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Selenium status alters tumour differentiation but not incidence or latency of pancreatic adenocarcinomas in *Ela*-TGF-α p53$^{+/-}$ mice

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Genetic predisposition and environmental factors act in concert in the pathogenesis of multi-factorial diseases. Selenoproteins represent fundamental antioxidative systems for the maintenance of cellular redox homeostasis, which is altered in various disease processes. Optimal function of selenoproteins requires availability of sufficient amounts of the essential trace element selenium, but in many countries the nutritive selenium supply is regarded insufficient. Supplemental selenium has been shown to have cancer-protective effects in a variety of experimental settings and clinical studies. Pancreatic carcinoma has so far not been tested as an end-point in such studies. We thus investigated the influence of supplemental nutritive selenium on pancreatic carcinogenesis in selenium-deficient animals by use of a genetically defined disease model. Over a period of 800 days, all animals ($n = 131$) in the study developed tumours. Within this time, the mean total tumour latency was not influenced by the selenium status (471 versus 472 days). Also, the mean latency of pancreatic carcinomas ($n = 83$) was not influenced (464 versus 466 days). In contrast, the percentage of pancreatic tumors within all tumours was lower in the selenium-deficient group (55 versus 70%). A highly significant difference in the differentiation grade of the pancreatic tumours was evident between the two groups: selenium-deficient mice ($n = 33$) developed predominantly undifferentiated anaplastic carcinomas (26 anaplastic versus 7 differentiated), whereas in the selenium-supplemented group ($n = 50$) mainly well-differentiated carcinomas were detected (20 anaplastic versus 30 differentiated). These data point at a new role of the trace element selenium in carcinogenesis.

Introduction

Environmental factors and genetic predisposition act in concert in the pathogenesis of multi-factorial diseases. Whereas oxidative stress is considered a major pathogenic determinant in various disease processes, selenoproteins represent fundamental antioxidative systems for the maintenance of cellular redox homeostasis. Optimal function of selenoproteins as antioxidative stress response factors requires availability of sufficient amounts of selenium, an essential trace element and environmental nutritive factor. In many countries, the average nutritive selenium intake does not allow maximum expression of selenoproteins and is therefore regarded insufficient (1).

Evidence from many ecological studies shows an inverse relationship between dietary intake of selenium and cancer risk (2–4). Moreover, studies examining the relationship of the individual selenium status and cancer showed an increased cancer risk for those individuals with low serum selenium values (1). Consequently, clinical data showed cancer-preventive properties of selenium, when added to the normal diet, in all and particularly in gastrointestinal cancers (4–6). Supplemental selenium has been found to reduce the incidence and mortality of liver cancer (7), stomach cancer (8) and colon cancer (6) in human interventional trials. In a large double-blind clinical trial with more than 1000 individuals, a significant decrease in total cancer mortality (56%) and overall cancer incidence (37%) resulted from supplementing the normal diet with 200μg of selenium daily (9). In this study, supplementation decreased the incidence of prostate, colon and lung cancers. Accordingly, the preventive effect was most prominent in those individuals that had low selenium levels at the start of treatment (10).

No controlled or randomized interventional study has been published proving the specific effect of selenium on pancreatic cancer, albeit data from ecological and case–control studies support a protective effect (11,12). Recently, a systematic analysis of published trials focussing on the effect of antioxidants in the prevention of gastrointestinal cancers including pancreatic cancer revealed no effect of beta-carotene, vitamin A, vitamin C and vitamin E given alone or in combinations (6). In contrast, these antioxidants may even increase overall mortality. Selenium, however, may represent an exception among the antioxidant supplements examined so far, potentially leading to reduction of gastrointestinal cancers in recent studies (6).

Effects of antioxidants on pancreatic cancer in animal studies were described by use of two models. In hamsters developing *N*-nitrosobis (2-oxopropyl)amine-induced pancreatic tumours, vitamin C supplementation decreased consistently the number of advanced ductular lesions (13), whereas β-carotene, vitamin E or sodium selenite (13–15) showed no effects. In rats, the incidence of azaserine-induced preneoplastic acinar lesions was lower in groups maintained on sodium selenite and also on β-carotene and vitamin C, whereas vitamin E had no effect (16). However, in other studies, sodium selenite had no effect in this model (17).

