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Vinblastine and 5-fluorouracil sensitivity of xenografts of four pancreatic ductal adenocarcinomas: is there a correlation with histological and cytological tumour differentiation?

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Abstract In a search for nuclear parameters which may predict chemosensitivity of ductal adenocarcinoma of the pancreas, the growth of four xenografted pancreatic carcinomas in response to chemotherapeutic agents was correlated with histological and cytological features of tumour differentiation. Histologically, the tumours were classified according to their ability to form glands into poorly (PaTu-2, PaTu-3), moderately (Panc-1) and well differentiated (PaTu-39) ductal adenocarcinomas. Cytologically, similar segregation of tumours was possible using the 'nuclear form factor', which was one of four nuclear parameters analysed by image cytometry on Feulgen stained tumour imprints. Histological and cytological differentiation correlated closely with tumour growth. One week after a single intraperitoneal injection of either vinblastine or 5-fluorouracil, both drugs inhibited the growth of PaTu-2 and PaTu-3 significantly. The growth of Panc-1 was only affected by vinblastine, while neither drug had an effect on PaTu-39. The results suggest that the response of pancreatic ductal adenocarcinoma to chemotherapeutic drugs may be, to some extent, predicted by histological and cytological differentiation features. However, within these lines, each tumour may show a specific response pattern.

Key words Pancreas carcinoma · Vinblastine · 5-Fluorouracil · Chemosensitivity · Nude mouse tumour

Introduction

Ductal adenocarcinoma of the pancreas is a common tumour and one of those neoplasms with the worst prognosis. At the time of diagnosis only 5–22% of patients have resectable tumours [20], usually in the head of the pancreas. However, even in these patients the 5-year survival rate is low and has not improved very much during recent years [9]. There is therefore a need for alternative of adjuvant treatment modalities such as radiotherapy and/or chemotherapy. It is well-known that the response rate of chemotherapy of pancreatic cancer is low [20]. As a consequence, a considerable percentage of pancreatic cancer patients receiving chemotherapy may suffer from the side effects of treatment without having any benefit. Limiting chemotherapy to that subset of patients who are most likely will benefit from it would therefore be of great help but predictive parameters that allow identification of this subset of patients are currently lacking. One possibility is to select the patients on the basis of the grade of differentiation given to the individual tumour, assuming that the least differentiated tumours may be most responsive to chemotherapy. This correlation has already been observed (in vitro) in other human tumour types such as head and neck carcinomas [16] and gastric carcinoma [13].

Ductal adenocarcinomas of the pancreas have been examined by their sensitivity to various cytotoxic drugs, using either the in vivo nude mouse xenograft model [11, 12, 19] or in vitro test [15]. However, these studies did not investigate the relationship between chemosensitivity and morphological differentiation of human pancreatic carcinoma. We therefore treated xenografts from four human pancreatic ductal adenocarcinomas showing either poor, moderate or high differentiation with two drugs, vinblastine and 5-fluorouracil (5-FU), known to have different mechanisms of action. Vinblastine acts by inhibiting the assembly of microtubules, while 5-FU interferes with DNA synthesis [4].

Materials and methods

Animals

Female nude mice (Swiss nu/nu, Iffa Credo, Belgium), 6-weeks-old, were housed in sterilized cages under controlled and patho-

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gen-free environmental conditions. The mice received sterilized food and tap water.

Tumour lines and xenografting

Three of the four pancreatic ductal adenocarcinomas used in this study (PaTu-2, PaTu-3, PaTu-39) were kept as serially transplanted xenografts for several years [6, 10]. Two of these tumours (PaTu-2 and PaTu-3) were also kept in cell culture [14]. The fourth tumour, Panc-1, was obtained as cell line from the American Type Culture Collection (ATCC, Rockville, Md., USA). Xenografts of Panc-1 were established as follows: a cell suspension, obtained from a cell culture monolayer by trypsinization, was inoculated subcutaneously in nude mice (10^6 cells per inoculation site). The resulting tumours subsequently underwent two transplantation passages before being used in the experiment. Xenografting was performed as follows: under ether anaesthesia, two small pieces of tumour tissue were implanted subcutaneously into the periscapular regions of each mouse, using a trocar.

