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Initial velocities in situ of G6PDH and PGDH and expression of proliferating cell nuclear antigen (PCNA): sensitive diagnostic markers of environmentally induced hepatocellular carcinogenesis in a marine flatfish (Platichthys flesus L.)

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Abstract

Flounder (Platichthys flesus L.) were sampled in areas of relatively low contamination, in the open sea, and in a region at the mouth of the highly contaminated river Elbe (Germany). Prognostic and diagnostic values of the in situ reaction velocities of glucose 6-phosphate dehydrogenase (G6PDH) and phosphogluconate dehydrogenase (PGDH) inside and outside altered hepatocellular foci and carcinomas were demonstrated histochemically. Reactions were monitored quantitatively in time using image analysis. Expression of proliferating cell nuclear antigen (PCNA) was detected in serial sections as a marker for cell proliferation using the immunogold–silver labelling technique. Enzymic parameters were correlated with PCNA expression and histopathology. A large variety of foci and tumours were found in the livers of fish from the contaminated area. Histopathological classification could only indicate tendencies with respect to diagnosis and prognosis. However, in all stages of carcinogenesis, from early foci to well-organized trabecular basophilic tumours and anaplastic carcinomas, significantly higher initial velocities of G6PDH but not of PGDH and a higher PCNA labelling index (43–65%) were found in altered hepatocytes in comparison with surrounding extrafocal tissue. Furthermore, the increased capacities of G6PDH and PGDH in basophilic foci and nodules were found to be useful prognostic parameters. Significantly increased PCNA expression in small satellites of carcinomas reflected their malignancy and was used to differentiate between early foci and invasive protrusions of carcinomas. It is concluded that G6PDH activity and PCNA expression are very useful as diagnostic and prognostic tools to detect early preneoplastic lesions and to analyse progression towards carcinomas in environmental monitoring. © 1998 Elsevier Science B.V.

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1. Introduction

Sensitive diagnostic and prognostic biomarkers of carcinogenesis in aquatic animals are needed urgently for risk assessment in monitoring programmes in environmental toxicology. Worldwide, increasing frequencies of liver cancer in fish are reported from areas exposed to anthropogenic discharges from industrial and agricultural sources (Falkmer et al., 1977; Christensen, 1980; Baumann et al., 1987; Black, 1988; Cormier et al., 1989; Gardner et al., 1989; Harshbarger and Clark, 1990; Kranz and Dethlefsen, 1990; Köhler et al., 1992; Vethaak and Jol, 1995). Marine bottom-dwelling flatfish are most affected by liver cancer and this is related to their close contact to sediments that accumulate hepatotoxic and carcinogenic compounds (Malins et al., 1985a; Malins et al., 1985b; Myers et al., 1990; Stein et al., 1990; Fabacher et al., 1991; Schiewe et al., 1991; Wahl et al., 1995). Uptake via the body surface and food is dependent on the class of compounds and their bioavailability (Couch and Harshbarger, 1985; Varanasi et al., 1987; Safe, 1990; Porte and Albaigés, 1993). Moreover, there is recent evidence for a direct transfer of carcinogens across the food net (Gardner et al., 1991).

Histopathological diagnosis of the multistep process of carcinogenesis in livers of flatfish from wild populations revealed striking similarities with respect to processes involved in experimentally induced chemical carcinogenesis in mammals (Bannasch et al., 1981a; Bannasch et al., 1981b; Bannasch et al., 1982; Pitot et al., 1989; Bannasch, 1990a; Bannasch, 1990b) and fish (Hendricks, 1982; Couch and Courtney, 1987; Hinton et al., 1988): yet, the biological significance of morphological alterations in livers of lower poikilotherms is still limited (Moore and Myers, 1994). Therefore, we have investigated whether metabolic alterations can be used for diagnosis and prognosis of carcinogenesis in livers of flounder. We monitored the reactions of a number of enzymes that show alterations in certain carcinogenesis models in mammals (Stumpf and Bannasch, 1994) with the use of image processing and analysis. Reactions were analysed in time and in situ in unfixed cryostat sections of livers against saturating substrate concentrations to calculate local initial velocities (Jonker et al., 1995). These metabolic parameters were correlated with pathological and quantitative morphological parameters and cell proliferation as determined in consecutive sections.

