CELLULAR RESPONSES IN FISH LIVER AS INDICATORS FOR TOXIC EFFECTS OF ENVIRONMENTAL POLLUTION

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ABSTRACT

Flounder (Platichthys flesus L.) were sampled along a decreasing pollutant gradient from the inner boundaries of the Elbe estuary to the mouth of the river and the northern Wadden Sea area including the Eider estuary during interdisciplinary studies comprising cellular pathology of the flounder liver (light-and electronmicroscopy, cytochemistry) and chemical analyses of heavy metals and organochlorines. The results were related to the "exposure time" or life span of the juvenile flounder in the different parts of the Elbe river and the Eider estuary. Prolonged stay after immigration into the contaminated regions led to specific differences in the nature and incidence of the liver lesions. The degree of severity of the liver lesions were closely correlated to the accumulation of hepatotoxic and carcinogenic substances (Hg, PCBs, OCS, d,y-HCH, DDE). Electronmicroscopic revealed subcellular responses indicating additive and antagonistic effects of different pollutant classes on cell organells. The experimental regeneration of the liver was significantly correlated with the elimination of lipophilic compounds (PCBs, HCB, OCS, d,y-HCH). Livers of flounder caught in the Eider estuary revealed no or only minor liver alterations; contaminant levels varied in low concentrations reflecting natural background levels. Our studies of normal and regenerating liver structure, as well as the pathological phenomena indicated a key role of the intracellular digestive and detoxifying system, - the lysosomal compartment-, in the cellular responses to anthropogenic xenobiotics. The lysosomal system functions as unspecific immune system of each eucaryotic cell and is specifically developed in central metabolic detoxifying organs, such as the fish liver. A test battery for measuring lysosomal alterations (lysosomal membrane stability, size and number) was applied in order to relate tissue and cellular pathology to fast and practicable cytochemical indices reflecting the biochemistry of the injured tissue for the biological effects monitoring. The results revealed a highly significant negative correlation between the stability of the lysosomal membrane and the degree of degenerative and preneoplastic liver lesions. Highly significant differences were identified with respect to lysosomal stability between the Elbe stations and the Wadden Sea stations independently from the season and/or reproductive stage of flounder. Investigations of cellular and cytochemical responses of the fish liver has to be regarded as an essential tool in fish disease studies for the identification of sublethal lesions induced by anthropogenic contaminants for the biological effect monitoring.
INTRODUCTION

The Elbe is one of the most polluted rivers in Europe and is characterized by a decreasing gradient of contaminant levels in water and sediments towards the mouth of the estuary and the northern Wadden Sea area (2,3,18). The interdisciplinary studies of the past years intended to link different methods of environmental research to test their sensibility to reflect early responses of the fish liver to anthropogenic stressors. These methods include the stepwise identification of pollution-induced changes on the organ, cellular and biochemical level by gross and microscopic (LM/TEM) morphology, cytochemistry / biochemistry (lysosomal stability, MFOs) and chemical analyses of representative contaminants.

Selected data on the cellular effects observed in the liver of flounder caught along a pollution gradient from the inner boundaries of the Elbe estuary (station 1) to the mouth (station 2), northward in reference areas at the Wadden Sea coast (station 3 and 5) and in the Eider estuary (station 4) are reported (Fig. 1). Based on the findings that the lysosomal digestive and detoxifying system of the liver cells play a key role in the cellular response to different contamination situations, a test battery measuring lysosomal perturbation was applied to the fish liver. Lysosomes are able to respond to a wide range of anthropogenic hepatotoxic and carcinogenic substances introduced into the aquatic environment, such as PAHs, organochlorines and heavy metals e.g. Cytochemical/biochemical techniques for measuring lysosomal changes were tested with respect to their sensitivity and practicability for the biological effects monitoring.

MATERIAL AND METHODS

During the initial phase of our studies different age groups of flounder (0, I, II, III) were investigated in order to detect early sublethal responses and their progression towards severe liver lesions in relation to the "exposure time" in the differently contaminated areas. Exclusively, flounder before the first sexual maturity were selected in order to exclude cellular and biochemical effects in the liver during the spawning cycle. Pieces of liver tissue were processed for LM and TEM. All data on contaminant concentrations (PCBs, HCB, d,y-HCH, OCS, Hg) referred to in this paper were already published (9). For the regeneration experiments flounder 1 year of age were caught in the highly contaminated Elbe region downstream of Hamburg and maintained under contaminant-free conditions up to forty days in a flow- through system. Liver parameters (liver cell size, lipid content, histopathology, ultrastructure) were measured after 5, 10, 20, 40 days of maintenance. Concentrations of HG, OCS, HCB, d,y-HCH, PCBs in the livers and muscle tissue were parally analysed.

