A murine tumor progression model for pancreatic cancer recapitulating the genetic alterations of the human disease

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This study describes a tumor progression model for ductal pancreatic cancer in mice overexpressing TGF-α. Activation of Ras and Erk causes induction of cyclin D1-Cdk4 without increase of cyclin E or PCNA in ductal lesions. Thus, TGF-α is able to promote progression throughout G1, but not S phase. Crossbreeding with p53 null mice accelerates tumor development in TGF-α transgenic mice dramatically. In tumors developing in these mice, biallelic deletion of Ink4a/Arf or LOH of the Smad4 locus is found suggesting that loci in addition to p53 are involved in antitumor activities. We conclude that these genetic events are critical for pancreatic tumor formation in mice. This model recapitulates pathomorphological features and genetic alterations of the human disease.

[Key Words: Pancreas; cancer; TGF-α; p53; Ink4a; tumor progression]

Received July 7, 2000; revised version accepted November 29, 2000.
that these mice develop pancreatic cancer similar to the human disease regarding cellular differentiation, growth characteristics, and genetic alterations.

Results

The Ras/Erk1/2 pathway is activated in premalignant lesions in TGF-α transgenic mice

To investigate whether Ras is activated in the pancreas of TGF-α transgenic mice, we have performed a Ras-affinity precipitation assay, which selectively detects GTP-loaded Ras (Taylor and Shalloway 1996). Elevated levels of active Ras are detectable shortly after birth and persist throughout adulthood in the pancreas of TGF-α transgenic mice as compared with littermate controls (Fig. 1A). To confirm Ras downstream signaling, immunohistochemical staining for phosphorylated Erk1/2 protein was performed.

Figure 1C shows strong staining for phosphorylated Erk1/2 in the pancreas of TGF-α transgenic mice as compared with littermate controls [Fig. 1B]. The immunoreactivity for active Erk is evident both in the nucleus and cytoplasm of duct-like cells in tubular complexes [Fig. 1D]. Fibroblasts and acinar cells surrounding the tubular complexes are negative for phosphorylated Erk protein [Fig. 1D]. These data show that the Ras/Erk signaling cascade is activated selectively in tubular complexes that represent the premalignant lesions in TGF-α transgenic mice.

Cyclin D1/Cdk4 activity is increased in premalignant lesions in TGF-α transgenic mice

Real-time PCR analysis was performed to investigate whether Ras signaling in the pancreas of TGF-α transgenic mice leads to up-regulation of cyclin D1. Cyclin D1 mRNA is increased up to 14-fold in the pancreas of mice with developed tubular complexes [180-day-old mice]. mRNA levels of cyclins D3 and E remain unchanged [Fig. 2A]. In mice with developing tubular complexes (28 d), immunohistochemical analysis shows staining for cyclin D1 [Fig. 2B], Cdk4 [Fig. 2C], and cyclin E [Fig. 2D] in a few clusters of acinar cells as well as in the surrounding fibroblasts. After 180 d, expression of cyclin D and Cdk4 is significantly increased but restricted to cells within tubular complexes, whereas cyclin E is not detectable anymore [Fig. B–J]. To investigate whether this up-regulation of cyclin D1 and Cdk4 results in increased activity of cyclin D1–Cdk4 complexes, immune complex kinase assays were performed. Figure 2K shows strong cyclin D1/Cdk4 activity in the pancreas of TGF-α transgenic mice, whereas GST-Rb(769–921) phosphorylation is barely detectable in the pancreas of littermate controls.

TGF-α promotes progression throughout G1, but not S phase, in premalignant lesions

To detect cell cycle progression, immunofluorescence analysis for PCNA and BrdU labeling were performed. BrdU incorporation is predominantly localized to cells in developing tubular complexes, with labeling indices of 15.5 ±3.4% [Fig. 3A]. At 180 d, fewer cells have incorporated BrdU in tubular structures, with a labeling index of 4.9 ±2.2% [Fig. 3B]. Nontransgenic controls show occasional staining for BrdU in ducts [Fig. 3C]. Although almost all cells within tubular complexes stain positive for cyclin D and Cdk4 [Fig. 2E,F], only few of these cells display nuclear immunoreactivity for BrdU. Similar results were obtained with PCNA staining [data not shown]. Cyclin E is not induced in tubular complexes.
mRNAs of cyclin E (Fig. 2A) and thymidine kinase 1 are not up-regulated in the pancreas of transgenic animals.