Flounder (Platichthys flesus L.) were sampled in a region close to the mouth of the highly contaminated river Elbe (Germany) where neoplastic lesions in up to 35% of the individuals were already registered by macroscopic inspection during annual surveys, while in the reference site of relatively low contamination neoplastic lesions from 0% to a maximal 12% of individuals were found. Histological diagnosis showed that neoplastic changes registered at the reference site were advanced stages of carcinogenesis, indicating that these animals had migrated from contaminated...
The majority of liver neoplasms diagnosed in flounder in the mouth of the river Elbe were of hepatocellular origin (73%), whereas other cell types of the liver appeared to become affected in later progressive stages, such as hepatocholangio-carcinoma (3%) and mixed-hepatocellular carcinoma and angiosarcoma (15%) (Köhler, unpublished). Parallel chemical analyses of the livers showed significantly increased levels of PCB congeners compared to the reference site (5.9-fold of tumour promoters like PCB 052, 3.4 of 101 and 2.4 of 153; and for the CYP450-inducers 118 and 138 2.5 fold for example), as well as of other hepatotoxic organochlorines and their metabolites (29 fold! of OCS, 4.5 of HCB; 3.6 of DDE; Safe, 1990). Heavy metal contamination measured in muscle tissue ranged between 1.2- and 1.8-fold of Pb and Cd, and between 6.4 and 6.8 fold of Cr and Hg (Wahl et al., 1995).

Two enzymes were selected to be tested as diagnostic tools in the present study on the basis of pilot experiments, in which the activity of a number of enzymes was localized that were reported to be altered in many cases of chemical carcinogenesis in livers of mammals (Moore and Myers, 1994; Hacker et al., 1991). These enzymes are glucose 6-phosphate dehydrogenase (G6PDH) and phosphogluconate dehydrogenase (PGDH), which are both involved in the NADPH-generating pentose phosphate pathway. Alterations of their activity are sensitive markers for early stages of certain forms of hepatocytic carcinogenesis in humans, rats and rodents before morphological changes are detectable (Pitot et al., 1989; Weber, 1977; Maronpot et al., 1989; Bannasch, 1990a; Bannasch, 1990b). In order to test the predictive value of variations in enzyme activities in various types of (pre)neoplastic foci, the expression of proliferating cell nuclear antigen (PCNA) was detected as a marker for cell proliferation in serial sections using immunogold–silver labelling. The expression of PCNA, a 36 kDa auxiliary protein for DNA polymerase plays an essential role in DNA synthesis. The protein is synthesized in the late G1- and S-phase (Bravo et al., 1987; Hall et al., 1990) and is therefore a cell cycle-related marker (Jones et al., 1993; Ortego et al., 1995).

In situ measurement of reactions of G6PDH and PGDH in time as quantitative reflection of activity was introduced recently as a tool in metabolic research (Jonker et al., 1995; Van Noorden and Jonges, 1995). This is one of the first applications of image analysis which enables a direct link between initial velocities of the NADPH-generating enzymes and expression of PCNA and histopathological changes in different tissue compartments in serial cryostat sections.

2. Materials and methods

2.1. Sampling and tissues

Adult female flounder (length 30–45 cm) aged between 7 and 12 years were sampled in the Elbe plume and in less contaminated offshore areas outside the spawning period. After short hauls with a dragnet (30 min, 70 mm mesh width) to minimize sampling stress, the fish were immediately dissected on board and
screened for macroscopically visible foci and tumours. Pieces of liver (total liver weight 4-5 g) were supercooled in hexane and deep frozen at −70°C. Parallel samples of the tissues or lesions, were fixed in 4% Baker’s formaline to be embedded in methacrylate (Merck, Darmstadt, Germany) to obtain high resolution photomicrographs of thin-sectioned lesions stained with Gill’s hematoxylin and eosin.

