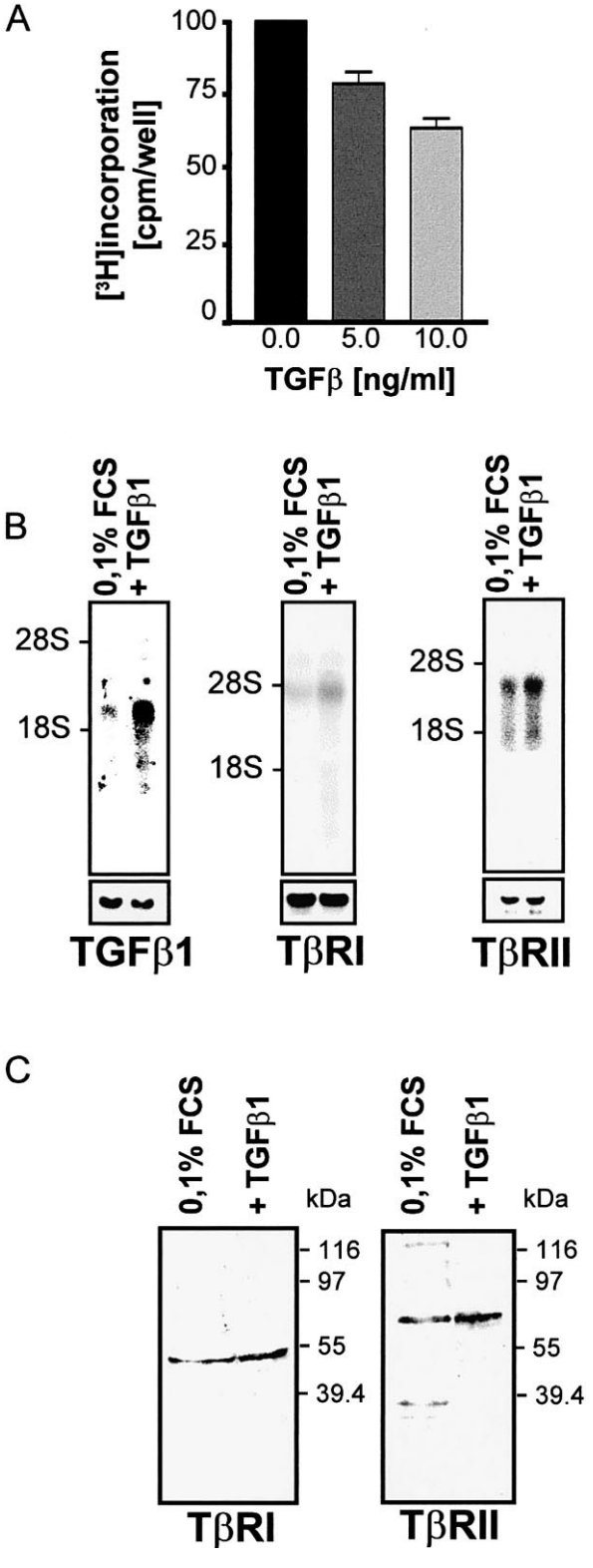


Fig. 3. Stimulation of cultured epithelial cells of the pancreas with $TGF\beta_1$ revealed reduced proliferation and altered mRNA levels. (A) Cell proliferation of primary epithelial cells, measured by [$^3$H]thymidine incorporation, was decreased by $TGF\beta_1$ in a dose-dependent manner. Thymidine incorporation in medium without $TGF\beta_1$ was set as 100%, inhibition by TGFβ was expressed in relation to the controls (mean ± S.E.M., $n = 3$). (B) Expression levels of $TGF\beta_1$ and TGFβ receptor type I and II were also increased under these in vitro conditions. $TGF\beta_1$ and TβRII mRNA were significantly elevated (13- and 2.9-fold), whereas the type I receptor exhibited only minor increase (1.7-fold). In the lower part, 18S rRNA hybridization is shown documenting equal amounts of RNA. (C) Similar changes were found for protein concentrations of TβRI and RII after $TGF\beta_1$ stimulation. Western blot analyses showed significantly elevated amounts of TβR type II and a lower increase in case of type I. ←