For the investigation lysosomal perturbations, flounder (17-25 cm, each 25 individuals, females) were caught along the contaminant gradient from the mouth of the Elbe river towards the northern Wadden Sea (Fig. 4). At two stations (2 and 3) seasonal variations of lysosomal stability were investigated every 2 month during 2 years in each 25 individuals. Lysosomal enlargement and increase in number was measured with a Kontron MOP Videoplan and the numerical and volume density was calculated. The test for lysosomal stability was applied according to Bitensky (1972) for N-Acetyl- β-hexosaminidase(5,13).
RESULTS

Prolonged duration of stay in the highly polluted part of the Elbe estuary, near the city of Hamburg (st. 1) was associated with dramatic increase of liver damage including steatosis, extensive necrosis and preneoplastic changes (megalocytic hepatosis, eosinophilic and hyperplastic foci) in 60 - 70% of the older age groups (Table 1). Compared to normal ultrastructure these lesions included: Extensive disorganisation of the RER, proliferation of a smooth membrane system associated with lipid droplets, atrophy of Golgi complexes and inhibition of lysosomal formation, apparent increase of lipofuscin granules, mitochondrial degeneration, and cytoskeletal perturbations (macrotubule formation in the RER lumina) (Table 2). The extension of pathological changes with age was accompanied by an age-dependent accumulation of hepatotoxic substances in muscle tissue (fresh weight) up to maximal levels of 3100 µg/kg PCBs, 1907 µg/kg HCB and 905 µg/Hg.

The flounder population permanently inhabiting the less polluted area near the mouth of the Elbe estuary (st. 2) showed only minor changes reflecting the morphological correlates to detoxification processes (increase of RER, proliferation of Golgi complexes, increased number of lysosomes). No further accumulation of contaminants above the age of 1 year were seen. Seasonal observations at this station revealed that in late summer a second type of liver lesion (compare table 1/st. 2) occurred closely resembling the tissue and cell phenomena characteristic for station 1. Besides, clear signs of an initial reorganisation and detoxification processes could be identified: An extensive development of tubular SER and reorganisation of RER as parallel stacks, the dissolution of macrotubular structures (paracrystals), enlargement of Golgi complexes and increase of lysosomes. Nearly identical patterns were observed during the experimental regeneration of the liver of flounder caught at the highly polluted area of the Elbe estuary (st. 1). Ranges of contaminant levels of flounder caught at station 2 only in late summer and autumn closely resembled annual ranges of station 1 flounder (Fig. 1). Dramatic oxygen deficiencies in the region near Hamburg likely led to downstream migration towards the mouth of the estuary thereby mixing of two differently contaminated flounder populations occurred. Livers of flounder caught in the reference area (st. 4) showed only minor changes (Table 1, 2). Hepatocytes displayed a characteristic compartmentation of mitochondria. Golgi complexes with few small lysosomes and an oval nucleus surrounded by parallel stacks of RER, and of large fields of reserve substances (mainly d-glycogen). Contaminant concentrations reflected natural background levels for Hg and only slightly enhanced levels for PCBs and correlated lipophilic substances.

Flounder caught at station 1, kept under contaminant-free conditions and fed ad libitum with uncontaminated food indicated initial and complete liver regeneration in 50% of the individuals after 20 days, and in 70% after 40 days. The signs of regeneration diagnosed at the light- and electronmicroscopic level (digestion of necrotic cells by apoptosis/lysosomes, initiation of detoxification processes/SER and lysosomes, reorganisation of cell structure, decrease of lipids, glycogen storage) were accompanied by a significant decline in the concentrations of PCBs and correlated lipophilic substances (Fig. 2). Binding of Hg in a non-toxic form, presumably by metal-binding proteins and lysosomes obviously occurred during the regeneration process.
Damage to the lysosomal membrane or overloading of the storage capacity by a variety of toxic compounds leads to increased fragility of the lysosomal membrane in mammals and invertebrates with subsequent release of degrading enzyme to the cytosol and catabolism of cell components leading to cell death (1, 13, 15, 19). Our findings in normal, regenerating and injured flounder livers suggested a basic role of lysosomes in adaptive responses to anthropogenic contaminants.