In the present study, 185 foci of altered hepatocytes, hepatocellular adenomas and carcinomas were diagnosed histopathologically in cryostat sections of 125 livers with neoplasia and 25 non-neoplastic livers from the contaminated site and 50 from the clean reference site. They were incubated later to demonstrate G6PDH and PGDH activity. A minimum of five cases of each lesion type and of extrafocal tissue, as well as of non-neoplastic liver from the contaminated site and from the clean reference site was analysed.

2.2. Histopathology.

Serial cryostat sections (10 μm thick) were cut on a motor-driven cryostat (Microm HM 500 OM, Biorad, Walldorf, Germany) at −25°C at low but constant speed to ensure reproducible section thickness (Van Noorden and Frederiks, 1992). Sections were kept at −25°C until used. Some of these sections were stained in Gill’s hematoxylin and eosin and Oil Red O (1E242; Chroma, König, Germany) to demonstrate lipids. Morphological diagnosis of lesions in these sections were linked with in situ enzyme studies and expression of PCNA in adjacent sections. Furthermore, sections (3 μm thick) were cut from methacrylate embedded samples of tissues with a rotation microtome (JB4, Sorvall microtomes, DuPont, Wilmington, Delaware, USA) and stained in a similar way as described above. Glycogen was demonstrated with the PAS reaction preceded by an aldehyde blockage in chlorous acid overnight, treatment in periodic acid (Merck) for 10 min and staining in Schiff’s reagent (Sigma Aldrich, Deisenhofen, Germany) for 20 min with subsequent bleaching of the sections in sulfurous acid for 2 min according to McManus (see Pearse (1985)).

Histopathological diagnosis was based on the criteria of experimental chemical carcinogenesis in mammals and comprised the staining properties of foci of altered hepatocytes, hepatocellular adenomas and carcinomas ranging from clear cells, eosinophilic/acidophilic and basophilic cells and their combinations which have been described as mixed cell foci (Bannasch et al., 1989). Furthermore, growth patterns (parenchymal, trabecular, anaplastic/dedifferentiated) and the growth behaviour (compressive/invasive) (Newborne, 1982), as well as maximum diameter of foci of altered hepatocytes, adenomas and carcinomas were recorded for the evaluation of early foci and sequential changes towards carcinomas.

2.3. Immunohistochemistry of PCNA

For immunohistochemistry of PCNA, serial cryostat sections (10 μm thick) were mounted on slides treated with BIOBOND (BR20, Plano, Marburg, Germany) as adhesive for tissue sections and fixed in 1% formaldehyde and 0.1% glutaraldehyde
in 0.2 M Sörensen’s buffer (pH 7.2) for 3 min at room temperature. After rinsing in Sörensen's buffer and aqua bidest, the sections were transferred to Lugol's solution (1% iodide and 2% potassium iodine in aqua bidest) in order to increase the reaction sensitivity and subsequently after three rinses of each 5 min in aqua bidest to an aqueous solution of 2.5% Na₂S₂O₃ for 3 min. Finally, the sections were transferred to TBS (Tris buffer saline; 0.05 M Tris +250 mM NaCl, pH 7.6) containing 0.05% Tween 20 (Merck). Blocking of unspecific background staining was performed in 5% goat serum (Dako, Glostrup, Denmark) in TBS at 20°C for 20 min.

PC10 anti-mouse PCNA antibodies (Dako) were used in dilution of 1:1000 (determined after titration) for incubation overnight at 4°C, because this antibody gives adequate labelling results in fish (Ortego et al., 1995). After rinsing in TBS and 0.05% Tween 20, the sections were incubated with the secondary goat anti-mouse IgG conjugated with 0.8 nm colloidal gold (Aurion, Wageningen, The Netherlands) in a dilution of 1:30 in 1% bovine serum albumin in TBS at 20°C for 60 min. After rinsing, additional stabilization of the immunogold-complex was performed by a fixation step in 2.5% glutaraldehyde in TBS for 15 min followed by three rinses in aqua bidest for 20 min each. Sections were stained in the dark room for 9 min in silver developer, freshly prepared according to Danscher and Nørgaard (1983). After rinsing in aqua bidest containing 2.5% Na₂S₂O₃, the sections were stained in Gill’s hematoxylin for 15 s and in eosin for 30 s before mounting in Euparal. For negative controls, the primary antibody was replaced by normal serum of the same species in identical concentrations; this resulted in weak cytoplasmic staining (data not shown). The immunogold–silver complexes of the positively labelled nuclei were visualized by epipolarized light using a Zeiss Axioskop (Oberkochen, Germany) and the labelling index was determined as the ratio of labelled nuclei and the total number of nuclei in a total of 1274 cells of 48 cases subdivided into eight groups of the different types of neoplasia, extrafocal tissue and reference tissue.

