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Advances in Brief

Enhanced Expression of the Type II Transforming Growth Factor β Receptor in Human Pancreatic Cancer Cells without Alteration of Type III Receptor Expression[1]

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Abstract

We have recently found that human pancreatic adenocarcinomas exhibit strong immunostaining for the three mammalian transforming growth factor β (TGF-β) isoforms. These important growth-regulating polypeptides bind to a number of proteins, including the type I TGF-β receptor (TβR-I), type II TGF-β receptor (TβR-II), and the type III TGF-β receptor (TβR-III). In the present study we sought to determine whether TβR-II and TβR-III expression is altered in pancreatic cancer. Northern blot analysis indicated that, by comparison with the normal pancreas, pancreatic adenocarcinomas exhibited a 4.6-fold increase ($P < 0.01$) in mRNA levels encoding TβR-II. In contrast, mRNA levels encoding TβR-III were not increased. *In situ* hybridization showed that TβR-II mRNA was expressed in the majority of cancer cells, whereas mRNA grains encoding TβR-III were detectable in only a few cancer cells and were present mainly in the surrounding stroma. These findings suggest that enhanced levels of TβR-II may have a role in regulating human pancreatic cancer cell growth, while TβR-III may function in the extracellular matrix.

Introduction

TGF-βs[3] are multifunctional polypeptides that influence cell growth and differentiation, adhesion, migration, angiogenesis, extracellular matrix formation, and immune functions (1, 2). The TGF-β gene superfamily consists of numerous regulatory polypeptides that include several TGF-β isoforms, as well as activins, inhibins, Müllerian inhibiting substance, and the bone morphogenetic proteins (1, 2). In mammalian cells only TGF-β_1, TGF-β_2, and TGF-β_3 are expressed. In contrast, TGF-β_4 and TGF-β_5 are found only in chickens and frogs, respectively (1, 2).

TGF-βs exert both positive and negative effects on cancer cell growth, depending on cell type and culture conditions. They bind with high affinity to the surfaces of many cell types. Cross-linking experiments with radiolabeled TGF-β have identified three primary binding proteins of M_r 53,000 (TβR-I), M_r 70,000 (TβR-II), and M_r 200,000–400,000 (TβR-III) (1–7). Recent cloning and biochemical studies indicate that TβR-II has intrinsic serine/threonine kinase activity which is dependent on the presence of TβR-I (4, 5). In turn, TβR-II is necessary for binding of TGF-β to TβR-I (5). In contrast, TβR-III is a membrane proteoglycan (also called betaglycan) that binds TGF-βs but is not believed to have direct signal transducing activity (3, 5–7).

Using isoform-specific, non-cross-reactive antibodies and specific cRNA probes, we have recently found that the three mammalian TGF-β isoforms, TGF-β_1, TGF-β_2, and TGF-β_3, are overexpressed in the cancer cells of human pancreatic adenocarcinomas.[4] To examine the possibility that TGF-βs may exert autocrine and paracrine effects within the pancreatic tumor mass, we assessed the expression of mRNA species encoding TβR-II and TβR-III in the normal and cancerous human pancreas. We now report that TβR-II but not TβR-III mRNA levels are increased in human pancreatic adenocarcinomas.

Patients and Methods

Normal human pancreatic tissue samples were obtained from 11 previously healthy individuals (5 female, 6 male; median age, 39 years; range, 18–57 years) through an organ donor program. Pancreatic cancer tissues were obtained from 9 female and 8 male patients undergoing surgery for pancreatic cancer. The median age of the pancreatic cancer patients was 63 years, with a range of 32 to 73 years. Surgical procedures consisted of either a partial duodenopancreatectomy (11 patients) or a left resection of the pancreas (6 patients). Freshly removed tissue samples were fixed in Bouin solution (3:1 ratio of picric acid and formaldehyde) for 12 to 24 h and paraffin-embedded for histological analysis. Tissues destined for RNA extraction were frozen in liquid nitrogen immediately upon surgical removal and maintained at −80°C until use.