Figure 2. (A) Relative mRNA levels for cyclin D1, cyclin D3, and cyclin E were evaluated in transgenic animals (TGF-α) by Real Time PCR and normalized to endogenous cyclophilin as described in Materials and Methods. Controls were performed with wild-type pancreas for each time point and target individually. One representative control is shown (WT). Immunostains were performed for cyclin D (B,E,H), Cdk4 (C,F,J), and cyclin E (D,G,F) in TGF-α transgenic mice with developing tubular complexes (at 28 d, B–D), or with developed complexes (at 180 d, E–G) and compared with wild-type controls (at 180 d, H–J). (B–D) Cyclin D, Cdk4, and cyclin E localize in small foci of developing tubular complexes and the surrounding fibroblasts. (E–G) Developed tubular complexes show strong immunoreactivity for cyclin D and Cdk4, whereas only single nuclei are positive for cyclin E. (H–J) Littermate controls show only occasional staining for either cyclin D, Cdk4, or cyclin E. (B–F) Bar, 50 µm. Autoradiograph of a representative immunocomplex kinase assay for cyclin D1/Cdk4 activity indicates increased phosphorylation of the *GST-Rb (769–921) substrate in pancreatic lysates from transgenic mice TGF-α at 180 d as compared with littermate controls (wild type).

Figure 3. Proliferating cells are detected by immunofluorescence analysis of BrdU labeling in the pancreas of TGF-α transgenic mice with developing tubular complexes [A] or developed complexes [B] and in littermate controls [C]. Arrowheads indicate BrdU-positive nuclei. (D,E) Comparison of immunohistochemical staining for p53 in the premalignant lesions in 180-day-old TGF-α transgenic mice [D] with littermate controls [E]. (F) Relative mRNA levels of p21^{Cip1}, p16^{Ink4a}, p19^{Arf}, and p27^{Kip1}. Controls were performed with wild-type pancreas for each time point and target individually. One representative control is shown (WT). (G) Cumulative tumor incidence of TGF-α, TGF-α/p53^{−/−}, TGF-α/p53^{+/−}, p53^{+/−}, and p53^{−/−}. Details are described in Materials and Methods.
[data not shown]. These data suggest that TGF-α is able to promote progression throughout G1, but not S phase, in premalignant lesions.

*p53 is induced in tubular complexes and acts as a tumor suppressor in TGF-α transgenic mice*

Activated Ras in primary cells is able to induce expression of cell cycle inhibitory molecules such as p16Ink4a, p21Cip1, or p53 and p19Arf. The premalignant lesions in TGF-α transgenic mice display increased nuclear immunoreactivity for p53 [Fig. 3D] as compared with controls [Fig. 3E].

p21Cip1 mRNA levels are increased more than eight-fold in 180-day-old TGF-α transgenic animals compared with littermate controls [Fig. 3F]. In contrast, mRNA levels of p16Ink4a, p19Arf, and p27Kip1 remain unchanged or even slightly decreased throughout adulthood [Fig. 3F]. Western blot analysis confirmed the increase of p21Cip1 expression and unchanged expression of p27Kip1 in transgenic mice [data not shown].

To determine the role of p53 in tumor progression, we mated TGF-α transgenic mice to p53-deficient mice [Jacks et al. 1994]. TGF-α transgenic, p53+/− and p53−/−, as well as the crossbred animals were born alive at the expected Mendelian ratio. Up-regulation of p21Cip1 mRNA is abolised in p53-null mice, although p27Kip1 mRNA is slightly induced [data not shown]. TGF-α transgenic mice [31 of 107, 29%] developed malignant pancreatic tumors with a mean tumor-free survival of 410 d. Heterozygous loss of p53 resulted in an increased incidence of pancreatic tumors [66 of 90 animals, 77.3%] and decreased mean tumor-free survival to 220 d in TGF-α/p53−/− animals. All 22 TGF-α/p53−/− mice developed pancreatic tumors within 120 d after birth [Fig. 3G]. p53+/− and p53−/− littermate controls developed mainly mesenchymal tumors with a frequency and latency as described previously [Fig. 3G; Jacks et al. 1994].

Tumor development in TGF-α/p53−/− and TGF-α/p53−/− mice precedes tumorigenesis in littermate control p53−/− and p53−/− animals. However, necropsy of 3 out of 90 TGF-α/p53−/− mice [3.3%] revealed both a large epithelial pancreatic tumor and extrapancreatic sarcoma [data not shown].