Tumour size was measured with a caliper and tumour volume was calculated with the formula: $a \times b^2 \times 0.5$ (a=maximal diameter, b=minimal diameter) [17]. For each of the four transplanted tumours, the growth rate was determined by calculating the mean tumour doubling time (TDT) from the growth curves of eight untreated tumours from the same transplantation passage as used for the experiments.

Morphological analysis

For histological examination 2–3 µm thick sections were cut from formalin-fixed, paraffin-embedded tumour tissue and stained with haematoxylin-eosin. Histological differentiation of the tumours was graded according to established criteria [10], the main determinator being gland formation. Cytological assessment was based on nuclear morphometry performed on imprints from untreated tumours derived from the same passage as the treated tumours. The tumours imprints were air-dried, fixed in formaldehyde and Feulgen stained. Of each imprint 170–200 randomly selected nuclei were examined using a computerised image cytometry system (VICOM Digital Image Processor) [5]. With a pixel resolution of 5.3 pixels/micron, four image features were determined for each nucleus: area, perimeter, minimal diameter, and form factor (defined as "perimeter^2/4 Π area", a circle having value 1).

Treatment

The experimental design to examine the effect of vinblastine and 5-FU was the same for each of the four tumours. Ninety-six mice were distributed on eight study groups, each comprising 12 mice (Table 1). All the grafts (two for each mouse) transplanted to 12 mice of a study group originated from the same tumour tissue. In each study group, the mice were randomized and equally divided into a 'treatment group' and a 'control group' (Table 1). Vinblastine and 5-FU were given as a single intraperitoneal injection, diluted in sterile water: vinblastine (Ely Lilly, Benelux) at a dose of 2 mg/kg and 5-FU (Roche) at a dose of 200 mg/kg. Related to body surface, these doses corresponded to values of 6 mg/m^2 and 600 mg/m^2, respectively [7]. The control mice received an equivalent volume of the drug solvent (sterile water). Drug administration was started, when tumour growth became obvious, i.e. when the tumour volume was greater than 30 mm^3 and the mean volume showed an exponential increase within 1 week. Tumour size was measured two to three times in the course of the experiment. The 'relative tumour volume' (RTV), defined as the ratio of the absolute tumour volume at a given day to the absolute tumour volume at day zero (start of treatment), was used to compare the tumour sizes in the treatment and control groups. The ratio of the mean RTV of treated tumours to the mean RTV of control tumours multipled by 100 (T/C%) was used as measure for treatment efficacy [1].

Statistics

The Mann-Whitney test (BMDP statistical software) was used to evaluate the difference between the mean relative tumour volume of treatment and control groups as well as the difference between the mean values of the morphometric nuclear features.

Results

Untreated tumours

The mean tumour doubling times of the untreated xenografts are given in Table 2. Histologically, PaTu-2 and PaTu-3 were found to be composed predominantly of solid carcinoma tissue, with a high mitotic activity (mi-

Table 1 Experimental design of the study (*VB*, vinblastine; *5FU*, 5-fluorouracil; *C*, control animals)

Tumour line	PaTu-2				PaTu-3				Panc-1				PaTu-39			
Treatment	VB	C	5FU	C	VB	C	5FU	C	VB	C	5FU	C	VB	C	5FU	C
No. of animals	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Table 2 Tumour characteristics and treatment effects (evaluated at day 7) *TDT*, mean tumour doubling time of untreated xenografts; *T/C%*, ratio of mean relative tumor volume (RTV) of treated tumours (T) to the mean RTV of control tumours (C) ×100; *P*, statistical significance of difference between mean RTV of treated and control tumours (Mann-Whitney test); *N.S.*=non significant ($P>0.05$)

	Grade	TDT (days)	Vinblastine		5-FU	
			T/C%	P	T/C%	P
PaTu-2	3	4.0	18.5	<0.01	33.9	<0.01
PaTu-3	3	3.5	56.5	<0.01	32.5	<0.01
Panc-1	2	7.5	68.6	<0.05	78.4	N.S.
Pa Tu-39	1	6.5	88.8	N.S.	92.2	N.S.

