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ARTICLE

Epidermal Growth Factor Receptor, Somatostatin and Bcl-2 in Human Pancreatic Tumor Xenografts

An Immunohistochemical Study

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Xenografted human pancreatic tumors (5 ductal adenocarcinomas, 1 leiomyosarcoma, altogether 26 samples) were investigated about their immunohistochemical expression of epidermal growth factor receptor (EGFR), somatostatin (SS) and bcl-2 protein. The expression of the EGFR varied from tumor to tumor. One originally negative carcinoma became immunoreactive during passagings, one tumor has lost its early positive expression, and in 3 cancer lines a phenotypically constant pattern was seen. SS immunoreactivity was practically absent in all tumor samples. Concerning bcl-2 expression, different staining patterns were observed among the carcinomas, but the leiomyosarcoma has retained its strong positivity during xenograftings. In the PZX-5 carcinoma line that was originally negative, the one-month Sandostatin treatment induced the strong expression of bcl-2 protein suggesting a development of an acquired resistance against programmed cell death in this tumor. (Pathology Oncology Research Vol 5, No 2, 146–151, 1999)

Keywords: pancreas tumor, xenogaft, somatostatin, bcl-2

Introduction

Epidermal growth factor (EGF) is recognized as a growth-stimulatory factor for pancreatic carcinoma.[4,20,28,30] In BOP-induced pancreatic carcinogenesis experiments in hamsters it exhibited a cocarcinogenic effect,[4] and the proliferation of different human pancreatic cancer cell lines has also shown to be stimulated by EGF.[7,20,30] In pigs EGF induced hyperplastic lesions in the large interlobular ducts, accompanied by increased number of PCNA-positive nuclei.[31] EGF acts through its receptor (EGFR), a 170 kD transmembrane glycoprotein. In normal human pancreas a weak immunoreactivity of EGFR was found only in the endocrine part of the organ, while the ductal system was free of EGFR. This pattern, however, changed in various diseases. In chronic pancreatitis, for example, both the centroacinar cells and the large ducts displayed strong cytoplasmic reaction.[10] Similarly, over 95% of ductal adenocarcinomas showed positivity, but there was no apparent relationship to tumor grade or histologic type.[10]

Somatostatin (SS) is found in the D cells of the Langerhans islets, but scattered SS-positive cells are interspersed among the ductal cells, as well. In the past decade it has become obvious that the pancreatic carcinomas of non-endocrine origin could also respond to hormonal manipulations. Synthetic analogs of SS may inhibit the growth of experimentally induced tumors[25,40] and xenografted pancreatic carcinomas.[16,18,29] The administration of a SS-analog Sandostatin (octreotide) has also been supported by a number of experiments.[17,26,35] In clinical studies prolonged survival could be achieved with combined treatment of octreotide and tamoxifen.[21] Moreover, somatostatin receptor mRNAs have recently been demonstrated in human pancreatic cancer samples.[5]

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Abbreviations: EGFR = epidermal growth factor receptor; SS = somatostatin; PBS = phosphate buffered saline; DAB = 3,3' diaminobenzidine; BOP = N-Nitrosobis(2-oxopropyl)amine

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Bcl-2 protein, a 25 kD product of the bcl-2 gene is known as an inhibitor of the programmed cell death (apoptosis) and it is expressed in various malignant tumors.[9,14,22,24,36,37] On pancreatic malignancies, however, only few published data are available. Wang et al.[34] reported 45% of immunostaining positivity in pancreatic neuroendocrine tumors. In ductal adenocarcinomas bcl-2 positivity ranged from 20-55%, but the correlation of these data with the prognosis is still unclear.[11,15,23]

Expression of the EGFR, somatostatin and bcl-2 have never been studied in human pancreatic cancer xenografts before. Our aim was to assess the immunoreactivity of these antigens in the early and late passages of our newly established human pancreatic tumor xenografts grown in artificially immunosuppressed mice.

Materials and Methods

Pancreatic tumor xenografts

Five human pancreatic ductal adenocarcinomas (PZX-2, PZX/5, PZX-11, PZX-16, PZX-20) and 1 pancreatic leiomyosarcoma line (PZX-7) have been established. Their detailed characteristics have been published elsewhere.[3,38,39] Briefly, human pancreatic tumors or pancreatic tumor metastases were inoculated subcutaneously into immunosuppressed CBA/CA mice and after taking the tumor fragments were further transplanted. These successfully established tumor lines have been maintained for 18 to 32 months. For immunohistochemical studies the archived original tumor and samples from the late passages have been used.