Among the biochemical mechanisms proposed for chemopreventive effects of selenium, a well-accepted hypothesis is that dietary selenium increases selenium-containing proteins, i.e. selenoproteins. On the other hand, low molecular weight selenocompounds were shown to be responsible for protective effects (4,18). The predominant biological form of selenium in mammals at low to adequate dietary levels is selenocysteine that is cotranslationally incorporated into selenoproteins (19). Selenocysteine is encoded by the UGA codon and is recognized as the 21st amino acid in protein (20). So far, 25 human and 24 mouse selenoprotein genes have been identified (21).

Despite rapid progress in understanding molecular mechanisms in pancreatic ductal adenocarcinoma (PDA), this cancer is still considered a fatal disease. Five-year survival rates for patients diagnosed with metastatic PDA are still less than 4%. Even among the 10–20% of surgically resectable PDA, recurrent and metastatic disease is prevailing (22). The high mortality of PDA is attributable to a lack of effective early detection methods and the poor efficacy of the therapies for advanced disease (23). As an alternative, preventive strategies in individuals with familial pancreatic carcinoma should be considered. At least one big prospective study with nearly 40 000 individuals from Finland showed a significant relationship between low serum selenium and pancreatic cancer risk (12).

To this end, we characterized the influence of the selenium status modified by nutritive sodium selenite on carcinogenesis by use of p53 hemizygous (p53$^{+/-}$) mice, in which transforming growth factor alpha (TGF-α) is over-expressed specifically in the pancreas leading to pancreatic hyperplasia followed by fibrosis and subsequently by invasive pancreatic carcinoma or invasive pancreatic adenocarcinomas (24,25). Our data showed that the selenium status alters tumour differentiation but does not affect its incidence or latency.

Materials and methods

Mice, diets and animal husbandry

Ela-TGF-α p53$^{+/-}$ mice used in this study have been described in detail (24,25). p53$^{-/-}$ mice were on a BALB/c background, kept as a hemizygous

Abbreviations: PDA, pancreatic ductal adenocarcinoma; TGF-α, transforming growth factor alpha.

line; *Ela*-TGF-α mice on a C57BL/6 background were bred as heterozygotes. Double transgenic F1 hybrids, *Ela*-TGF-α $p53^{+/-}$, were used in the present study. For the production of selenium-deficient mice, the parental mouse lines were depleted of selenium for three generations by feeding a commercially available (MP Biomedicals, Aurora, OH) selenium-depleted (mean basal selenium: 22 μg/kg), semi-purified diet (26), which contained torula yeast, sucrose, lard, minerals and vitamins. For selenium supply, the same diet supplemented with 300 μg/kg selenium as sodium selenite was used for the adequately selenium-fed mice. The two parental and the tumour-prone experimental F1 mouse lines were kept in parallel on the two diets. All mice were kept at the GSF animal facilities in groups of up to five in type II polycarbonate cages on wood shavings (Altromin, Lage, Germany) at 20–24°C, 50–60% humidity 20 air exchanges per hour and a 12/12-h light/dark cycle. Sterile filtered water was given to them *ad libitum*.

Necropsy, tumour sampling and histology

F1 mice were checked daily for clinical signs of illness (27) and killed for necropsy after reaching the hyper-acute phase of disease. All tumours and organs showing gross pathological changes were fixed in 4% buffered formalin and embedded in paraffin. All animal experiments were performed in compliance with the German animal welfare law and have been approved by the Institutional Animal Care and Use Committee and by the government of Upper Bavaria.

Tumour nomenclature and grading

Haematoxylin and eosin stained slides were analysed by two pathologists (G.H. & L.Q.-M.). Tumour description and grading was performed according to the consensus report and recommendations for mouse models of pancreatic exocrine cancer (28).

Selenium analysis

Serum samples were analysed by means of a Perkin Elmer graphite furnace atomic absorption spectrometer (4100 ZL) with $Mg(NO_3)_2$ and $Pd(NO_3)_2$ (each 0.2%) as matrix modifier. The samples also contained 0.3% HCl and 0.4% Triton X100. Solid samples were dissolved in HNO_3 for 10 h at 170°C in a pressure digestion system (Seif, Unterschleissheim, Germany) and measured by Inductively coupled plasma-atomic emission spectrometry in a Spectro Ciros Vision-System (SPECTRO Analytical Instruments). Sample introduction was based on hydride generation with HCl (10%) and $NaBH_4$ (1% in 0.3% NaOH) using Argon as plasma and introduction gas. All selenium contents are expressed as microgram per kilogram wet mass.