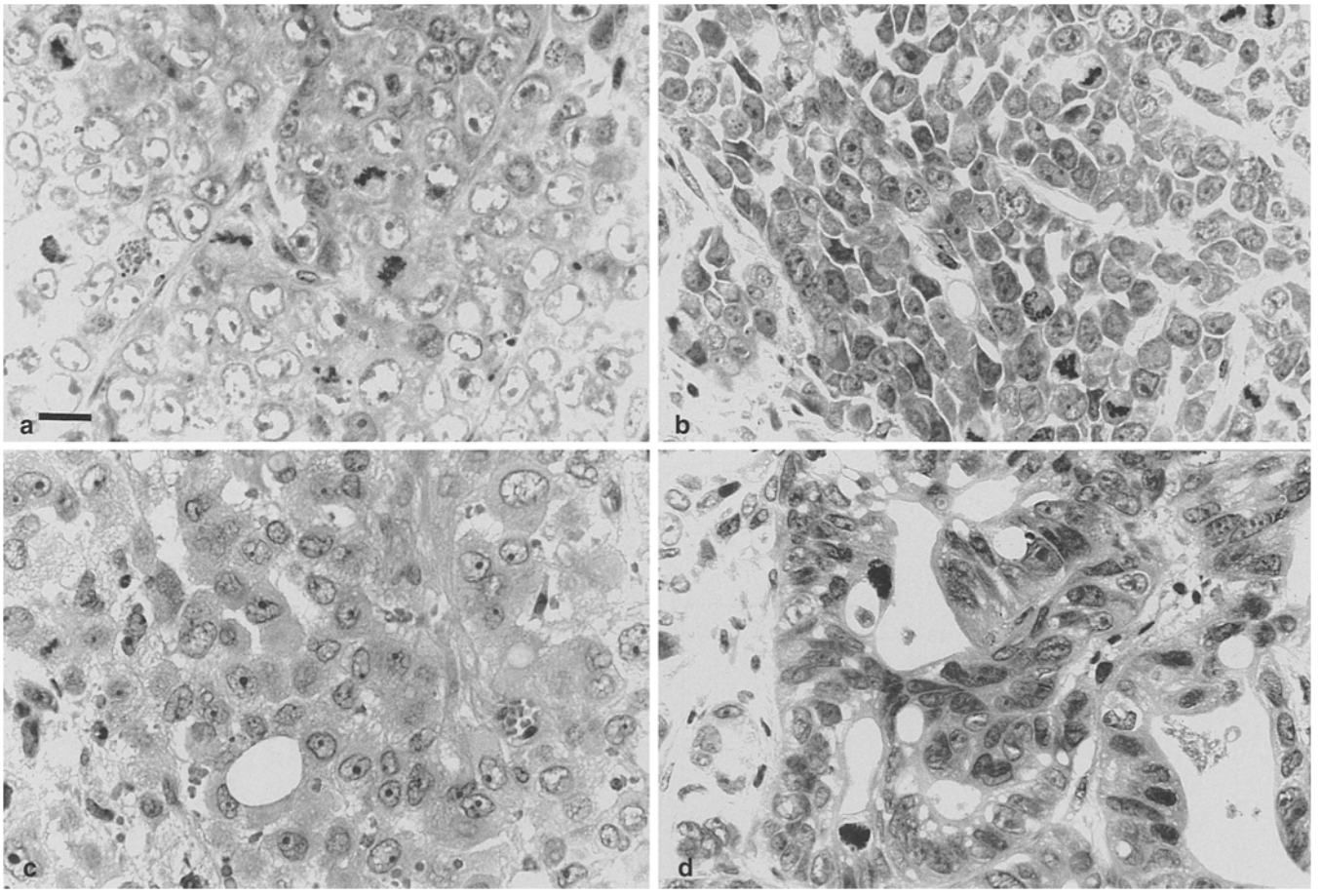

Fig. 1a–d Histological appearance of xenografted pancreatic ductal adenocarcinomas treated with vinblastine or 5-FU. **a**, PaTu-2; **b**, PaTu-3: Poorly differentiated carcinomas with solid architecture and many mitoses. **c**, Panc-1: Carcinoma with occasional gland formation. **d**, PaTu-39: Differentiated adenocarcinoma with well-developed glands. Haematoxylin-eosin staining. Bar=40 μm

