Tumour Suppressor Protein (p53), Apoptosis Inhibiting Protein (Bcl-2) and Proliferating Cell Nuclear Antigen (PCNA) Expressions in a Rat Pancreatic Tumour Model

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Abstract. Background: Previous studies have shown that, cultured rat pancreatic carcinoma cells, derived from azaserine-induced acinar tumours, yield tumours with a ductal phenotype. Materials and Methods: In order to find out the molecular characteristics of this tumour model, tumour suppressor protein (p53), apoptosis inhibiting protein (Bcl-2) and proliferating cell nuclear antigen (PCNA) expressions were analysed in rat pancreatic and subcutaneous tumours, as well as in normal rat pancreas. Results: Immunoreactivity for p53 protein was found in 86% of intrapancreatic tumours and in 100% of subcutaneous tumours. The average fraction of positive carcinoma cells was over 50%. Normal rat pancreas showed only slight positive or negative staining for p53. Bcl-2 did not show positive immunoreactivity in rat tumour samples. For PCNA all tumour samples showed positive staining. Also normal pancreas of 6-week-old animals were clearly positive, whereas the samples of the older animals were only slightly positive. Conclusion: Possible mutations in the p53 tumour suppressor gene and a strong expression of PCNA were shown in carcinoma cell line-induced rat pancreatic tumours. These features of the rat pancreatic tumour model resemble human pancreatic carcinoma and may favour the use of this model in pancreatic cancer studies.

Deletions and point mutations in the tumour suppressor gene coding for p53 protein appear to be one of the most common abnormalities demonstrated in human cancer (1, 2). Previous studies have also shown mutated p53 expressions in different pancreatic cancer cell lines, as well as in pancreatic tumours and their metastases (3-6).

Nitrosamine-induced hamster and azaserine-induced rat experimental pancreatic carcinomain models are the most widely used rodent models in the studies concerning pancreatic cancer (7-10). The pancreatic carcinomas in hamsters are considered as most similar to human ductal adenocarcinomas in regard to the phenotype of the tumours and the molecular similarities (11-13). Both p53 tumour suppressor gene and Ki-ras oncogene mutations have been found in hamster pancreatic carcinoma, a finding not identified in rat models (12, 13). Cultured rat pancreatic carcinoma cells (DSL-6A/C1), originally derived from azaserine-induced acinar tumours, yield pancreatic tumours with a ductal phenotype. This tumour model has been well documented (14-16) and we have previously demonstrated that these tumours showed intense staining for cytokeratins (15). To investigate possible molecular similarities between the rat tumour model and human pancreatic cancer, the expressions of mouse specific p53 (CM5) protein, human apoptosis inhibiting protein (Bcl-2) and proliferating cell nuclear antigen (PCNA) were analysed in experimental pancreatic and subcutaneous tumours, as well as in normal rat pancreas.

Materials and Methods

Tumour cell line and transplantation. The tumour cell line DSL-6A/C1 (ATCC CRL-2132) was obtained from an acinar cell carcinoma (DSL-6) of a rat pancreas (14). Cell culture, inoculation technique, the establishment and characteristics of subcutaneously as well as intrapancreatically transplanted tumours have been reported previously (14-16).

Animals and tissue samples. Male Lewis rats were obtained from Charles River Laboratories (Sulzfeld, Germany). For intrapancreatic as well as subcutaneous carcinoma cell transplantation, 5-week-old rats (±1 week) with initial weights of about 100 g were used. Normal pancreatic samples were collected at ages between six and 24 weeks. The animals were kept under standard laboratory conditions and fed a chow diet. All animal procedures were approved by the Animal Care and Use Committee of the University of Kuopio, Finland.
At autopsy, pancreatic tumours (n=14), subcutaneous tumours (n=10), as well as normal pancreatic tissue (n=10), were removed. Samples were fixed in 10% buffered formalin, embedded in paraffin and sectioned for conventional microscopic examination and for immunohistochemical studies.

**Immunohistochemistry.** For immunohistochemical demonstration a polyclonal rabbit p53 protein (mouse specific) antibody (Novocastra Laboratories, UK), a monoclonal mouse anti-human bcl-2 oncoprotein (Dako, Golstrup, Denmark) and a monoclonal mouse anti-proliferating cell nuclear antigen PC-10 (Dako) were used. Formalin-fixed, paraffin-embedded specimens were cut, 5 μm sections were deparaffinized, rehydrated and washed for 5 min with phosphate-buffered saline (PBS). The sections were rinsed in distilled water and heated in a microwave oven for 2x5 min in 0.01 M citrate buffer (pH 6.0) and then the slides were rinsed in Tris-buffered saline (pH 7.4). Endogenous peroxidase was blocked by incubation for 5 min with 5 per cent hydrogen peroxide. After treatment with the blocking goat serum (to reduce any non-specific binding of the conjugated second antibody) the samples were incubated overnight at +4°C with primary antibodies. Dilution for p53 was 1:1250, bcl-2 1:400 and for proliferating cell nuclear antigen (PCNA) 1:90 in PBS (pH 7.2) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide. After washes with PBS (pH 7.2), a secondary antibody in PBS was added followed by incubation for 30 min at room temperature. Slides were washed twice with PBS and incubated in a pre-formed avidin-biotinylated peroxidase complex (ABC, Vecastatin Elite kit) (Vector, CA, USA) for 40 min. The reaction products were visualized by immersing the slides in diaminobenzidine tetrahydrochloride (DAB) (Sigma, Paisley, UK) and finally counterstained with Mayer’s haematoxylin. For p53 demonstration the microwave retrieval method was used for 5x5 min. Both negative (samples processed without primary antibody) and positive controls were demonstrated to be negative or positive for each antigen and when scoring the expressions of each antigen the results were compared with both control samples.