Sandostatin-treatment

In the 4th passage of PZX-5 tumors a hormonal treatment (octreotide acetate, Sandostatin; NOVARTIS) was performed. The tumor-bearing animals have been treated with 2x500 μg/kg body weight Sandostatin given intraperitoneally for 1 month.

Immunohistochemistry

The 5-μm-thick sections from formalin-fixed, paraplast-embedded materials were deparaffinised, e hydrated and endogenous peroxidase was blocked by 3% H_2O_2 for 10 min. After rinsing with PBS, primary antibodies raised against the following antigens were applied (all antibodies were from BioGenex): epidermal growth factor receptor (polyclonal, epitope specific, Cat. No. AR335-5R) for 2 hours; somatostatin (polyclonal, Cat. No. AR042-5R) for 30 min and bcl-2 protein (monoclonal, Cat. No. AM287-5M) for 30 min. Before applying bcl-2 primary a 5 min microwave antigen retrieval procedure was performed (Meditest MFX 800-2) in 0.01 M citrate buffer (pH 6.0). As a detection system a Super Sensitive MultiLink kit was used utilizing biotin-streptavidin (B-SA) formula and DAB as a chromogen. For positive controls normal pancreas (SS), breast cancer (EGFR) and normal tonsil (bcl-2) were used.

Intensity of the reactions was scored at a semiquantitative scale: – no reaction, ± vague reaction, + mild reaction, ++ moderately strong reaction, +++ strong reaction.

Results

The degree of differentiation and the immunohistochemical results are summarized in *Table 1*.

The various adenocarcinomas displayed different EGFR patterns. In the PZX-2 the original tumor proved to be negative, but after the 5th passage the majority of the tumor cells became positive. Conversely, in the PZX-11 tumors the originally strong positivity *(Figure 1.)* gradually disappeared. In two tumor lines, namely PZX-16 and PZX-20 the early and the late transplants exhibited an unaltered pattern: complete negativity in the former, and retained positivity in the latter *(Figure 3.)*. As it was expected, the leiomyosarcoma (PZX-7) did not express any immunohistochemical positivity for EGFR. All the samples of the PZX-5 line expressed strong EGFR positivity (varying between 75 to 95% of the cells), and this pattern has remained unchanged after a 1-month treatment with the SS-analog (*Figs. 5,6*).

Somatostatin immunoreactivity was practically absent in all tumor samples studied both in the early and late passages, just occasionally some vague positive cells were present in individual samples. No alterations were seen after the Sandostatin treatment.

Like the EGFR immunoreactivity, the bcl-2 staining pattern has varied from tumor to tumor. The PZX-2 line was completely negative, while in the PZX-16 and PZX-20 lines both the early and the late xenografts exhibited a positive reaction. The originally positive PZX-11 tumor *(Figure 2.)* has lost this staining property during passaging the tumor samples from the 3rd and 9th generations turned out to be completely negative. The PZX-7 leiomyosarcoma has retained its strong positivity during passagings (*Figure 4.*).

An interesting phenomenon was observed in the PZX-5 tumor. The original and the control untreated xenograft tumors from the 4th generation were negative for bcl-2. After 1-month of Sandostatin treatment, however, all but one xenografted tumors expressed strong positivity in the majority (70%) of the carcinoma cells (*Figs. 7,8*). This suggests that bcl-2 protein expression could be induced by hormonal treatment. On the other hand, this immunohistochemical expression was unrelated to the effect of the hormone: both the regressive and the unresponsive tumors exhibited positive staining.

There was no clear-cut relationship between differentiation and expression of EGFR or bcl-2 protein. In the poorly differentiated carcinomas EGFR immunoreactivity was usually absent, but similar findings were also found in well differentiated tumors (PZX-2/0, PZX-16). For bcl-2, negative immunostaining was equally observed both in well differentiated (PZX-2) and moderately differentiated tumors (PZX-5/controls), while it was expressed in a Grade III carcinoma (PZX-16/7).