totic figures averaged over 30 per HPF) (Fig. 1a and b). These tumours were considered to be poorly differentiated carcinomas (grade 3). In Panc-1, the tumour tissue was in many parts solid, but occasionally also showed a glandular component (Fig. 1c). The mitotic activity was less than 30 mitoses per HPF. Panc-1was therefore graded as moderately differentiated (grade 2). PaTu-39 disclosed well-developed mucin-producing duct-like glands. There were less than 20 mitoses per HPF. PaTu-39 was therefore classified as well differentiated (grade 1) (Fig. 1d and Table 2).

The cytological results obtained by nuclear morphometry (Fig. 2 and 3) were as follows: As to area and perimeter, PaTu-2 had the largest (166.6 μm^2/45.8 μm), PaTu-39 the lowest (103.2 μm^2/38.2 μm), and PaTu-3 and Panc-1 intermediate values (144.6 μm^2/43.2 μm and 142.2 μm^2/42.7 μm, respectively). The values of PaTu-3 and Panc-1 did not differ significantly from each other, but differed from the values of PaTu-2 and PaTu-39 ($P<0.05$). The results of the minimal nuclear diameter followed a pattern similar to that of area and perimeter, except that the value of PaTu-3 (11.8 μm) lay between those of PaTu-2 (12.7 μm) and Panc-1 (10.9 μm) and differed significantly from both ($P<0.05$). PaTu-39 again showed a value (9.4 μm) significantly lower than those of the other tumours ($P<0.05$). With respect to the nuclear form factor, PaTu-2 and PaTu-3 had the smallest value (1.05), while PaTu-39 had the largest value (1.17), implying that the form of its nuclei showed the strongest deviation from a circle. Panc-1 had an intermediate value (1.10), significantly different from the other values ($P<0.05$).

Effects of treatment

The treatment effects on tumour growth at day 7 are summarized in Table 2, Figs. 4 and 5. Concerning vinblastine, PaTu-2 was the most sensitive tumour (T/C%=18.5, $P<0.01$) followed by PaTu-3 (T/C%=56.5, $P<0.01$) and Panc-1 (T/C%=68.6, $P<0.05$), while there was no significant growth inhibitory effect on PaTu-39 (T/C%=88.8). 5-FU exerted a significant growth inhibition on PaTu-2 (T/C%=33.9, $P<0.01$) and PaTu-3 (T/C%=32.5, $P<0.01$), but had no significant effect on Panc-1 (T/C%=78.4) and PaTu-39 (T/C%=92.2). Between day 0 and day 7, there was a mean animal weight gain of 11.2% and 11.4% in the two control groups, and

