Induction and expression of cyclin D3 in human pancreatic cancer

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Abstract Purpose: Cyclins play a key role in the control and regulation of the cell cycle. The role of cyclins in the pathogenesis of pancreatic cancer is largely unknown. Methods: Using Northern blot analysis, polymerase chain reaction (PCR) and immunohistochemistry, we examined the expression of cyclins D1, D2, and D3 in human pancreatic cancer and studied the induction of these cyclins by growth factors in pancreatic cancer cell lines. Results: We now report that cyclin D1 and D3 mRNAs are expressed in human pancreatic cancer cell lines, and that the expression of cyclin D3 is enhanced in pancreatic cancer cells by amphiregulin, a member of the epidermal growth factor family. Cyclins D1 and D3 are also expressed in normal and malignant pancreatic tissues. However, while the normal pancreas and pancreatic cancers express cyclin D2 as determined by reverse-transcriptase PCR, we could not detect cyclin D2 mRNA by either Northern blot analysis or reverse transcriptase PCR in the two pancreatic cancer cell lines. Immunohistochemical analysis revealed the expression of cyclin D3 in pancreatic cancer cells. Conclusions: These findings suggest that D-type cyclins are differentially expressed in pancreatic cancer and that the aberrant activation of the EGF receptor in human pancreatic cancer by amphiregulin may lead to the progression of the cell cycle via induction of cyclin D3 expression, thus contributing to the growth advantage of these transformed cells.

Key words Cell cycle · Cyclin · Growth factor · Cancer · Pancreas

Introduction

D-type cyclins comprise a family of three members, cyclin D1 being identified as the putative proto-oncogene PRAD1 which is located on chromosome 11q13. As the cell cycle is controlled primarily during the G1 phase, D-type cyclins and their cyclin-dependent kinases are required for the regulation and progression of the cell cycle (Hunter and Pines 1994). Cyclin D1 associates primarily with cyclin-dependent kinases 4 and 6, and thus leads to the phosphorylation of the retinoblastoma protein (Hunter and Pines 1994; Grana and Reddy 1995). Experimental studies involving the selective overexpression of cyclin D1 in transgenic mice resulted in mammary hyperplasia and carcinomas in the lactating mouse (Mueller et al. 1997). Furthermore, cyclin D1 has recently been demonstrated to be overexpressed in a number of malignancies, including pancreatic carcinoma and esophageal cancer (Gansauge et al. 1997; Hinds et al. 1994). Moreover, amplification of CCND1/PRAD1 has been reported in leukemia and breast cancer, while the overexpression of cyclin D1 in human malignancies is associated with advanced disease and poor prognosis (Gansauge et al. 1997; Hinds et al. 1994). Based on independent analysis, several groups have reported that the expression of D-type cyclins is highly growth-factor-dependent, e.g., in human fibroblasts cyclin D1 mRNA levels are induced by platelet-derived growth factor (Surmaez et al. 1992). The second member of the family of D-type cyclins is cyclin D2 which is encoded by the CCND2 gene and which is located on chromosome 12p13 (Hunter and Pines 1994; Grana and Reddy 1995). In a small series of colorectal cancers,
amplification of cyclin D2 has been reported (Leach et al. 1993), and amplification of the cyclin D3 gene has been reported in a glioblastoma cell line (Hunter and Pines 1994; Wang et al. 1995; Kuchiki et al. 2000).

Pancreatic carcinoma is a devastating disease with poor prognosis (Warshaw and Fernandez-Del Castillo 1992). Recently, a number of genetic and molecular alterations have been reported, such as K-ras gene mutation, deletion of DPC-4 and p53 gene mutation (Hahn et al. 1996; Berrozpe et al. 1994). In addition, human pancreatic carcinomas overexpress a number of growth factors, e.g., epidermal growth factor and amphiregulin of the EGF family and platelet-derived growth factor of the PDGF family (Ebert et al. 1994, 1995; Yokoyama et al. 1995). While the K-ras gene mutation is considered to be an early event in pancreatic carcinogenesis, its presence is not associated with poor prognosis (Berrozpe et al. 1994). In contrast, the overexpression of growth factors is often correlated with poor prognosis, pointing to a role of growth factor-dependent autocrine and paracrine loops in the pathogenesis of this disease (Ebert et al. 1994, 1995; Yokoyama et al. 1995). However, while amphiregulin and transforming growth factor-alpha, which are aberrantly expressed in human pancreatic ductal carcinomas, both stimulate the growth of pancreatic cancer cells in vitro (Ebert et al. 1994; Yokoyama et al. 1995), the overexpression of platelet-derived growth factors in pancreatic carcinoma is not associated with the stimulation of pancreatic cancer cell growth by PDGF in vitro (Ebert et al. 1995). In an effort to assess the expression of cyclins D1, D2, and D3 in pancreatic cancer and to further elucidate the pathways which are associated with amphiregulin-dependent growth stimulation of pancreatic cancer cells in vitro, we analyzed the expression and induction of D-type cyclins by growth factors in pancreatic cancer. We now report that D-type cyclins are differentially expressed in pancreatic carcinoma and that cyclin D3 mRNA levels are increased by amphiregulin in a human pancreatic cancer cell line.