Discussion

During the last decades numerous molecular alterations have been described in pancreatic carcinomas, but their stability throughout the successive transplantations has not been investigated. In humans, overexpression of different molecules (EGF, EGFR, p53, cyclin-D1 or Ki-67) was found in 44 to 69% of cases, but their prognostic significance is low.[6]

EGFR is present in a wide range of normal and malignant tissues. In our study its expression varied from xenograft to xenograft, and there were no unequivocal changes in the early and late passages. Although Lemoine et al.[10] investigating 84 cases of human pancreatic carcinomas found a 95% immunohistochemical positivity in malignant cells, other reports claimed about 43% of expression.[28] Primary tumors and metastatic foci exhibited practically the same immunostaining (43 and 46%, respectively).[28] This kind of expression seems to be neither of prognostic nor of differential diagnostic value, because EGFR immunoreactivity is also strong in chronic pancreatitis.[10] Since the normal pancreatic duct are negative for EGFR, its expression in disease may be a secondary, nonspecific phenomenon. According to our data, the 1-month Sandostatin treatment did not influence the high receptor expression in the tumor samples.

As for the bcl-2 antiapoptotic protein expression, positive and negative xenografts were found in our series, and with exception of one tumor line (PZX-11), the staining pattern remained unchanged during passaging. The PZX-11 line, however, has lost its strong immunohistochemical positivity after the early transplantations. The most important finding appeared in PZX-5 tumors. The controls did not express bcl-2 protein, but after Sandostatin treatment 6 of 7 tumors

Table 1. **Summary of the immunohistochemical results**

Code		*Grade*	*EGFR*	*SS*	*bcl-2*
PZX–2/0		I	–	–	–
2/5		I	+ 70%	–	–
2/10		I	±	–	–
PZX–7/0		III	–	–	++ 80%
7/2		III	–	–	+ 60%
7/4		III	–	–	++ 70%
7/5		III	?	–	+++ 95%
PZX–11/0		I	++ 60%	–	++ 70%
11/3		III	±	–	–
11/9		III	–	–	–
PZX–16/3		I	–	–	+
16/7		III	–	–	++ 80%
PZX–20/0		I	++ 75%	–	+ 90%
20/5		I	+ 40%	–	+ 30%
PZX–5/1	I	II	+ 75%	–	–
5/4	KS	II	++ 75%	–	–
5/4	K6	II	+++ 95%	±	–
5/4	K8	II	+ 80%	+	–
5/4	K9	II	aspec.	–	+
PZX–5/4	SS1	II	++ 95%	–	–
5/4	SS3	II	++ 80%	±	+ 60%
5/4	SS5	II	+ 70%	–	+ 50%
5/4	SS7	II	++ 90%	–	+++ 80%
5/4	SS8	II	+++ 70%	±	+++ 80%
5/4	SS9	II	++ 70%	–	++ 85%
5/4	SS10	II	+ 85%	–	++ 70%

became strongly immunoreactive, the proportion of the positive cells ranged between 50% to 85%. This means that the hormonal treatment *induced* bcl-2 expression in an originally negative, non-endocrine pancreatic carcinoma. This result seems to be interesting both theoretically and practically.

In the literature there is a plethora of reports dealing with bcl-2 in various malignant tumors including small cell lung cancer,[24,37] soft tissue sarcomas,[14] endometrial carcinoma,[9,22] neuroblastoma,[13] and ovarian cancer,[36] but there are only a few articles on non-endocrine pancreatic

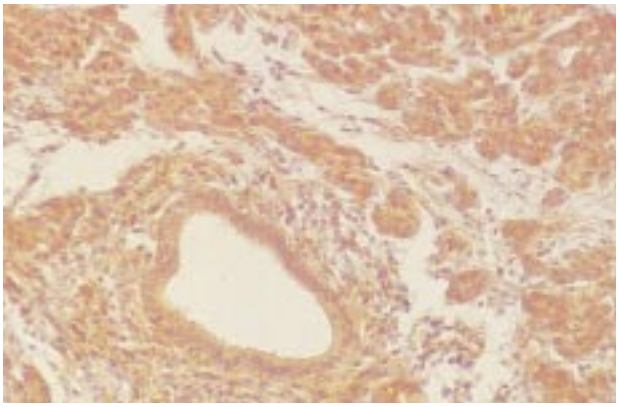

Figure 1. *PZX-11, original tumor. Strong epidermal growth factor receptor (EGFR) positivity is shown in the cytoplasm of the tumor cells. (x 100)*