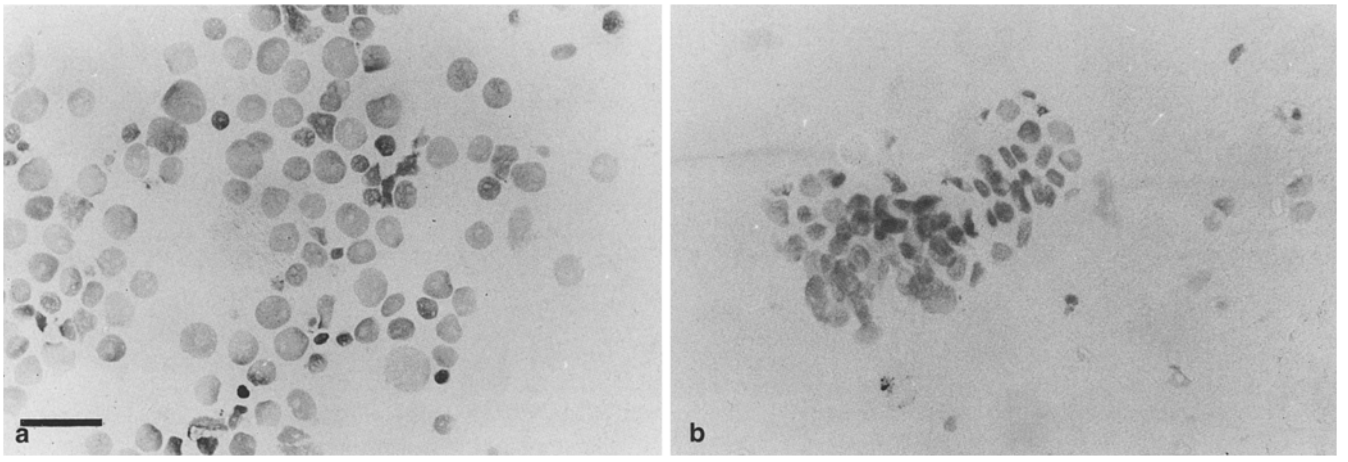

Fig. 2a, b Cell imprints of two untreated pancreatic ductal adenocarcinomas (**a**, PaTu-2; **b**, PaTu-39) used in the experiment. Feulgen staining. Bar=40 µm

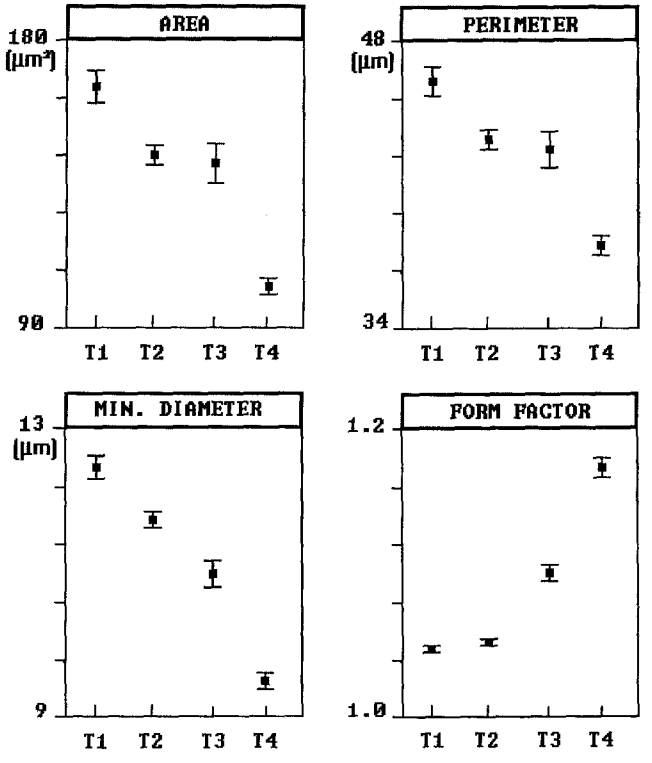

Fig. 3 Nuclear morphometric parameters, measured on imprints of untreated xenografts. Values are expressed as mean±s.e.m. (T1, PaTu-2; T2, PaTu-3; T3, Panc-1; T4, PaTu-39)