Statistical analysis

Mean tumour latency was calculated with the Log-Rank test (proc lifetest, SAS 9.1). Tumour type proportions in the selenium-deficient and selenium-adequate groups were calculated by an exact randomized version of the Fisher test (29). Data are expressed as mean ± standard deviation.

Results and discussion

Previous animal studies on pancreatic carcinogenesis were based on chemically induced models (13–17). In contrast, in the present study, we used a genetically defined model of spontaneous pancreatic car-

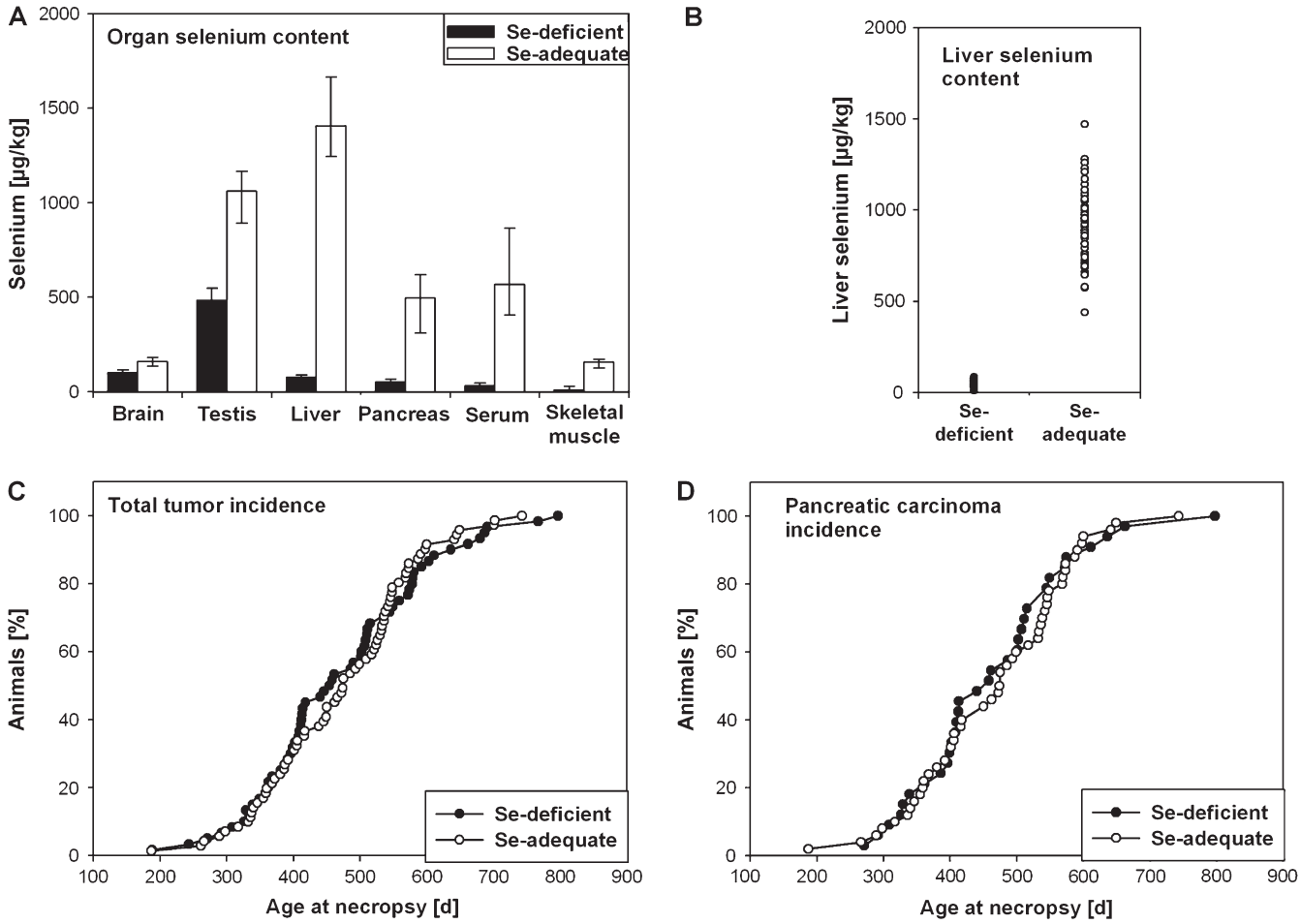


Fig. 1. Selenium status and tumour latency. (**A**) selenium content of brain, testis, liver, pancreas, serum and skeletal muscle from selenium-deficient and adequately selenium-fed mice (testis: $n = 4$, all other organs: $n = 8$; all organs: $P < 0.01$). (**B**) Selenium status of the experimental groups of mice. Mean selenium content in the livers of the selenium-deficient group was 48 μg/kg (Se deficient, $n = 60$) as compared with 923 μg/kg ($P < 0.0001$) in the selenium-adequate group (Se adequate, $n = 71$). (**C**) Incidence of all tumours detected in selenium-deficient and selenium-adequate mice within 800 days of observation. Effects of the selenium status in the two groups were not detected. (**D**) Incidence of pancreatic carcinomas in the two groups shown in (C). Mean tumour latency for the selenium-deficient group was 471 ± 128 days ($n = 60$) for all tumours shown in (C) and 464 ± 117 days ($n = 33$) for pancreatic carcinomas shown in (D). These data were nearly identical to those observed in the selenium-adequate group for all tumours (472 ± 113 days, $n = 71$) or for pancreatic carcinoma (466 ± 112 days, $n = 50$). Each symbol represents one mouse. Solid circle, selenium deficient; open circle, selenium adequate. (All selenium contents: wet mass.)