2.4. Enzyme assays

For the histochemical detection of G6PDH and PGDH activity, the tetrazolium salt methods as described by Van Noorden and Frederiks (1992) were used at a modified incubation temperature of 22°C (instead of 37°C), the maximal natural environmental temperature of flounder. Before incubation, cryostat sections were adjusted to a temperature of 22°C for at least 10 min and all media and equipment were kept at this temperature during the experiment because preliminary studies had indicated that G6PDH reactions in fish liver are highly sensitive to temperature differences as small as 1–2°C. The incubation medium for the demonstration of PGDH (EC 1.1.1.44) activity according to Jonges and Van Noorden (1989) contained 18 g polyvinyl alcohol (PVA, weight average M, 70,000–100,000, Sigma) in 100 ml 0.1 M phosphate buffer (pH 8.0) and 10 mM gluconate-6-phosphate (Boehringer, Mannheim, Germany), 0.8 mM NADP (Boehringer), 0.45 mM methoxyphenazine methosulphate (Merck), 5 mM MgCl₂, 5 mM sodium azide and 5 mM tet-
ranitro BT (Sigma). The media were freshly prepared just before incubation, exactly as described in detail by Van Noorden and Frederiks (1992).

Tetranitro BT was added after being dissolved in a heated mixture of dimethylformamide and ethanol (final dilution of each solvent in the medium was 2%). The incubation medium for the demonstration of G6PDH (EC 1.1.1.49) activity was similar to that of PGDH but phosphogluconate was replaced by 10 mM glucose 6-phosphate (Merck) and the pH of the medium was 7.45. Control reactions were performed in the absence of substrate and in the presence of coenzyme. These enzyme histochemical methods have been rigidly validated for quantitative purposes. The amount of coloured final reaction product is a proper measure of enzyme activity to be able to subtract the conversion of endogenous substrates from the test reactions (Butcher and Van Noorden, 1985).

2.5. Image analysis and processing

Enzyme reactions in situ were measured by monitoring reduction of tetranitro BT into its coloured formazan over an incubation period of 5 min for G6PDH and 10 min for PGDH. Images were captured of a selected area in a section during incubation with 15 s intervals for G6PDH and 30 s intervals for PGDH. To avoid interference of stray light, glare and out-of-focus error, a setup described by Jonker et al. (1995) was adopted. A stainless steel ring (0.5 mm thick) was set on the slide with the section and filled with incubation medium (test or control) at the beginning of the incubation (T = 0) and a cover glass was placed on top. For each selected area analysed, reaction rates were calculated by subtraction of the control reaction from the test reaction obtained in consecutive sections. A 3-chip CCD colour video camera was (Sony, ATV Horn, Aalen, Germany) connected to the Zeiss Axioskop light microscope and coupled via a frame grabber (maximal size 786 × 512) to an image analysis system with the KS 300 software package (Kontron, Eching, Germany). The camera signal and set up were adjusted according to the recommendations of Chieco et al. (1994). Tissue sections were viewed in white light with a ×10 objective (NA 0.03), a stabilized power supply, an infrared blocking filter and a monochromatic filter of a wavelength of 585 nm (Van Noorden and Frederiks, 1992).