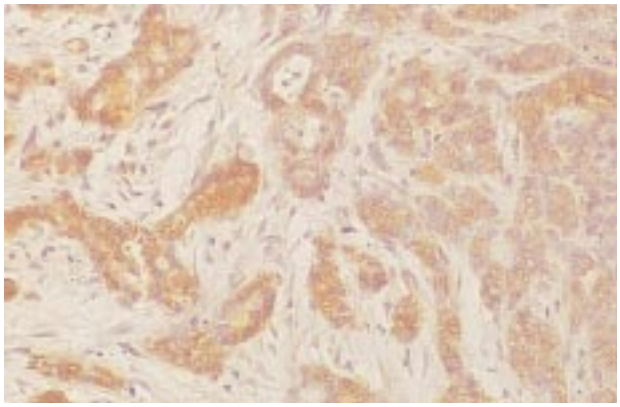

Figure 2. *PZX-11, original tumor. The tumor cells express strong bcl-2 immunoreactivity (x 100)*

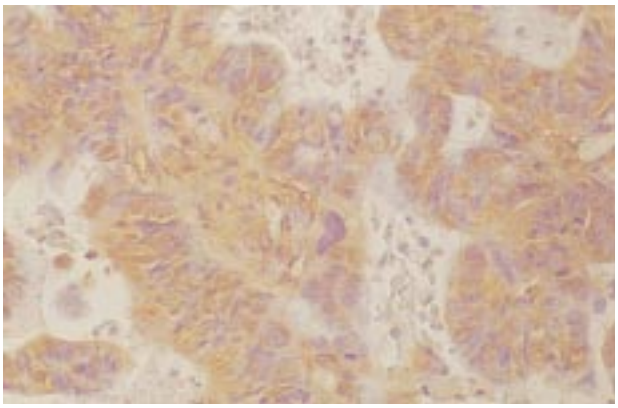

Figure 3. *EGFR positivity in the original tumor of the PZX-20 line (x 400)*

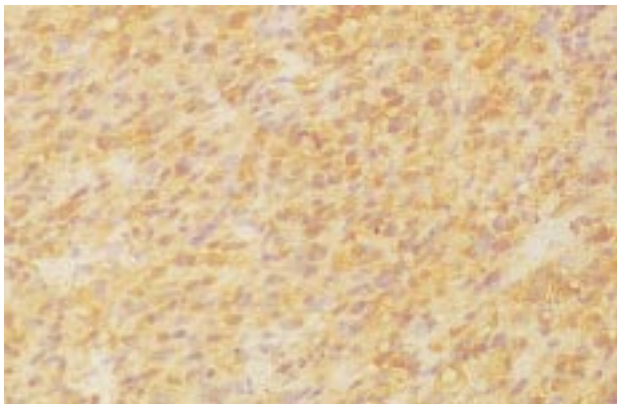

Figure 4. *Pancreatic leiomyosarcoma xenograft (PZX-7), 5th passage. The tumor cells express strong bcl-2 positivity. (x 400)*

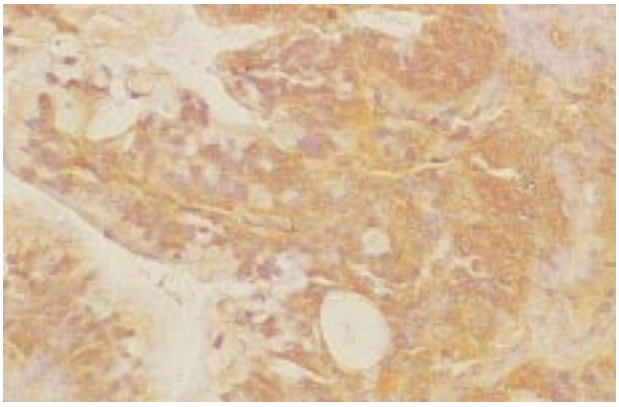

Figure 5. *PZX-5 tumor line, 4th passage. The tumor cells are strongly positive for EGFR (x 400)*

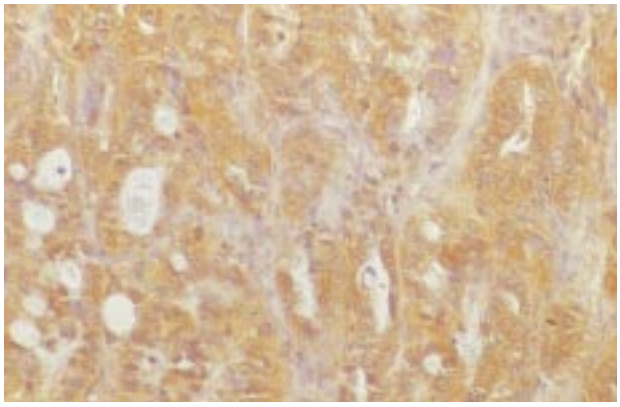

Figure 6. *PZX-5 tumor line, 5th passage, after Sandostatin treatment. There is no change in the strong EGFR positivity (x 200)*