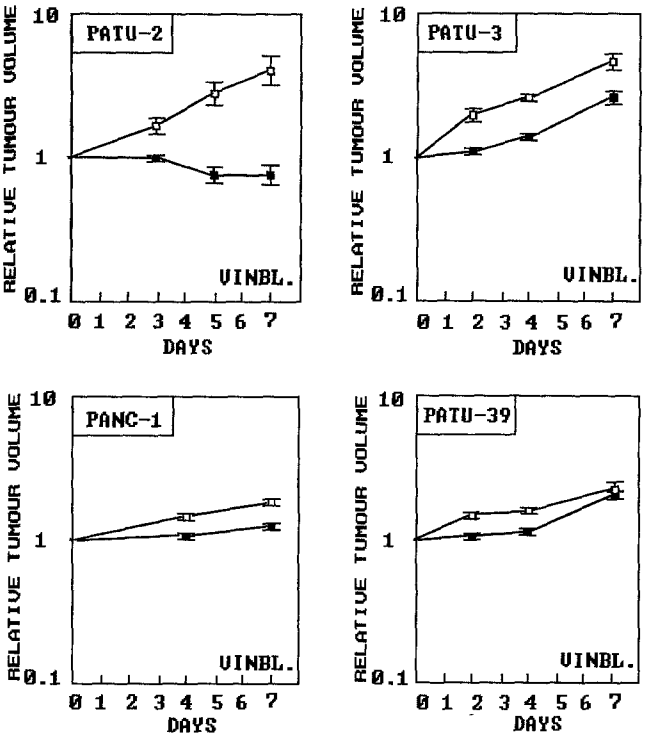

Fig. 4 Growth curves of vinblastine-treated (■) and untreated (□) xenografts of four pancreatic ductal adenocarcinomas (one single i.p. injection of 2 mg/kg at day 0). Tumour size is expressed as mean±s.e.m. of the relative tumour volume

a mean weight loss of 1.6% in the 5-FU treated animals and a mean weight gain of 1.6% in the vinblastin treated animals.

Discussion

Pancreatic ductal adenocarcinoma belongs to the group of neoplasms with a bad prognosis. At the time of diagnosis these tumours are usually so far advanced that they cannot be cured by resection [20] and palliative treatment by chemotherapeutic drugs is not very helpful. Less than 20% of patients with ductal adenocarcinomas have shown a response to 5-FU, the most widely used cytotoxic agent, and even combining 5-FU with other drugs such as mitomycin or streptozotocin has only slightly increased the response rate [2]. These results imply that most pancreatic carcinoma patients who receive chemotherapy will not profit from it. If, however, prognostic factors were identified which would allow to predict the response to an individual tumour to chemotherapeutic drugs, a selection of the few patients who may

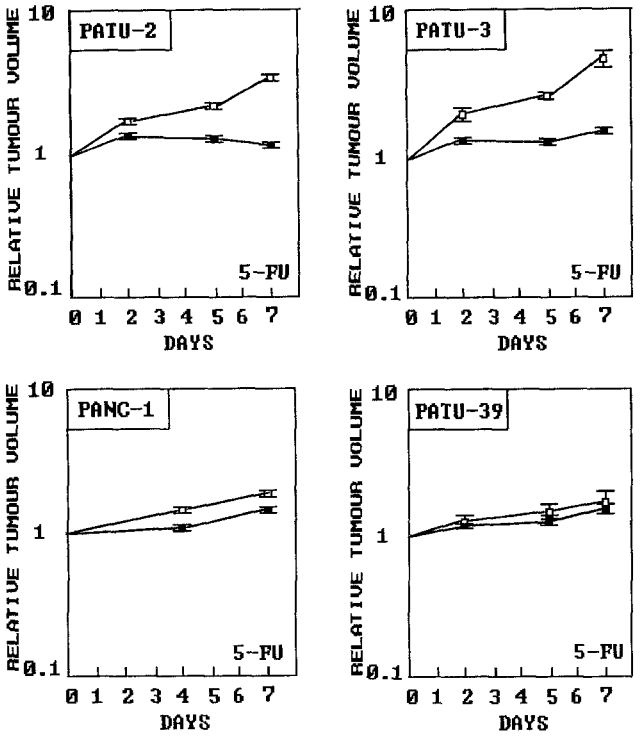

Fig. 5 Growth curves of 5-FU-treated (■) and untreated (□) xenografts of four pancreatic ductal adenocarcinomas (one single i.p. injection of 200 mg/kg at day 0). Tumour size is expressed as mean±s.e.m. of the relative tumour volume

benefit from chemotherapy might become possible. To this end we investigated the relationship between morphological tumour differentiation and chemosensitivity of xenografts from four different human pancreatic ductal adenocarcinomas, which were experimentally treated with a single intraperitoneal injection of vinblastine or 5-FU. We found that the tumours with no or only abortive gland formation and the largest and most circular nuclei showed the best response to the applied drugs. The dosage which was chosen for vinblastine and 5-FU is comparable to that which is used clinically [2]. However, we administered the drugs only once to minimize the influence of toxic side effects on the animals which may indirectly influence tumour growth.