cinoma as described (25). Moreover, in cancer intervention studies with selenium supplementation as therapeutic principle, the preventive effect was most prominent in those individuals that were selenium deficient or had low selenium levels at the start of treatment (10), indicating that selenium replenishment rather that supplementation was most effective. Based on these findings, we used mice with low selenium status and compared their tumour pattern with that of mice replenished with non-toxic amounts of sodium selenite sufficient to support maximal tissue activities of selenoproteins (designated below adequately selenium-fed mice). Sodium selenite was chosen as selenium source, since, in contrast to selenomethionine, it cannot be incorporated non-specifically into proteins. Instead, selenium from selenite is predominantly incorporated into selenoproteins (30).

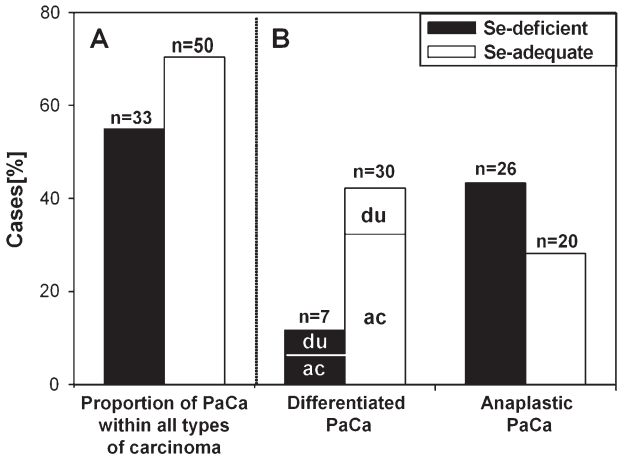
At the start of breeding, the experimental F1 generation of mice, the selenium content of pancreas, liver, serum and skeletal muscle of mice of the third parental selenium-depleted generation was (all wet mass) 53.6, 72.932.3 and 14.0 μg/kg, respectively (Figure 1A). These levels were 10- to 20-fold lower than those of the adequately selenium-fed mice and confirmed successful deprivation of selenium in these mice. In contrast, selenium contents of brain and testis were 95.3 and 467.2 μg/kg wet mass, respectively. The fact that they were only about 2-fold lower than in adequately selenium-fed mice points to the high hierarchy of these two organs with respect to selenium retention even after three generations of reduced supply (31). Moreover, it confirms earlier findings showing that selenium resorption from food and its storage and retention in vital organs is very efficient, particularly in conditions of low selenium intake. Selenium is also effectively transmitted from mother to offspring via the milk, most likely in the archaic view of preservation of the species (32).