Pancreatic carcinomas display a ductal phenotype, grow invasively into the surrounding tissue, and metastasize

Some pancreatic tumors form ductal structures in dense connective tissue [Fig. 4A–C4]; others show frequent mitotic figures surrounded by sparse fibrosis [Fig. 4D–F]. p53−/− and p53−/− littermate controls develop sarcomas and lymphomas [Fig. 4G–I; Jacks et al. 1994]. Furthermore, we observed malignant ascites, local invasive growth with duodenal obstruction, and metastasis to the liver and lung in TGF-α transgenic mice crossbred to p53-null mice [Fig. 4M–O]. Double staining of serial sections with cytokeratin 8/18 and CD45-LCA indicates epithelial differentiation of the pancreatic tumors [Fig. 4B,E]. Both pancreatic tumors are positive for the ductal marker cytokeratin 19, whereas surrounding fibroblasts express vimentin [Fig. 4C,F]. In contrast, lymphoma from p53−/− deficient littermate controls express CD45-LCA and vimentin to a lesser degree but do not show any immunoreactivity for cytokeratin 8/18 and 19 [Fig. 4G,J]. In wild-type pancreas, cytokeratin 8/18 is expressed both in acinar and ductal cells, whereas cytokeratin 19 is restricted to ducts [Fig. 4J–L].

Pancreatic tumors in TGF-α/p53−/− mice segregate the wild-type p53 allele and acquire homozygous deletions of the Inka4/Arf locus

Acceleration of tumor formation in TGF-α/p53−/− and TGF-α/p53+/− transgenic mice identifies the p53 protein as a rate-limiting step during tumorigenesis. To elucidate whether heterozygous p53-null mice lose the inherited wild-type p53 allele during tumorigenesis, we have applied loss of heterozygosity [LOH] analysis to laser-captured microdissected tumor specimen from F1 and F2 Cl57Bl/6–Balb/c hybrid mice informative for polymorphic genetic markers. The residual wild-type p53 allele is segregated in almost all of the tumors arising from TGF-α/p53−/− mice [Table 1]. Interestingly, tumors still take more than 45 d to develop in TGF-α/p53−/− mice, suggesting that in addition to persistent activation of Ras and inactivation of p53, other genetic events have been acquired during tumorigenesis. To reveal the basis for this delayed onset, LOH analysis was performed with known tumor suppressor loci. Both alleles of the Inka4/Arf locus were deleted in 5 out of 15 tumors, and in one case a heterozygous deletion was found, indicating a strong selection pressure for loss of functional p53 and Inka4/Arf. In addition, we found LOH of the Smad4 locus in four tumors. The well-characterized tumor supressor locus Rb1 was affected in only two of these tumors [Table 1].

Discussion

Mice overexpressing TGF-α under the control of the elastase promoter develop pancreatic tumors originating from cells within tubular complexes after 1 yr of age [Wagner et al. 1998]. In the pancreas of these transgenic mice, persistent stimulation of the EGF receptor by TGF-α increases GTP loading of Ras and phosphorylation of Erk selectively in tubular structures at 180 d. At 28 d of age these tubular complexes start to develop interspersed between large areas of normal-appearing pancreas. Some cells in these areas seem to be programmed to respond positively to growth activation by Ras signaling and are positive for cyclin D1. At 180 d, numerous tubular complexes form three-dimensional accumulations of ductular structures. Almost all of these cells show nuclear accumulation of cyclin D1. Neither acinar cells nor fibroblasts are positive for cyclin D1 or Cdk4, suggesting that increased
Figure 4. Histological analysis of two representative pancreatic tumors (A–F) is compared with thymic lymphoma (G–I) and wild-type pancreas (J–L). Each tissue was stained with hematoxylin and eosin. (B,E,H,K) Expression of CD45-LCA (red fluorescence) and cytokeratin 8/18 (green fluorescence) was analyzed by double-immunostaining. (C,F,I,L) Expression of vimentin (red fluorescence) and cytokeratin 19 (green fluorescence) was analyzed by double-immunostaining. Note the histological appearance of pancreatic tumors with ductal structures (asterisk in A) and frequent mitotic figures (arrowhead in D). Pancreatic tumors express cytokeratin 8/18 (B,E) and 19 (C,F) in contrast with the lymphoma, which stains for CD45-LCA (H). Vimentin staining is restricted to fibroblasts and lymphoma (C,F,I). Acinar and duct cells stain for cytokeratin 8/18 in the normal pancreas (K), whereas cytokeratin 19 is restricted to ducts (asterisk in I,L). Pancreatic tumors metastasize to the liver (M) and lung (N) and infiltrate the duodenal wall (O). Metastasis (M,N) originates from the tumor shown in D–F. Bars, 100 µm in H&E stains, 50 µm in immunostains; (O) bar, 160 µm.
transgenic mice, p21Cip1 is induced up to sevenfold at which is not up-regulated in the pancreas of TGF-
p21Cip1, or p19Arf and p53 (Macleod et al. 1995; Serrano p21Cip1 is complex. In addition to activation through Roberts 1999). The molecular basis for the induction of provide a logical explanation for the G1 arrest (Sherr and