In a previous study we demonstrated that histological tumour features such as gland formation were associated with growth and prognosis of ductal adenocarcinoma [10]. Similarly, nuclear morphometric parameters such as area, perimeter, minimal diameter and form factor were found to correlate with growth of xenografted pancreatic carcinomas [6]. Other studies have shown that nuclear area is useful in discriminating between chronic pancreatitis and pancreatic adenocarcinoma [18, 21] and correlates well with the prognosis of pancreatic carcinomas [8, 10]. In this study we confirm and further establish the close correlation between histological features of differentiation, morphometric nuclear features and growth kinetics in pancreatic ductal adenocarcinomas. Thus, the fastest growing tumours, PaTu-2 and PaTu-3, were the least differentiated carcinomas with almost solid (anaplastic) architecture and large circular nuclei, while the slowest growing tumour, PaTu-39, was a well-differentiated adenocarcinoma with abundant mucin-producing glands as well as irregularly shaped nuclei of variable size. Panc-1 combined an intermediate histological differentiation and nuclear form factor with a slow xenograft growth rate. Nuclear area and perimeter also discriminated between the fast growing PaTu-2 and the slow growing PaTu-39, but failed to separate PaTu-3 from Panc-1.

Analysis of the sensitivity of the four pancreatic carcinomas to the treatment with vinblastine and 5-FU reveals on the one hand a general response pattern and on the other a tumour-specific response. The general response pattern indicates that PaTu-2 and PaTu-3, the two fastest growing (poorly differentiated) tumours, are more sensitive to both drugs than the two slower growing tumours Panc-1 (moderately differentiated) and PaTu-39 (well-differentiated). If these results are assigned to patients it would imply that those patients who, on biopsy, are found to have a poorly differentiated ductal adenocarcinoma are more likely to respond to chemotherapy than patients with well-differentiated carcinoma. So far there is no clinico-pathological study which has tested this hypothesis. However, it is of interest in this respect that poorly differentiated ductal adenocarcinoma occurs at a frequency of 10–20% [10], which compares well with the response rate of pancreatic carcinoma to 5-FU alone or in combination with other drugs [2].

Correlations between tumour differentiation and chemosensitivity have also been shown for other human cancers. Using the in vitro succinate dehydrogenase inhibition test on tissue fragments, a statistically significant difference in sensitivity between poorly and well-differentiated tumours was found for head and neck squamous cell carcinomas [16] and gastric carcinomas [13] after incubation with different drugs. In colorectal cancer xenografts it was found that a tumour line derived from a poorly differentiated adenocarcinoma showed a better response to three of four tested agents than well-differentiated tumours. Moreover, tumours with a short doubling time had a better response to two of four tested agents than more slowly growing tumours [22]. In breast carcinoma it was demonstrated that tumours with a large nuclear area were more responsive to chemotherapy than tumours with a small nuclear area [3]. From all these data it can be concluded that histological and cytological differentiation in carcinomas obviously reflects to some extent the tumour sensitivity to chemotherapy. As most of the drugs applied interfere with DNA metabolism, the greater chemosensitivity of fast growing and undifferentiated neoplasms is best explained by the fact that these rapidly dividing tumours are, in general, more sensitive to drugs interfering with DNA metabolism or mitotic cell division than well-differentiated neoplasms.

Comparison of the responses of PaTu-2 and PaTu-3 to vinblastine and 5-FU in detail reveals that the effect of 5-FU on both tumours is similar, while vinblastine inhibits

growth of PaTu-2 more than PaTu-3. This difference in response to vinblastine of tumours which are otherwise comparable regarding xenograft growth and differentiation shows that the sensitivity of an individual tumour to a certain drug can only be partly predicted from structural and biological features. It is of interest that, concerning the morphometric parameters area, perimeter and minimal diameter, PaTu-3 resembles Panc-1 more closely than PaTu-2. However, it is uncertain whether these nuclear features could reveal more subtle differences of sensitivity to certain drugs than the nuclear form factor.

In conclusion, this study supports the notion that differentiation of pancreatic ductal adenocarcinoma as assessed by histological examination and nuclear morphometry may predict the sensitivity to cytotoxic drugs such as 5-FU and vinblastine to some extent. However, as the number of tumours examined is small, our results have to be regarded as preliminary.

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