For the analysis of the reactions in time, grey value images were stored at intervals of 15 or 30 s on magnetic optical discs (Sony, Japan) using a macro recorded in the Kontron KS 300 image analysis software. Conversion of grey values to absorbance values, analysis of absorbance in time in selected areas in the sections and editing of the serial images for data visualisation were performed on a Macintosh 8100 computer using the public domain NIH image software program (written by Wayne Rasband at the National Institute of Health and available electronically via Internet by anonymous ftp from zippy.nlm.nih.gov or from Library 9 of the Mac < App forum on CompuServ and on floppy disc from NTIS, 5285 Port Royal Rd, Springfield, VA 22161, part number PB93-504868). Initial velocities were calculated by determining best-fitting curves with the software program Mac Curve Fit (Jonker et al., 1995). As a rule, second order polynomials were used mathematically
described as \( y = ax^2 + bx + c \) where \( b \) represents the initial velocity of the enzyme reaction at \( T = 0 \). For standardization and validation, a series of grey filters were used and their images were applied to convert grey values to absorbance values. Absorbance values were converted in absolute units of enzyme activity on the basis of the Lambert–Beer law: \( A = \varepsilon cd \) in which \( A \) = absorbance, \( \varepsilon = 19.000 \) (molar extinction coefficient of tetranitro BT formazan, 43), \( c \) = concentration of formazan and 1 mole of formazan is produced by 1 mole of substrate (glucose 6-phosphate or phosphogluconate) and \( d = 0.001 \) cm (section thickness). Initial velocities are expressed as \( \mu \)mol substrate converted per cm\(^3\) tissue per min.

Cytophotometric end point absorbance measurements were taken after 5 min of incubation in serial sections reacted for G6PDH and after 10 min for PGDH activity using the image analysis system Zeiss/Kontron KS 300 with the above described camera set up. Grey value measurements and conversion to absorbance values were performed with a sequence of functions recorded and executed by a macro in the KS 300 software. Photomicrographs of the distribution of formazan in the different types of lesions were taken with identical illumination and exposure time.


Differences between initial velocities of enzymes and PCNA labelling indices in different types of neoplastic lesions were tested with the distribution-free Kruskal–Wallis test for more than two groups and with the post hoc test according to Nemenyi for \( k > 3 \) and varying \( n \) in the subgroups and the chi-squared test. Correlations were analysed with the Spearman’s rank test and \( p < 0.05 \) was taken as the level of significance.

3. Results

3.1. Histology and histopathology

3.1.1. Histology of normal liver

The morphology of normal liver of flounder (\( Platichthys flesus \) L.) is largely similar to that of the teleost liver as described by Elias and Bengelsdorf (1952). Circular sinusoids with endothelium are surrounded by 6–10 hepatocytes (average diameter, 15 \( \mu \)m) with microvilli protruding in the space of Disse (Fig. 1 (a)). The organization is not in lobuli or acini as in mammalian liver (James, 1989).

3.1.2. Hepatocellular carcinogenesis

Liver tumours of flounder caught in highly contaminated areas show striking resemblance to experimentally induced tumours in mammals (Myers et al., 1987; Köhler et al., 1992). A clear sequence of cellular changes can be identified by staining properties of altered hepatocytes from early putatively reversible clear cells, acidophilic foci towards basophilic persistent foci progressing towards baso-
Fig. 1. Histology of flounder liver embedded in methacrylate and stained with hematoxylene and eosin, ×600. (a) Normal liver of flounder caught at the reference site showing well-organized tissue (H = hepato-cytes; S = sinusoid). (b) Transition of clear cell focus (CC) into a basophilic focus (BC) with lipid accumulation in the extrafocal liver tissue. Small arrows mark the borders of the focus. (c) Well-organized trabecular liver cell adenoma with small basophilic cells (BC). (d) Anaplastic liver cell carcinoma with enlarged neoplastic cells containing giant nuclei (arrowed).

philic trabecular adenomas and anaplastic carcinomas depending on the experimental period (Bannasch, 1976; Farber, 1980; Newborne, 1982; Bannasch et al., 1989).

Altered hepatocytes in flounder liver appeared at first in perisinusoidal and peribiliary zones which displayed early toxipathic lesions, like lipid accumulation, hydropic degeneration of hepatocytes and bile ducts and lytic necrosis. Initial stages of altered hepatocytes could only be localized with certainty by enzymic
Fig. 2. (a) Size of foci and nodules in relation to the growth pattern from well-organized (parenchymal) to trabecular, glandular and anaplastic patterns during progression towards malignancy. (b) Size of foci and nodules in relation to the staining properties from well-organized (parenchymal) cells, early clear and acidophilic cells to mixed type and persistent basophilic cell type. The total number of foci and nodules investigated is 185.

markers (G6PDH) and could then be analysed for morphological changes, whereas mixed cell and basophilic foci which were continuous with the extrafocal tissue were more easily localized. Transition of the clear vacuolated cell type to the ba-
sophilic type of focus was common in flounder liver (Fig. 1 (b)) suggesting that the clear cell type might be a precursor stage of the basophilic type comparable to mammalian carcinogenesis.