pancreatitis in rats $TGF\beta_1$ and $TGF\beta_2$ were significantly overexpressed with a maximum on the second day [15,19]. In agreement with data from the literature on experimental glomerulonephritis and wound scarring [30,31], we have shown that this transient fibrosis can be dramatically altered by inhibiting the biological activity of $TGF\beta_1$, one of the most prominent regulators in extracellular matrix turnover in many tissues [19]. The injection of neutralizing antibody against $TGF\beta_1$ during induction of acute pancreatitis reduced the mRNA level of $TGF\beta_1$ and the expression and deposition of collagen type I, type III and fibronectin [19], suggesting a successful inhibition of TGFβ activity. Most recently, it was shown that pancreatic stellate cells, which were regulated by TGFβ, represents the main producer of extracellular matrix [32]. The process of collagen deposition was paralleled by increased expression of matrix metalloproteases (MMPs), which are also regulated by $TGF\beta_1$. Especially MMP-2 and MMP-3 [33] were involved in the subsequent degradation of the ECM. Therefore $TGF\beta_1$ seems to be one factor leading to a coordinated action of collagen production, deposition and following degradation during pancreatic regeneration.

The results shown here demonstrated that $TGF\beta_1$ enhanced its signal transduction activity by upregulation of the expression of its own receptors during regeneration after acute pancreatitis. TβRI and TβRII mRNA levels were remarkably increased on day 2 after induction of pancreatitis, whereas protein levels reached their maxima on day 3, respectively.

These data correlate well with the elevated expression of collagen with its maximum at day 3 as shown before [19], supporting the regulatory influence of TGFβ via its receptors on collagen contents. Immediately after their expression maxima collagen, $TGF\beta_1$ and the TGFβ receptor mRNA concentrations declined to control values. For the first time we show that TGFβ is able to promote the expression of its own receptors, a process which is well known in growth factor signaling via receptor-tyrosine kinases, such as basic fibroblasts growth factor (bFGF) or hepatocyte growth factor (HGF), but not described for receptor-serine/threonine kinases. Stimulation of fibroblasts and epithelial cells with bFGF or HGF led to elevated expression of the FGF receptor 2 or c-met, the HGF receptor respectively [34–36].

Neutralization of $TGF\beta_1$ activity caused decreased mRNA and protein concentrations of TGFβ receptor type I and type II during regeneration after experimentally induced acute inflammation. These results were confirmed by in vitro data using cultured primary epithelial cells. In this experiment, $TGF\beta_1$ positively regulated its own synthesis and the expression of its receptors. Cultured pancreatic cells upon TGFβ stimulation exhibited a lower increase in receptor expression than observed in vivo. These small differences may be explained by more defined conditions in cell culture, where we analyzed only the TGFβ effects on epithelial cells. In contrast to the cell culture model, cells in a complete organ are influenced by complex interactions of growth factors, cytokines and different cell types leading to intensified signals.

As shown by others, TGFβ stimulation leads to enhanced expression of Smad proteins, the intracellular mediators of the TGFβ pathway [37–39]. Altogether these results implicate that $TGF\beta_1$ amplified its biological effects by a self-stimulated production. Additionally, the increased production of its receptors and intracellular signaling mediators potentiated the sensitivity of the TGFβ cascade, at least in epithelial cells.

Pancreatic epithelial cells seem to contribute to regenerative processes by autocrine and paracrine TGFβ-induced mechanisms. Autocrine stimulation of epithelial cells led to reduced proliferation, while paracrine stimulation of stellate cells induced production of ECM components. In line with these data, the neutralization of $TGF\beta_1$ during pancreatitis, as reported before [19], led to a slightly accelerated regeneration of the pancreas, due to reduced transient fibrosis and non-inhibited epithelial proliferation.

In addition to regeneration after inflammation, Oft and colleagues recently showed that in late-stage events of carcinogenesis, a cell-autonomous signaling via TGFβ is required for the invasive growth of tumor cells, most probably mediated by autocrine loops involving TGFβ and TGFβ receptors [40].

In summary, these data suggest the important role of $TGF\beta_1$ as a key regulator in control of cell proliferation and induction of enormous expression and synthesis of collagen and other ECM components observed in pancreatitis. The TGFβ effects on epithelial pancreatic cells are potentiated by autocrine and/or paracrine stimulated growth factor synthesis and TGFβ-induced expression of its cellular receptors.

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