The selenium content of the liver was chosen as reference for the selenium status at the time of necropsy of the experimental F1 mice. Mean concentration in this organ (wet mass) was 48.0 ± 16.9 μg/kg in selenium-deprived mice and 923 ± 224 μg/kg in adequately selenium-fed mice (Figure 1B). At the hyper-acute phase, 100% ($n = 131$) of experimental mice showed malignant tumours. The mean latency for all tumour types in the selenium-deficient group and in the adequately selenium-fed group was 471 ± 128 and 472 ± 113 days, respectively. The mean latency for pancreatic carcinomas was 464 ± 117 in the selenium-deficient group and 466 ± 112 days in the adequately selenium-fed group, indicating that the selenium status had no effect on tumour incidence or tumour latency in *Ela*-TGF-α $p53^{+/-}$ mice. These data are summarized in Figure 1.

Interestingly, the percentage of pancreatic carcinomas among all types of tumours detected in the mice was 70.4% and thus higher in the selenium-adequate group as compared with 55.0% in the selenium-deficient group; however, this difference was not statistically significant ($P = 0.07$). This suggests that other tumours than pancreatic carcinomas may exhibit a different pattern of reaction in response to the different selenium levels (Figure 2). The other tumours found in the mice comprise mostly haematopoietic tumours, some mammary and bone tumours and few cases of other tumours of the lung, liver, skin and muscle as described previously for p53 hemizygous mice (33,34).

As a major finding, the differentiation grade of the pancreatic carcinomas showed highly significant differences between the two experimental groups. In the selenium-deficient group, the proportion of differentiated pancreatic carcinomas was 21.2% and significantly ($P < 0.001$) lower as compared with 60.0% in the adequately selenium-fed group. Also within the differentiated carcinomas, there was a non-significant trend towards a lower proportion of acinar type carcinomas ($P = 0.07$) in the selenium-deficient group (57.1 versus 76.7%). The majority of pancreatic carcinomas in the selenium-adequate group were diagnosed as well-differentiated acinar (Figure 3A) and ductal (Figure 3B) tumours. Interestingly, most well-differentiated tumours additionally showed significant proportions of pleomorphism (Figure 3C). Representative cases of pancreatic carcinomas as well as the histological appearance of tumour metastases are shown in Figure 3.

These findings highlight the implication of selenium in tumour differentiation. Low levels of selenium and subsequently also low



C

Selenium-	n (all PaCa)	n (diff. PaCa)	p-value
deficient	33	7	0,0005
adequate	50	30	

Fig. 2. Tumour differentiation grade in the experimental groups. Morphology of pancreatic adenocarcinomas was analysed by two pathologists and categorized as differentiated or anaplastic. Differentiated tumours, in turn, were divided into acinar or ductal phenotype. (**A**) The percentage of pancreatic carcinomas within all types of tumours found in the mice was higher in the selenium-adequate group compared with the selenium-deficient group, although the difference was not significant ($P = 0.07$). (**B**) Although the selenium status did not influence tumour incidence or latency, the tumour differentiation grade was significantly distinct. In the selenium-adequate group, the majority of tumours was well-differentiated pancreatic adenocarcinomas mostly of the acinar type, whereas in the selenium-deficient group, most of the tumours were anaplastic carcinomas. Statistical analysis (**C**) was performed using an exact randomized version of the Fisher test (29). Duct, ductal; ac, acinar; PaCa, pancreatic adenocarcinoma).

levels of selenoproteins may therefore impair differentiation programs in the tumour or in tumour precursor cells as shown for the maturation program of spermatozoa during spermiogenesis (35,36). Therefore, it may be speculated that minimal changes in cell metabolism and redox state affect differentiation or maturation processes. Whether similar mechanisms account for the effects of an experimentally low selenium status on tumour differentiation in tumour-prone mice remains elusive.

The origin of PDA (37) in pancreatic cancer is still unresolved. Recent data suggest that cancer precursors in PDA arise from stem cells having the unique potential of self-renewal and differentiation into multiple lineages. In agreement with this assumption, reactivation of embryonic programs emerging during the development of pancreatic tumours has been shown recently in both gene expression studies and functional assays (38–40).