According to the Union Internationale Contre Cancer classification (8) there were one grade 1, six grade 2, nine grade 3, and one grade 4 duct cell adenocarcinomas. All the studies were approved by the Ethics Committee of the University of Ulm (Ulm, Germany) and by the Human Subjects Committee at the University of California (Irvine, CA).

HepG2 human hepatocellular carcinoma cells (HB 8065) were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

***In Situ* Hybridization.** Tissue sections were placed on poly-L-lysine-coated plates, deparaffinized, incubated at 23°C for 10 min with 1 μg/ml proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and for 10 min with 0.5× standard saline citrate (1× standard saline citrate = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), as previously reported (9). Tissue sections were then prehybridized and hybridized with cRNA probes specific for TβR-II and TβR-III as previously reported (9). The sections were washed and digested with RNAse A (20 μg/ml; Sigma Chemical Co., St. Louis, MO), dehydrated by immersion in graded alcohol, coated with NTB2 autoradiography emulsion (Eastman Kodak Co., Rochester, NY), and exposed for 7 days (9). Following development, the slides were counterstained with Mayer's hematoxylin. To verify the specificity of the *in situ* hybridization reaction, control slides were treated either with RNAse or with the respective sense probes. In both cases, this resulted in loss of the *in situ* hybridization signals.

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[3] The abbreviations used are: TGF, transforming growth factor; TβR-I, TβR-II, TβR-III, types I, II, and III TGF-β receptor, respectively; poly(A)$^+$, polyadenylated; cRNA, complementary RNA; cDNA, complementary DNA.

[4] H. Friess, Y. Yamanaka, M. Büchler, H. G. Beger, L. I. Gold, and M. Korc. Enhanced expression of transforming growth factor β isoforms in human pancreatic cancer, submitted for publication.

cRNA antisense and sense probe synthesis for *in situ* hybridization studies was performed as previously described (9), using [α-^{35}S]UTP (Amersham International, Arlington Heights, IL). cDNAs encoding sequences corresponding to TβR-II and TβR-III genes were produced by amplification with the polymerase chain reaction of single-stranded cDNA that was reverse transcribed from HepG2 RNA. The cDNAs were then subcloned into a pGEM3Zf vector (Promega, Biotechnology, Madison, WI). HepG2 cells were shown to express high levels of TβR-II and TβR-III mRNA (4, 7). Furthermore, authenticity of the reverse transcriptase-polymerase chain reaction products was confirmed by sequencing. The TβR-II cRNA probe consisted of a 477-base pair fragment of human TβR-II cDNA, corresponding to nucleotides 42–519 (4). The TβR-III cRNA probe consisted of a 721-base pair fragment of the human TβR-III cDNA, corresponding to the nucleotides 1734–2455 of the corresponding rat cDNA sequence (6, 7). Details of this cloning procedure will be published elsewhere.[5]

Northern Blot Analysis. Total RNA was extracted by the guanidine isothiocyanate method, fractionated on 1.2% agarose/1.8 M formaldehyde gels, and stained with ethidium bromide for verification of RNA integrity and loading equivalency (9, 10). In the case of HepG2 cells, poly(A)$^+$ RNA was prepared following isolation of total RNA, using oligodeoxythymidine column chromatography (10). Fractionated RNA was electrotransferred onto Nylon membranes (GeneScreen, Du Pont, Boston, MA) and cross-linked by UV irradiation. The filters were then prehybridized, hybridized, and washed under high stringency conditions appropriate for antisense riboprobes (TβR-II, TβR-III) or the cytoplasmic 7S cDNA probe, which was used as an internal control (9, 10).

The antisense probes used for Northern blot analysis were radiolabeled with [α-^{32}P]CTP (Amersham International). A 0.19-kilobase *Bam*HI fragment of the mouse 7S cytoplasmic cDNA that cross-hybridizes with human 7S RNA (9, 10) was labeled with [α-^{32}P]dCTP (Amersham International) using a random primer labeling system (Boehringer-Mannheim, Indianapolis, IN). All blots were exposed at −80°C to Kodak XAR-5 film with Kodak intensifying screens, and the intensity of radiographic bands was quantified by laser densitometry (Ultrascan XL; LKB), as previously reported (9, 10).