Mixed cell and basophilic foci changed into basophilic adenoma with increasing size with a well-organized trabecular structure and were clearly separated from extrafocal tissue (Fig. 1 (c)). Transient stages of mixed-type nodules towards purely basophilic types by increasing basophilia of the tumour cells accompanied by complete loss of an organized tissue structure in anaplasia was diagnosed as carcinomas which consisted of extremely enlarged basophilic pleomorphic cells with giant nuclei (Fig. 1 (d)) which invaded into the extrafocal parenchyma. Satellite-like small groups of basophilic cells, similar to early basophilic foci were localized in the extrafocal tissue close to the border of carcinomas. Correlating the size of nodules to the growth pattern showed that size of neoplasms was positively correlated with anaplasia (Fig. 2 (a), p < 0.0001; Spearman's correlation test).

During tumour progression, the proportion of basophilic cells increased as reflected by the appearance of mixed cell foci and exclusively basophilic nodules in relationship with the size of the nodules: their percentage ranged from 42% in nodules smaller than 1 mm to 64% in nodules 1–2 mm and up to 79% in nodules larger than 2–15 mm (Fig. 2 (b)). Due to the fact that foci and tumours of altered hepatocytes displaying all variations of staining properties and sizes occurred as well, the histopathological classification indicated only tendencies with respect to diagnosis and prognosis and was ridden with uncertainty.

For example, foci smaller than 1 mm can already display all variations of staining properties including basophilia. This raises the question whether small basophilic foci result either from an ordered sequence from clear/acidophilic cells through mixed cell foci, or are carcinomas in situ or protrusions (satellites) of invasively growing carcinomas. In order to acquire a better insight in these matters, metabolic changes in foci were taken into consideration as described in the next part of the study.

3.2. Extrafocal lesions.

Extrafocal liver lesions that are generated during toxic liver injury leading to carcinogenesis ranged from megalocytosis, nuclear polymorphism, accumulation of lipid (Fig. 1 (b)) and occasional fibrillar structures (macrotubules, Köhler, 1989; Köhler, 1990) with up to four nuclei per cell indicating inhibition of cell division in later phases. Progressive liver injury was accompanied by hydropic degeneration of hepatocytes and bile duct epithelial cells, necrosis and cholangio-fibrosis. which is reported to occur during medium and high carcinogen exposure in mammals in severe cases of necrosis (Bannasch, 1976). Loss of reserve substances (lipids, glycogen) as signs of starvation, and immigration and proliferation of macrophages were observed around the tumours in the later stages of carcinogenesis.
3.3. Metabolic alterations and cell proliferation

3.3.1. Reactions of G6PDH and PGDH in time

During the first part of the incubation period (180 s for G6PDH and 540 s for PGDH) test reactions in all types of lesions and livers tested in the presence of saturating substrate concentrations (10 mM) were virtually linear (Fig. 3 (a), (b)). Significantly higher initial velocities of G6PDH were found in healthy livers of animals caught at a less polluted reference site as compared with initial velocities in livers of animals caught at the contaminated site without neoplastic changes \((p < 0.05, \text{two-tailed}; \text{Fig. 4 (a), Fig. 5})\). Equally low initial velocities were recorded in extrafocal tissue of neoplastic livers of flounder caught in the contaminated area. However, in all stages of carcinogenesis from early foci, to well-organized trabecular basophilic tumours and anaplastic carcinoma significantly higher initial velocities of G6PDH were found in altered hepatocytes than in sur-