Among the redox-active selenoproteins, the cytosolic glutathione peroxidase (GPx1) is the only selenoprotein able to protect cells from excessive amounts of reactive oxygen species (41). Other redox-active selenoenzymes such as the phospholipid hydroperoxide glutathione peroxidase (PHGPx) and the cytosolic (Txnrd1) and mitochondrial (Txnrd2) thioredoxin reductases are considered to act on a more subtle level and regulate redox-reactive signals. In contrast to GPx1, PHGPx, Txnrd1 and Txnrd2 are indispensible for mammalian development (42–44). Knockout embryos fail to develop as a consequence of disturbed redox balance and most likely subsequent deregulation of developmental programs. It might be speculated that perturbed activities in these redox-regulating selenoenzymes influence development of pancreatic tumours, thereby directing pathogenesis towards a less differentiated and more aggressive phenotype. Since selenium deprivation affects all selenoproteins, a specific role of single selenoproteins can only be achieved by genetic models (knockout of individual

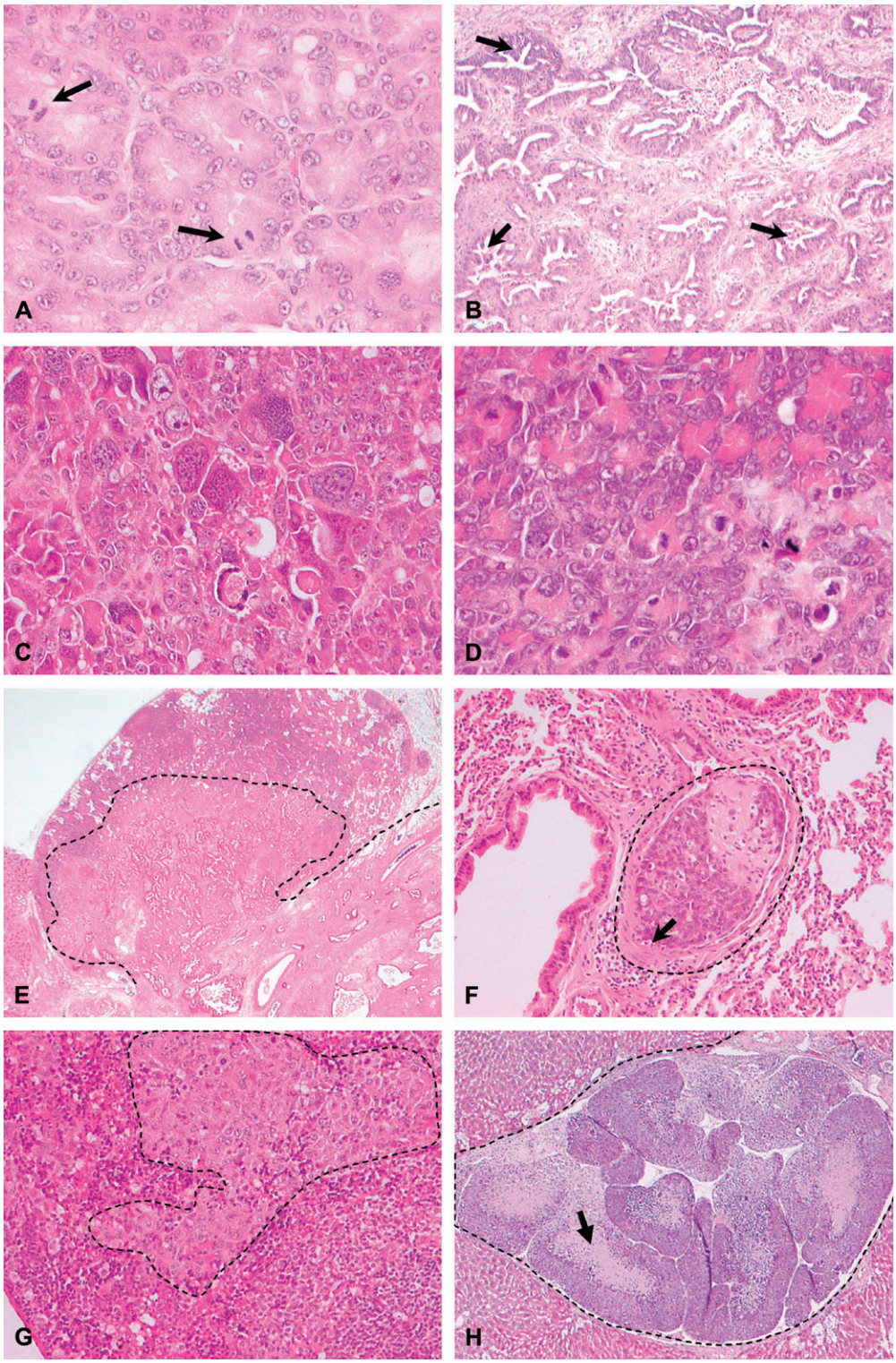


Fig. 3. Morphological analysis of pancreatic carcinomas. (**A**) An acinar cell carcinoma showing a well-differentiated acinar arrangement of the tumour cells is depicted. Cytologically, the tumour is characterized by round to oval nuclei, a single prominent nucleolus and abundant eosinophilic cytoplasm with only mild pleomorphism and moderate mitotic activity (arrows) [400× haematoxylin and eosin (H and E)]. (**B**) The invasive ductal adenocarcinoma consists of enlarged irregular ducts with epithelial folding into the lumen (arrows). A high degree of cytological atypia is present, including hyperchromasia and high mitotic activity (160× H and E). Ductal neoplastic growth was accompanied in many cases by acinar malignant proliferation. (**C**) Shows an area of poorly differentiated carcinoma, which in most cases was alternating with areas of well-differentiated acinar tumours. The typical features are cellular crowding, pleomorphism, an increased mitotic activity, although the acinar cellular origin can be still recognized (400× H and E). (**D**) In contrast to the well-differentiated types of pancreatic tumours, the cell of origin of some neoplasias, so-called "anaplastic" carcinoma cannot be defined. These tumours show high degree of cellular pleomorphism. High rate of mitoses, apoptotic cells, giant cells and multinucleated cells as well as cytoplasmatic vacuolization could be observed (500× H and E). (**E**–**H**) Local invasion and/or distant metastasis of pancreatic carcinomas are shown. (E) Demonstrates the local invasion of a ductal carcinoma into the regional lymph node (15× H and E). (**F**) A distant metastasis into the lung of an acinar carcinoma is shown. Note the presence of a neoplastic thrombus in the lung blood vessel (arrow) (160× H and E). (**G**) Invasion by contiguity of an anaplastic carcinoma into the adjacent spleen is depicted (160× H and E). (H) Liver metastasis of an anaplastic carcinoma of the pancreas (400× H and E). Note the cohesive growth of the cells with variable cell sizes (including giant cells) numerous mitoses, apoptosis and highly necrotic areas with masses of cell debris (arrow). Dotted lines in (E–H) demark the border between metastasis and normal tissue.