Statistical Analysis. Results are expressed as median and range or as mean ± SE. For statistical analysis the unpaired Student's *t* test was used. Significance was defined as $P < 0.05$.

Results

Quantification of TβR-II and TβR-III mRNA Moieties. To confirm the specificity of the hybridization signals obtained with the TβR-II and TβR-III riboprobes, poly(A)$^+$ mRNA prepared from HepG2 cells was used as a positive control. As seen in Fig. 1, TβR-II and TβR-III migrated approximately as 5.5- and 6.0-kilobase bands, respectively, when HepG2 poly(A)$^+$ RNA was used. Both mRNA species were readily detectable on Northern blots of total RNA isolated from normal human pancreas. The significance of the lower faint band observed with the TβR-III riboprobe in both the poly(A)$^+$ mRNA of HepG2 cells and the total RNA of the pancreatic samples is not known (Fig. 1).

Northern blot analysis of total pancreatic RNA showed low levels of TβR-II mRNA in all normal pancreatic tissues (Figs. 1 and 2). However, in some of these samples the band corresponding to TβR-II mRNA was visible only on the original autoradiograph (Fig. 2). In contrast, with one exception, all of the pancreatic cancer samples showed increased TβR-II mRNA levels (Fig. 2). Densitometric analysis of the Northern blots indicated that by comparison with the normal pancreas, there was a 4.6-fold increase ($P < 0.01$) in the mRNA levels encoding TβR-II (Fig. 3). TβR-III mRNA levels were similar ($P = 0.39$) in normal and cancerous pancreatic tissues (Figs. 2 and 3). For reasons that are not readily evident, the same sample that had low TβR-II mRNA levels exhibited TβR-III mRNA levels that were lower than in the normal controls (Fig. 2). However, none of the cancer samples exhibited any aberrant TβR-II or TβR-III transcripts.

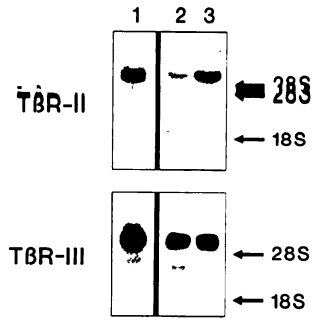

Fig. 1. Northern blot analysis of TβR-II and TβR-III mRNA. Poly(A)$^+$ mRNA (5 μg) from HepG2 cells (*Lane 1*) and total RNA (20 μg/lane) from two normal human pancreatic tissue samples (*Lanes 2* and *3*) were size fractionated, blotted, and hybridized as described in "Patients and Methods." Hybridization was carried out with 1 × 10^6 cpm/ml of [α-^{32}P]CTP-labeled TβR-II (*top*) and TβR-III (*bottom*) cRNA probes. Exposure times were 14 days for TβR-II and TβR-III in HepG2 cells and 7 days (TβR-II) and 4 days (TβR-III) in the normal human pancreas.

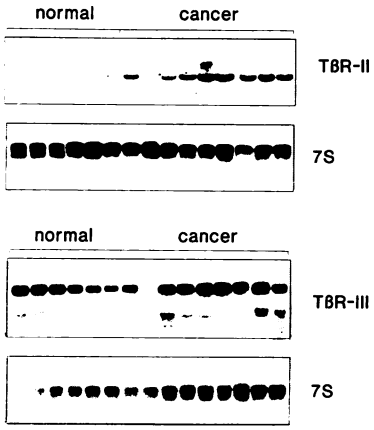

Fig. 2. Northern blot analysis of TβR-II and TβR-III mRNA in normal and cancerous human pancreas. Total RNA (20 μg/lane) was size fractionated and hybridized as described in Fig. 1. Two different blots are shown for TβR-II and TβR-III. However, the same samples were run in the same order in both blots. TβR-II mRNA (*top*) was present at low levels in the normal pancreas (*Lanes 1–6*) and at increased levels in 8 of 9 pancreatic carcinomas. In contrast, mRNA levels encoding TβR-III (*bottom*) were similar in the two groups. 7S mRNA was used to confirm equivalent RNA loading. Exposure times were 7 days for TβR-II and 4 days for TβR-III.