The main ubiquitin ligase for p53 is the oncogene Mdm2. p19Arf appears to protect p53 from degradation by binding directly to Mdm2 [Pomerantz et al. 1998; Weber et al. 1999].

Although it is clear from in vitro studies that overexpression of Ras leads to the induction of p19Arf, we cannot detect an up-regulation of p19Arf in the pancreas of TGF-α transgenic mice.

Fibroblasts derived from p53−/− mice were able to overcome the G1 arrest mediated by Ras and form transformed foci [Serrano et al. 1997].

In mating experiments we show a dramatically accelerated tumor formation in TGF-α/p53−/− mice resulting in pancreatic cancer development before the age of 120 d. TGF-α transgenic mice, hemizygous for p53-deficiency, display accelerated disease, and almost 100% of these tumors segregate the wild-type p53 allele. Loss of p53 accelerates tumor formation, however, it takes 45 d to develop cancerous lesions in TGF-α transgenic mice null for p53. In the pancreas of these mice, persistent activation of Ras and cyclin D1/Cdk4 is observed shortly after birth. This suggests inactivation of additional genes coding for growth-arrest proteins to be required during tumorigenesis. LOH analysis revealed frequent homozygous losses of the Ink4a/Arf locus in TGF-α/p53−/− mice. The deletion also includes Ink4b. The Ink4a/Arf locus encodes the two related putative tumor suppressors p16Ink4a and p19Arf [Serrano et al. 1996]. p16Ink4a represents a potent tumor suppressor in humans and is frequently inactivated in pancreatic neoplasms [Caldas et al. 1994]. In addition, inherited mutations of exon 1α implicate p16Ink4a in familial tumor susceptibility [Gruis et al. 1995]. However, the final proof of p16Ink4a as a tumor suppressor in mice awaits the specific inactivation of the p16Ink4a gene. The loss-of-function approach strongly suggested that p19Arf is the major tumor suppressor in mice [Serrano et al. 1996; Kamijo et al. 1997]. Deletions of the Ink4a/Arf locus occurred in 6 of 15 tumors in TGF-α/p53−/− and TGF-α/p53−/− mice. This result was unexpected from previous findings, indicating a mutually exclusive manner of p53 and p19Arf inactiva-
tion in c-myc-induced lymphomagenesis [Eischen et al. 1999]. All tumors retained other polyomorphic markers, showing that the effect cannot be simply explained by chromosomal instability. These data therefore indicate that the Ink4a/Arf locus is synergistic to p53 in tumor suppression. Similarly, human pancreatic cancer involves homozygous deletion of the region 9p21-p22, resulting in inactivation of the p14Arf and p15INK4b genes [Naumann et al. 1996]. Similar to human pancreatic cancer, LOH of the SMAD4/DPC4 locus occurred with a lower frequency only in our tumor model [Hahn et al. 1996]. Recent data suggest functional inactivation of the TGF-β-Smad4 pathway in the presence of activated Ras, which might explain a reduced selection pressure for LOH at the Smad4 locus [Kretzschmar et al. 1999]. Furthermore, the Rb1 locus was infrequently affected by LOH. This is consistent with the low number of Rb mutations in human pancreatic cancer.