Materials and methods

Fetal bovine serum (FBS), trypsin EDTA solution, and penicillin-streptomycin solution were from Sigma Chemicals (Deisenhofen, Germany); Dulbecco’s minimal essential medium (DMEM) and RPMI 1640 medium were from Gibco-BRL (Eggenstein, Germany); Hybond membranes were from Amersham (Braunschweig, Germany); PGE1/2F vector was from Promega Biotech (Mannheim, Germany); reverse transcriptase was from Boehringer Mannheim (Mannheim, Germany); Taq polymerase was from Gibco-BRL; [alpha-32P]dCTP (3000 Ci/mmol) was from Amersham; human recombinant amphiregulin and platelet-derived growth factor were from R&D Systems (Wiesbaden, Germany); oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). All other materials were obtained from Sigma Chemicals and were of molecular biology grade.

Cell culture

CAPAN-1, CAPAN-2, and PANC-1 human pancreatic cancer cells were obtained from the American Type Culture Collection (Rockville, Md., USA). CAPAN-1 cells were grown in RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin, and 1% glutamine. CAPAN-2 and PANC-1 cells were grown in DMEM containing 10% FBS, 1% penicillin-streptomycin, and 1% glutamine. Agonists were added after culturing the cells in serum-free media, containing 0.1% BSA, insulin, and transferrin (5 μg/ml), and antibiotics for 48 h (Ebert et al. 1994).

Tissue samples

Pancreatic cancer tissues (six female, four male) were obtained from patients undergoing pancreatic surgery. Normal pancreatic tissues were obtained from five individuals (two female) through an organ donor program. The median age of the patients with pancreatic cancer was 61.5 years (range: 48–73). The median age of the organ donors was 36 years (range: 25–54). Immediately after surgical removal, tissue samples were frozen in liquid nitrogen. The tumor samples were classified as pancreatic ductal adenocarcinomas according to the TNM classification for pancreatic tumors (Warshaw and Fernandez-Del Castillo 1992).

PCR and Northern blot analysis

Oligonucleotide primers were purchased from MWG-Biotech. Primer sequences were adapted according to Toledo et al. (Toledo et al. 1995). Repeated RT-PCR analysis revealed a PCR product of the expected size. Primer sequences were (Toledo et al. 1995): cyclin D1: 5'-AGGAAAGAACAAG ATCA-3', 5'-GTGTGTGAAGG-ACAGGAT-3'; cyclin D2: 5'-TCATGACTTCATTGAGCA-3', 5'-GTCGTCTCTCTCTCC-3'; cyclin D3: 5'-ACATGATTCCC TGCCCTT-3', 5'-ACAGGCCCCCTACTCGAGT-3'. cDNAs were synthesized from total RNA (2 μg/sample) isolated from human pancreatic cancers (n = 4), normal pancreatic tissues (n = 5), and pancreatic cancer cell lines (n = 2), using oligo(dT) and reverse transcriptase. Following inactivation, 2 μl of the reaction mixture were incubated in buffer containing 1.23 mM concentrations each of dATP, dCTP, dGTP, and dTTP, 600 nM each of oligonucleotide primers, and 10% dimethyl-sulfoxide in buffer consisting of 16.6 mM [NH4]2SO4, 67 mM Tris-HCl (pH 8.0), 6.7 mM MgCl2, 10 mM β-mercaptoethanol, and Taq polymerase (Ebert et al. 1994). Reaction cycles consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C were repeated 35 times. The PCR products were size fractionated on 1% agarose gels and visualized by ethidium bromide (Ebert et al. 1994). A 1.4-kb EcoRI fragment of the human cyclin D1 cDNA, a 550-bp PstI fragment of the human cyclin D3 cDNA, which were kindly provided by Dr. Beach (Cold Spring Harbour Laboratory, New York), and a 190-bp BanHI fragment of the mouse 7S cDNA that cross hybridizes with human 7S RNA were randomly labeled with [alpha-32P]dCTP (Ebert et al. 1994). Total RNA was extracted from both cancer cell lines and human pancreatic tissues and blotted onto nylon membranes. The blots were prehybridized, hybridized, and washed under high stringency conditions for a cDNA probe, as previously described (Ebert et al. 1994). The blots were exposed at ~80°C to Kodak XAR-5 film with Kodak intensifying screens, and the intensity of the radiographic bands was determined by laser densitometry (Ultrascan XL; Pharmacia LKB Biotechnology, Uppsala, Sweden).

Immunohistochemistry

The presence of human cyclin D3 was assessed using paraffin-embedded tissue sections obtained from ten patients with pancreatic cancer undergoing pancreatic surgery. The human tissues were fixed in Bouin’s solution and paraffin embedded. Two cyclin D3 antibodies were used: the anti-cyclin D3 antibody (H-292) is an affinity-purified rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1–292 representing full-length cyclin D3 of human origin and was used at a 1:100 dilution (Santa Cruz, Calif., USA). The specificity of the antibody was confirmed by Western blotting, immunoprecipitation, and