Localization of TβR-II and TβR-III by *in Situ* Hybridization. In the normal pancreas, *in situ* hybridization grains corresponding to TβR-II mRNA were detectable at a low density in some acinar and ductal cells (Fig. 4*A*). A focal pattern of distribution was evident within the stroma (Fig. 4*A*). *In situ* hybridization grains corresponding to TβR-III mRNA were located mainly in the stroma but also occasionally in acinar cells (Fig. 4*C*) and ductal cells (data not shown).

In pancreatic cancer tissues, *in situ* hybridization grains encoding TβR-II were markedly increased by comparison with the normal pancreas (Fig. 4*B*). These grains were evident at high levels in the majority of the cancer cells forming the duct-like structures within the tumor but at relatively low levels in the surrounding stroma (Fig. 4*B*). In contrast, only a few cancer cells exhibited *in situ* hybridization grains encoding TβR-III (Fig. 4*D*). Instead, this mRNA moiety was mainly expressed in the stroma of the tumors (Fig. 4*D*).

Discussion

Loss of responsiveness to the inhibitory actions of TGF-βs has been proposed to confer a growth advantage to cancer cells (1–3). It is intriguing, therefore, that so many different cancer cell types express TGF-βs. Elevated levels of TGF-β_1 have been reported in gastric (11), breast (12), thyroid (13), and brain (14, 15) cancers. Furthermore, TGF-β_1 expression has been associated with increased cell proliferation in gastric, thyroid, and brain cancers (11, 13–15) and with

[5] R. L. Baldwin, M. S. Kobrin, and M. Korc, manuscript in preparation.

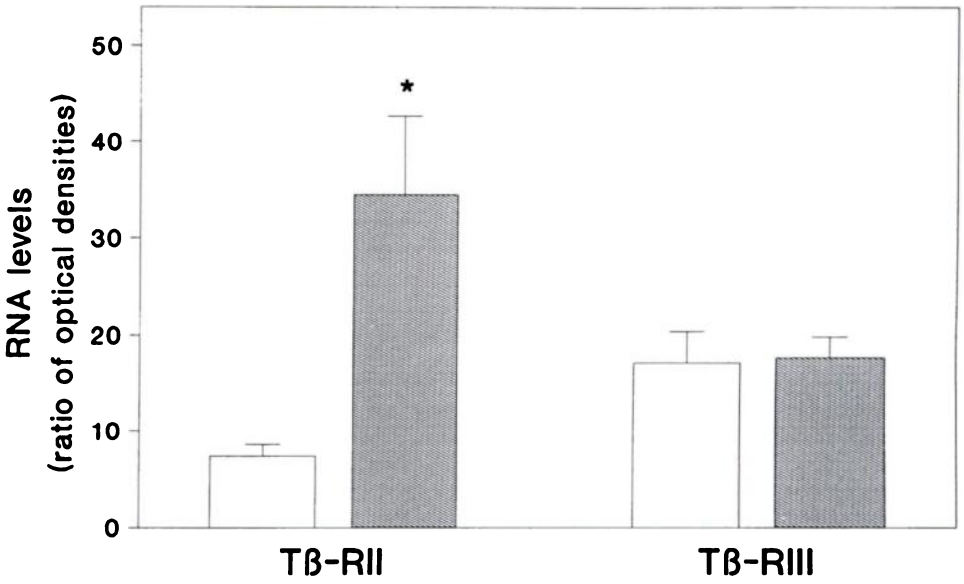

Fig. 3. Densitometric analysis of Northern blots. The ratio of absorbances of TβR-II or TβR-III with the corresponding 7S signals was calculated. *Columns*, mean for the calculated ratios; *bars*, SE. □, normal pancreas; ▨, cancerous pancreas. *, $P < 0.01$ when compared with the respective control.

disease progression in breast cancer (12). These observations raise the possibility that the bifunctional characteristics of TGF-βs allow these polypeptides to enhance cancer cell growth *in vivo*. For example, prostatic cancer cells that overproduce TGF-β_1 exhibit faster tumor growth and more extensive metastases than identical tumor cells that do not express TGF-β_1 (16). It is not clear whether this is due to a direct effect of TGF-β_1 on the cancer cells or to an indirect effect, inasmuch as TGF-βs stimulate angiogenesis and are potent suppres-