The tumor spectrum of p53-deficient mice is dominated by lymphomas and sarcomas [Jacks et al. 1994]. This spectrum shifted to ductal pancreatic cancer in crossbred animals. Only 3 out of 90 mice developed pancreatic tumors and sarcomas. APC-mutated [Min] mice null for p53 develop pancreatic acinar cell adenocarcinomas [Clarke et al. 1995]. Our data complement this observation that p53 is critical in the protection of pancreatic epithelial cells by causing cell cycle arrest.

Carcinogenesis is a multistep process; to reach the malignant phenotype, multiple alterations affecting several levels of growth control are required. Here we report a sequence of genetic events in a murine pancreatic cancer model that recapitulates pathomorphological features and genetic alterations of the human disease.

Materials and methods

Transgenic mice

The EL-TGF-α-hGH transgenic mice [line #2261-3] and p53-deficient mice have been described [Sandgren et al. 1990; Jacks et al. 1994]. Crossbreeding and inbreeding of congenic C57BL/6×EL-TGF-α-hGH transgenic and Balb/c×p53-deficient mice resulted in F1, C57BL/6×Balb/c progeny of TGF-α/p53+−/− and in F2 progeny of TGF-α/p53+−/− mice. Mice were sacrificed for analysis at given time points or with obvious signs of wasting disease. The tumor incidence of TGF-α−/− mice was followed up to 360 and 180 d, respectively, and evaluated for tumor indices were calculated as fraction of BrdU-positive cells in relation to the total cell number and expressed as percentage ± SEM. For each time point, tubular complexes of four sections separated by 200 µm of at least three individual animals were evaluated.

Activation assay for Ras, immunocomplex kinase assay, and Western blot

Activation assay for Ras was performed as described recently [Taylor and Shalloway 1996] with a modified, Mg2+-containing lysis buffer [MLB: 25 mM HEPES at pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.05% sodium deoxycholate, 1 mM sodium vanadate, 10 µg/mL aprotinin, 0.5 Μ mol/mL leupeptin]. Cdk4 activity assay was performed as described [Kerkhoff and Rapp 1997].

Quantitative RNA analysis

mRNA expression was evaluated by RT–PCR analysis using Real Time PCR [TaqMan, PE Applied Biosystems] [Gibson et al. 1996]. Reverse transcription of 2µg total RNA was performed in duplicate and further processed independently. PCR reaction (denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min; Sybr Green PCR Core Reagents, PE Applied Biosystems) was performed in triplicate and normalized to the endogenous cyclophilin mRNA level for each reaction. Target cDNA in transgenic mice was quantified using the delta-delta-CT method. Primer sequences are available on request.

Histopathological analysis

Hematoxylin/eosin and immunohistochemical analysis were performed as described previously [Wagner et al. 1998]. Sections were stained with anti-p53 [1:2000, rabbit polyclonal], anti-phospho-Erk1/2 [1:200, New England Biolabs], anti-cyclin D [1:50], anti-cyclin E [1:40], anti-Cdk4 [1:50], anti-PCNA [1:200, all Santa Cruz Biotechnology], anti-cytokeratin 8/18 [1:400, Progen], anti-vimentin [1:40, ICN], and anti-Cdk4-LCA [1:50, Pharmingen]. Double labeling for cytokeratin 19/vimentin and cytokeratin 8/18/Cdk4-LCA was performed overnight at 4°C in PBS/1.0% Triton X-100/2.0% BSA followed by sequential incubation with fluorochrome-labeled anti-goat [Alexa 568]/anti-mouse [Alexa 488] and anti-guinea pig [Alexa 488]/anti-rat [Alexa 568] antibodies and nuclear counterstain with Hoechst dye.

For in vivo BrdU labeling, animals were injected with BrdU (100 mg/kg body weight) intraperitoneally 3 h prior to sacrifice. Immunostaining for BrdU was performed using a monoclonal anti-BrdU antibody [1:400, Boehringer Mannheim]. Labeling indices were calculated as fraction of BrdU-positive cells in relation to the total cell number and expressed as percentage ± SEM. For each time point, tubular complexes of four sections separated by 200 µm of at least three individual animals were evaluated.

Acknowledgments

We thank E. Sandgren for the generous gift of the transgenic mouse line EL-TGF-α-hGH [no. 2261-3] and R.D. Palminter for the hGH poly[A] probe. We thank R. Rittelmann for excellent technical support and S. Aigner for assistance with manuscript preparation. We thank H. Häcker and all members of the labo-
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*Genes Dev.* 2001, 15:

Access the most recent version at doi:10.1101/gad.184701

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