![Graphs showing reactions of G6PDH and PGDH over time.](image-url)
Fig. 4. Initial velocities of G6PDH (a) and PGDH (b) activity (median, 95% confidence interval) in flounder liver without neoplasms from reference site (A) and contaminated site (B) and with neoplasms from the contaminated site U to Z. U = extrafocal liver tissue, V = early clear cell/acidophilic foci (<1-2 mm ⌀), W = basophilic trabecular foci and nodules (2-7 mm ⌀), and carcinoma (7-12 mm 닷) with X = degenerative center of carcinoma, Y = basophilic rim of carcinoma, and Z = satellites (<1 mm 닷).

rounding extrafocal tissue (Fig. 4 (a) and Fig. 5). While early foci, basophilic rims of late stages of carcinomas and satellites showed similar elevated initial velocities of approximately 20 μmol G6P converted per min per cm³ tissue, well-organized trabecular basophilic tumors displayed significantly higher initial velocities (p < 0.05, two-tailed, Fig. 3 (a), Fig. 4 (a)).

Comparable, but less pronounced differences in initial velocities of PGDH in the different lesion types were found. Initial velocities of PGDH of contaminant-exposed livers without tumors were significantly lower than those of the healthy animals caught at the reference site (p < 0.05, Fig. 4 (b), Fig. 5). In contrast to G6PDH, initial velocities of PGDH in early foci did not differ significantly from the extrafocal tissue. Trabecular basophilic nodules showed highest initial velocities for PGDH (Fig. 4 (b)) similar to G6PDH (Fig. 4 (a)). Degenerative centers of carcinomas showed identical initial velocities as extrafocal tissues, while relatively high initial velocities were found at their rims and in satellites around the carcinomas as compared with extrafocal tissue surrounding the tumors (p < 0.05, one-tailed).
Formazan was homogeneously distributed in tissue compartments of healthy and diseased livers and in neoplastic changes up to the stage of adenomas (Fig. 5). In progressive anaplastic carcinomas different local metabolic compartments could be identified on the basis of initial velocities of G6PDH and PGDH (Fig. 4). Zones showing low velocities similar to those in extrafocal tissue were found in degenerating areas or centres of tumours while the cells at the rim and in the so-called satellites of the same tumours showed high metabolic rates (Figs. 3 and 4). Initial velocities of G6PDH were always higher than that of PGDH for all tis-
sue types. Livers without neoplasms and extrafocal tissue of livers with neoplasms showed a 2.5–2.9-fold higher initial velocity of G6PDH than of PGDH. This ratio increased to 4–4.5 in lesions with the exception of the centers of carcinomas.

The comparison of various diagnostic techniques to identify neoplastic changes showed significant differences with respect to their sensitivity. For the precise localization and quantification of altered foci and nodules during carcinogenesis, alterations of the G6PDH activities appeared to be the most reliable and sensitive techniques as compared with histopathological criteria and gross inspection (Fig. 6).

3.4. PCNA

Highly significant differences in PCNA labelling were found ($p < 0.0001$). In healthy livers from the reference site, in exposed livers without neoplasia as well as in extrafocal tissue of neoplastic livers, 3.5–9.6% were positive for PCNA (Table

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>Geometric mean</th>
<th>Range min–max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy liver</td>
<td>3.5</td>
<td>0–8.3</td>
</tr>
<tr>
<td>Non-neoplastic</td>
<td>9.6</td>
<td>0–25</td>
</tr>
<tr>
<td>Extrafocal</td>
<td>7.7</td>
<td>0–17</td>
</tr>
<tr>
<td>Early focus</td>
<td>42.8</td>
<td>34.5–58</td>
</tr>
<tr>
<td>Trabecular tumor</td>
<td>44.0</td>
<td>40–50</td>
</tr>
<tr>
<td>Carcinoma centre</td>
<td>11.2</td>
<td>0–26</td>
</tr>
<tr>
<td>Carcinoma rim</td>
<td>50.8</td>
<td>36–66</td>
</tr>
<tr>
<td>Satellite</td>
<td>64.7</td>
<td>55.5–74</td>
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</table>
1). PCNA expression increased significantly in early foci and in trabecular basophilic nodules \((d < 0.05\) two-tailed, Table 1). Hepatocellular carcinomas displayed a low mean-labelling index in areas of degeneration, but the periphery of carcinomas and satellites showed highest mean PCNA labelling index. Satellites around hepatocellular carcinomas showed a significantly higher labelling index of PCNA than the small foci not associated with carcinomas \((p < 0.001,\) one-tailed).