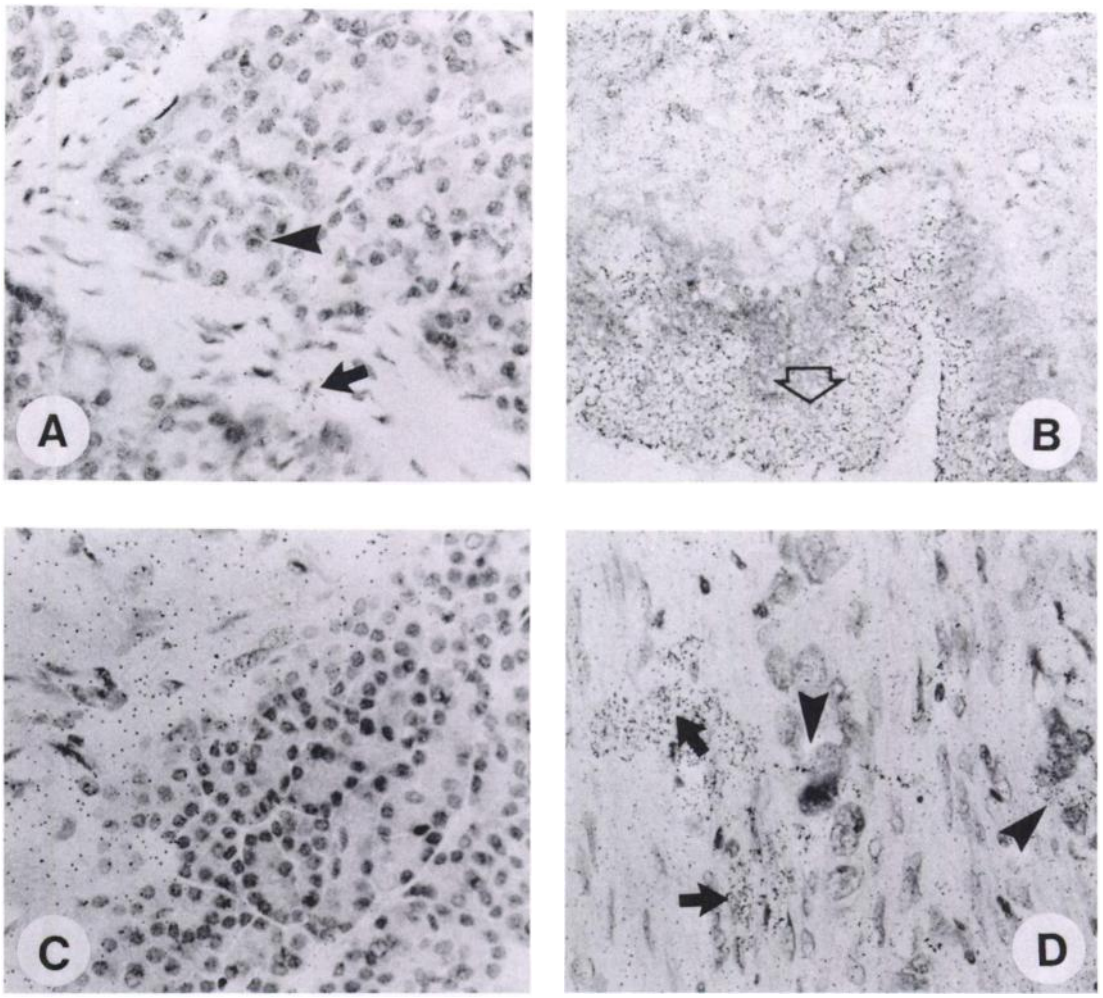

Fig. 4. *In situ* hybridization. In the normal pancreas, *in situ* hybridization grains encoding TβR-II mRNA were present in a few acinar cells (*A*, *arrowhead*) and in the stroma (*A*, *arrow*). In contrast, *in situ* hybridization grains encoding TβR-III mRNA (*C*) were mainly present in the stroma but also in some acinar cells. In the cancerous pancreas, the cancer cells forming the duct-like structures (*B*, *open arrow*) showed a marked increase in TβR-II mRNA. *In situ* hybridization grains encoding TβR-III mRNA were mainly found in the stroma surrounding cancer cells (*D*, *arrows*) and were less frequent in the cancer cells (*D*, *arrowheads*). × 100. Exposure times were 7 days for TβR-II and TβR-III.