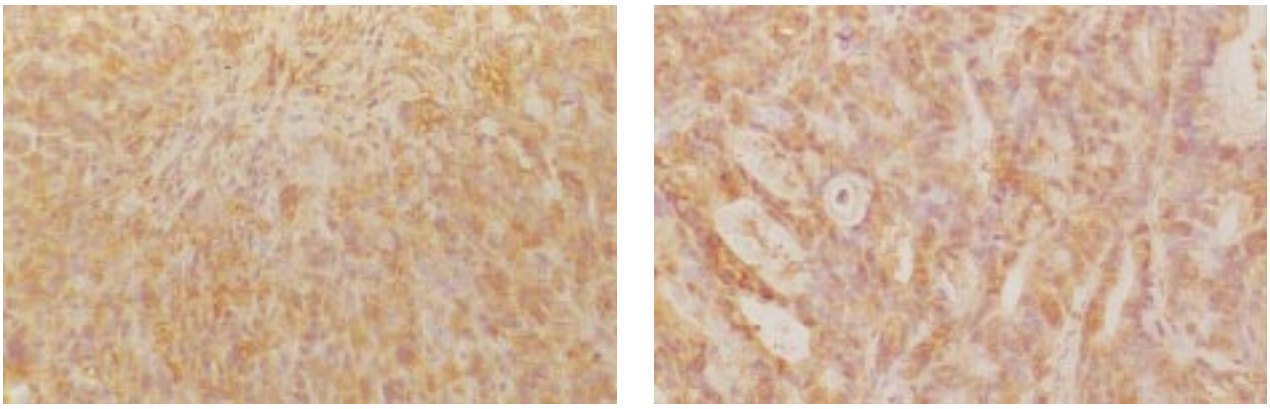

Figure 7–8. *PZX-5 tumor line, 5th passage, after Sandostatin treatment. The majority of the tumor cells express bcl-2 positivity that was not present in the untreated controls (x 200)*

carcinoma.[11,15,23] In normal human pancreatic tissue bcl-2 positive cells are present in moderate number usually randomly distributed among the acinar and islet cells, and the ductal cells exhibit rare or no positivity.[1,8] Human pancreatic cancer samples expressed 20–55% bcl-2 positivity,[11,15,23] but the prognostic impact of bcl-2 is still not clear. Ohshio[15] could not observe any survival benefit among patients with negative immunostaining, but, Mäkinen[11] and Sinicrope[23] have found that the tumors with bcl-2 overexpression are less aggressive, especially the well differentiated carcinomas.

The growth of a malignant tumor is essentially determined by the balance between the cell loss and cell proliferation. Many proapoptotic and antiapoptotic mechanisms participate in these processes. One protein (e.g. bcl-2) can hardly be marker alone, since other proteins are able to modify its effect.[19]

Another reason that makes the interpretation of bcl-2 immunoexpression difficult is that it could be induced by a number of external factors. In rats the ligation of the pancreatic duct results in a significantly increased bcl-2 expression in the ductal cells when the acinar cells are gradually vanishing.[32] Conversely, in human pancreatic cancer cell lines its expression was down-regulated after treatment with an exogenous cdk inhibitor (butyrolactone).[33] Many well documented experiments clearly demonstrated that the bcl-2 expression was also profoundly altered under hormonal effects. Uterine leiomyoma cells exhibited different expression in the different phases of menstrual cycle: progesterone caused an increase, while estrogen resulted in a decrease.[12] Teixeira et al. have reported that estrogen administration to an estrogen-receptor positive human breast cancer cells (MCF-7) markedly induced the presence of the 8.5 kb bcl-2 mRNA transcripts and this finding was accompanied by an increased resistance against adriamycin.[27] Similarly, in a hormone dependent human prostatic cancer cell line an increased expression of bcl-2 protein was observed after androgen treatment.[2] To our knowledge, however, the observation that a somatostatin analog can induce bcl-2 positivity in an originally bcl-2 negative pancreatic carcinoma has not been noticed earlier.

Although our finding is theoretically interesting, there are some questions to be answered. First, it reinforces again the earlier observations that human pancreatic cancer is *responsive* to hormonal manipulations. Secondly, in addition to steroid hormones, bcl-2 expression could also be induced by a somatostatin analog, a polypeptide. Thirdly, this expression of the bcl-2 may imply a "defense" of the pancreatic cancer cells against the "noxious" effects rendering the tumor cells more resistant to the hormonal treatment.

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