selenoprotein genes) or by use of specific inhibitors. The thioredoxin 1/thioredoxin reductase 1 system might be especially interesting in this respect. Thioredoxin reductase 1 has recently been shown to be a key enzyme in the tumour phenotype and the tumorigenicity of lung carcinoma cells (45). Beside its essential role for embryogenesis (46), thioredoxin 1 is involved in the redox regulation of p53, whose DNA-binding activity is controlled by the thiol redox status of some critical cysteinyl residues in its DNA-binding domain (47,48). The redox state of these residues appears to be regulated by thioredoxin (49). Oxidative stress promotes nuclear translocation of thioredoxin 1 and activates various kinases phosphorylating p53 resulting in stabilization and activation of p53 in the nucleus (50). Thioredoxin-dependent redox regulation of p53 thus couples oxidative stress response and p53-dependent DNA repair and apoptosis. If this reflects a pathogenetic pathway in our model has to be resolved in the future.

In the APC^{min} model of colon carcinoma, mice hemizygous for SelenoproteinP (SepP), a selenium transport protein, develops more malignant tumours than APC^{min} mice, which are wild-type for SepP (L.Schomburg, personal communication). In addition, men homozygous for the Ala/Ala mutation of manganese superoxide dismutase (MnSOD), an enzyme functionally linked with the selenoprotein Txnrd2, have an increased risk for high-grade prostate cancer. Moreover, there is also a positive correlation between low/baseline selenium levels in these patients and the development of more aggressive cancer (51). Taken together, these findings indicate that a lack in selenium/selenoprotein function possibly in combination with a lack in other redox-regulating factors drives tumours to a more malignant phenotype.

In summary, using a genetically defined mouse model of pancreatic carcinogenesis, our data suggest that selenium in physiological concentrations does not prevent or decelerate PDA but significantly alters the differentiation status of the tumour. These findings therefore point at a new role for the trace element selenium in cancer development and cancer cell differentiation, which may serve as basis for intervention or treatment strategies. To further clarify the role of specific selenoproteins in pancreatic carcinogenesis, future studies are aimed at using specific selenoprotein knockout models in combination with newly developed models of pancreatic carcinogenesis.

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