**Scoring of slides.** The staining was assessed using objective magnifications 4x and 40x. The antigen expression in adenocarcinoma cells was scored using arbitrary units 0, 1, 2 and 3 in order of increasing intensity (0 = no staining, 1 = weak or slight staining, 2 = intermediate staining and 3 = intense or heavy staining). As a second parameter the fraction of nuclei and/or cytoplasm positive for the examined proteins was analysed by averaging the fraction of these cells throughout the samples.

**Results**

Positive immunostaining for p53 was found in 12 out of the 14 samples of intrapancreatic tumours. Staining was always intermediate or weak and concentrated in the carcinoma cell cytoplasm and nuclei (Figure 1). The fraction of positive cells was over 10%. The size of the tumours had no influence on staining intensity. In three samples the cytoplasm of desmoplastic cells was slightly positive. Also the cytoplasm of the islets of Langerhans as well as intraluminal contents showed slight positive staining. Four samples showed positive staining in the peritumoral area. Normal acinar and ductal cells in the samples were negative, as well as inflammatory cells in the peritumoral area. Subcutaneously induced tumours showed the same staining intensity and distribution as the pancreatic tumours, but the fraction of positively stained cells was larger (>50%). The normal rat pancreas was negative for p53 staining although in some samples the cytoplasm of islets of Langerhans, as well as intraluminal contents were slightly positive.

Bcl-2 expression was not found in pancreatic tumours, subcutaneous tumours or normal rat pancreas.

PCNA positivity was found in all experimental tumours (Figure 2). Nuclear staining was intermediate or intense and the number of positive stained carcinoma cell nuclei was over 90% in 50% of the samples. Cytoplasmic staining was found in 50% of the samples and the intensity was usually weak. The desmoplastic stroma as well the vascular cells were negative. Frequently the normal acinar and islet cell nuclei were positive, and also some ductal cells were found to be positive. The normal pancreata of six week old animals showed positive staining in the ductal, acinar and islet cell nuclei. Number of positive cells was 10% and the staining intensity was intermediate. However normal pancreata of older animals (>12 weeks) showed less intense staining and the number of positively stained cells was lower.

**Discussion**

The results from the present study showed that p53 protein expression was positive in rat pancreatic and subcutaneous tumours. Also intensive staining for PCNA was found in tumour samples, as well as in normal pancreas of younger animals. Bcl-2 expression was negative in all rat samples.

A transplantable tumour model designated as DSL-6 was derived in 1986 from an azaserine-induced primary acinar carcinoma of the Lewis rat pancreas (14). DSL-6A/C1 is a pancreatic carcinoma cell line, derived from this primary acinar tumour. The cells are tumorigenic in Lewis rats and produce duct-like tumours when inoculated subcutaneously (14) and intrapancreatically (15). Immunohistochemical studies of the cell line and the re-grafted tumours have demonstrated expression of several ductal markers, including cytokeratin 19 (14, 15). Interestingly, the cultured cells initially produced amylase, but the production of exocrine enzymes ceased after 1-2 weeks in culture. That is to say, that this cultured cancer cell line yield tumours with a ductal phenotype and has lost its structural, as well as immunohistochemical acinar cell markers, at least partially, while acquiring duct cell markers during culture and regrafting.

The relevance of the animal model to the human is always a question. Therefore comparative studies between human and experimental tumour models are necessary in elucidating differences and similarities between them. Although the cause of pancreatic cancer is unknown, a series of molecular changes are known to be associated with it, such as frequent mutations in the Ki-ras oncogene and p53 tumour suppressor gene (17, 18). Among experimental pancreatic cancer models, a
Figure 1. Intermediate staining intensity for p53 in intrapancreatic tumour sample taken 6 weeks after tumour cell inoculation. Scale bar = 200 μm.

Figure 2. Intermediate and intense staining for PCNA in subcutaneously induced tumour taken 12 weeks after tumour cell inoculation. Scale bar = 200 μm.
nitrosamine-induced hamster model shows morphological, immunophenotypic and molecular (Ki-ras and p53 mutations) similarities with human disease. The results presented in this study showed mutated p53 expression also in a rat tumour model.

PCNA is a marker of cell proliferation. It is necessary for DNA synthesis and it also participates in DNA repair (18). Interestingly, PCNA immunoreactivity has also been demonstrated without cell proliferation in association with neoplasia (19). This may be mediated by growth factors. In human pancreatic cancer the PCNA labelling index increased with histologically malignant grading and pathological stage, but did not correlate with lymph-node status (20). According to our study, all rat tumour samples showed intermediate or intense staining for PCNA. In normal pancreatic tissue, PCNA expression was stronger in younger animals. This finding may reflect true organ cell proliferation, as well as organ growth in growing animals.

The Bcl-2 protein has a role in the inhibition of apoptosis and its overexpression is found in human follicular lymphoma (21). In human pancreatic cancer, bcl-2 protein was expressed in 53% of samples (6). However, bcl-2 was negative in all of our rat samples. The reason may be that the antibody did not recognise this particular epitope in rat samples.

Our results have shown mutations in the p53 tumour suppressor gene and strong expression of proliferating cell nuclear antigens in carcinoma cell line-induced rat pancreatic tumours. These features of the rat pancreatic tumour model resemble pancreatic carcinoma seen in humans and may favour the use of this carcinoma model in pancreatic cancer studies.

References


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