4. Discussion

Our results imply that metabolic changes and cell proliferation indices can be used as sensitive diagnostic and prognostic markers of toxic liver injury and carcinogenesis in the liver of flounder. Reduced G6PDH and PGDH activities were found in extrafocal liver tissue, degenerative areas of carcinomas and in contaminant-exposed livers without neoplasms as compared with the high NADPH-generating capacity in healthy livers of flounder from the reference site (Fig. 4). Reduction in activity of both enzymes (Hinton et al., 1988; Ono et al., 1963) and a low PCNA labelling index has been found in chemically induced precancerous conditions or in extrafocal liver in fish and rats (Farber and Sarma, 1987; Paolini et al., 1991).

Very early foci consisting of few hepatocytes were found easily because of their increased G6PDH activity before the foci could be clearly identified by morphological criteria. This is in accordance to the concept of using alterations of enzymes as early markers during carcinogenesis in mammals (Bannasch et al., 1981b; Bannasch et al., 1982; Bannasch, 1990a; Bannasch, 1990b). Evidence of the "preneoplastic" nature of early foci with high G6PDH activity is as follows:

1. Regularly, small foci with increased activity of G6PDH and elevated expression of PCNA appeared prior to development of tumours.
2. The basophilic cell type observed in transitional stages in early foci was also found in adenomas and carcinomas.
3. So-called early foci co-occurred with progressive stages of liver cell adenoma and carcinoma.
4. High initial velocities of G6PDH and high PCNA labelling index found in early foci persisted in hepatocellular adenomas and carcinomas.

The appearance of a basophilic cell type in foci and nodules in flounder liver was characterized by highest levels of both G6PDH and PGDH. In line with this finding, Moore et al. (1986) found highest activities of G6PDH in well-organized adenomas of rats. Elevated G6PDH activity with increasing basophilia has been documented in experimental carcinogenesis of estuarine fish species as well (Couch and Courtney, 1987; Hinton et al., 1988; Hinton et al., 1992). The initial transformational step towards basophilia leads to a persistent stage of a cellular pheno-
type in chemically-induced carcinogenesis which has the potential to progress to carcinomas at least in mammals (Newborne, 1982).

Immunohistochemical labelling of cell proliferation in flounder liver parallel with the enzyme studies revealed that satellites around carcinomas could be discriminated by high levels of PCNA labelling from early foci, the precursor stages of basophilic trabecular adenomas. The high proliferating rate in satellites indicated their invasive potential and their malignancy as described for mammalian carcinogenesis by Wolf and Dittrich (1992). When the satellite-like small groups of basophilic cells were followed in serial sections, it appeared that they were small protrusions of large carcinomas invading the extrafocal liver parenchyma (Table 1).

In flounder, the coincidence of increased NADPH-generating capacity and proliferative activity in foci and nodules in toxically injured liver tissue that itself showed a low capacity to generate NADPH and a low proliferation rate suggests a clonal adaption of initiated hepatocytes which became resistant to inhibitory effects of cell division by contaminants. Apparently, there are regulation mechanisms that facilitate adaption of selected altered cell populations to the toxic environment and guarantee proliferation and survival in a toxic environment by changes of NADPH-dependent metabolism of xenobiotics (Farber, 1990; Buchmann et al., 1985).

On the basis of the present quantitative in situ study of G6PDH and PGDH reactions in time, it can be concluded that end point measurements of enzyme reactions can be applied in epizoological studies because different capacities of enzymes in the various types of lesions are reflected by the amount of formazan precipitated after a set incubation time. G6PDH appeared to be the more reliable marker for the very early stages of altered hepatocytes in the multistep process of carcinogenesis. Further, increased velocities of NADPH-generating enzymes (G6PDH, PGDH) and PCNA labelling index demonstrated the potential of certain basophilic cell populations to develop towards carcinomas and to invade extrafocal tissue. Therefore, localization and quantification of metabolic alterations are very useful diagnostic and prognostic markers for altered foci in epizoological studies.

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