sors of immune function. Thus, TGF-βs may enhance tumorigenesis by increasing tumor blood supply and by inhibiting antitumor immunological mechanisms (1, 2). TGF-βs may also enhance the metastatic potential of cancer cells by increasing the expression of adhesion molecules and extracellular matrix components (1, 2).

For TGF-βs to exert direct effects on cancer cells *in vivo*, these cells must be able to express receptors that bind TGF-βs. Recently, we found that TGF-β_1, TGF-β_2, and TGF-β_3 are overexpressed in pancreatic adenocarcinomas,[4] a malignancy that is associated with greater than 90% mortality within the first year following diagnosis (17). In the present study we have determined that TβR-II mRNA levels are increased in the pancreas of patients with pancreatic cancer by comparison with the normal human pancreas. By *in situ* hybridization, TβR-II mRNA expression was markedly increased in the cancer cells but not in the surrounding stroma. These findings suggest either increased TβR-II gene transcription rates or increased TβR-II mRNA stability in pancreatic cancer cells *in vivo*. In view of the pivotal role of TβR-II in mediating signal transduction by TGF-βs and the ability of this receptor to bind all three TGF-β isoforms, these observations suggest that TGF-βs may act in an autocrine and paracrine manner to excessively activate the overexpressed TβR-II in pancreatic cancer cells *in vivo*.

The exact functions of TGF-βs and TβR-II in pancreatic cancer cells are unknown. In cultured human pancreatic cancer cell lines high concentrations of TGF-β_1 inhibit proliferation but up-regulate c-*myc* expression (18). This observation raises the possibility that TGF-β_1 may also increase c-*myc* expression in pancreatic cancer cells *in vivo*, thereby directly giving these cells a growth advantage. Pancreatic cancer cells also overexpress the epidermal growth factor receptor, c-*erb*B-2, c-*erb*B-3 (9, 19), epidermal growth factor, and TGF-α (9). Therefore, the extent of tumor cell proliferation and invasion and the advantage that these cells may derive from the overexpressed TβR-II may depend on a combination of these regulatory signals.

In contrast to our findings with TβR-II, TβR-III mRNA levels were not increased in the pancreatic cancer tissues. Nonetheless, the *in situ* hybridization studies indicated that some TβR-III mRNA was present in a few cancer cells. Interestingly, in both the normal and cancerous pancreas, this mRNA species was most prominent in the stroma. These findings are consistent with previous observations that TβR-III is the predominant TGF-β binding protein in a number of mesenchymal cells, including fibroblasts (14). Although TβR-III may not have the capacity to act as a signal transducer, it may serve to enhance the presentation of TGF-βs to TβR-II (3). It is conceivable, therefore, that TGF-βs produced by the cancer cells may exert paracrine effects on the pancreatic stroma following binding to TβR-III. The differential localization of TβR-II and TβR-III, in conjunction with the selective increase in the levels of TβR-II and the abundance of the TGF-βs, may also lead to alterations in epithelial-mesenchymal interactions within the tumor mass, thereby contributing to the desmoplastic reaction around the cancer cells.

It has been suggested that the products of the *p53* and retinoblastoma tumor suppressor genes may participate in TGF-β-mediated signal transduction pathways (3). Recently, cultured pancreatic cancer cells as well as pancreatic carcinomas have been found to exhibit a high frequency of mutations in the *p53* tumor suppressor gene (20, 21). It is possible, therefore, that the overexpression of TβR-II and the presence of mutations in the *p53* gene may lead to perturbations that abrogate negative constraints on cell growth. Thus, a lack of negative growth-regulatory signals, when combined with enhanced stimulatory growth signals mediated via the overexpressed tyrosine kinase receptors (9, 19), may give pancreatic cancer cells an additional growth advantage.

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