The observation of the seasonal variations of lysosomal stability at two sites (Stations 2 and 3) indicated decreased lysosomal membrane stabilities during winter. This fact coincided with the appearance of maturing female flounder in the catches from October until March. Therefore, the seasonal data were sorted according to the activity and pause in gonadal growth and vitellogenesis. During the two years of investigation, significant differences could be statistically identified between the phases of activity and pause in vitellogenesis, with respect to lysosomal stability at both stations (Fig.3). Nevertheless, the differences in lysosomal stability between the differently contaminated flounder populations of these two stations (ICES C.M.1990/E:29) remained statistically significant, independently of the phases of vitellogenic activity. Electron microscopic studies revealed the participation of the lysosomal compartment in the processes of vitellogenesis in liver.

The results of lysosomal perturbations in flounder liver caught along a suspected pollution gradient in summer 1989 are summarized in Figure 4a-d. Highly significant differences between the three stations were found with respect to lysosomal stability with lowest membrane stability in the Elbe estuary and increasing values towards the northern station 5. A similar northward gradient could be observed with respect to lysosomal enlargement and numerical density. Electron microscopic studies affirmed the pronounced differences in lysosomal cytochemistry by lysosomal morphology at the three stations. In healthy flounder livers, a few small lysosomes with mainly autophagic activity were located near pericanalicular Golgi complexes. These ultrastructural findings were accompanied by a considerable membrane stability of up to 40 minutes in some individuals. No pathological alterations could be identified in these livers.

In contrast, liver cell lysosomes of flounder caught in the Elbe estuary drastically increased in number and size. There was a considerable accumulation of pigmented material (melanin, lipofuscin), injured cell components and unsaturated neutral lipids. A highly significant negative correlation ($r = -0.63$ to $r = -0.80$, n=25) was found between the enlargement of lysosomes and the number of lysosomes at Stations 2 and 3 (Fig 4a, b), indicating that new lysosomal formations were retarded and that lysosomes displayed an increased rate of fusion. Also, highly significant but positive correlations were calculated for lysosomal size and for pathological accumulation of unsaturated neutral lipids ($r = +0.68$, n=25), confirming our anticipation that lipid accumulation was intralysosomal. Interestingly, no correlation between lysosomal stability, size, number with the lipid content could be statistically identified at these stations.

Highly significant correlations were found between the lysosomal membrane stability and the degree of histopathological liver lesions (Fig. 4c, d) at all stations ranging from normal and minor reversible changes (stages 1 and 2) to necrosis, fibrosis, cytoskeletal changes, megalocytic hepatosis, caryomegaly, lipid accumulation (stages 3 and 4) to heavy steatosis, fat necrosis and cirrhosis (stage 5) and eosinophilic, clear cell and basophilic foci (stage 6) (Table 3, ICES C.M. 1990/E:29). As shown in
Figure 5 normal or slightly and reversible altered livers displayed a high stability of the lysosomal membranes. The onset and progress towards degenerative and preneoplastic changes is reflected by the breakdown of the membrane stability of liver lysosomes.

**DISCUSSION**

The Elbe, one of the most polluted rivers in Europe is characterised by a decreasing gradient of contaminant levels in water and sediments towards the mouth of the estuary and the Wadden Sea. Prolonged duration of stay after immigration into the differently polluted regions led to significant differences in the nature and incidence of liver lesions in flounder. The degree of severity of liver lesions was closely correlated to the accumulation of certain characteristic hepatotoxic and - carcinogenic contaminants (Hg, PCBs, HCB, OCS, d,y-HCH and DDT; 9). Electronmicroscopy revealed subcellular responses indicating additive and antagonistic effects on cell organelles due to the specific contamination situation (12). Our results suggest that specific pathological responses of the cell and patterns of contaminants may function as markings in migrating fish populations.

The morphometric, histological and chemical studies indicated that transfer of flounder caught in a highly contaminated area of the Elbe into a contaminant-free environment induced increased activity of biotransformation and detoxification in fish liver cells (SER/MFOs; lysosomes). It is concluded that metabolically resistant or slowly metabolised contaminants analysed exemplarily in this flounder population apparently induced the considerable hepatotoxic effects observed (10).

Lysosomes are able to accumulate and sequester a wide range of organic and inorganic compounds as well (for review see 1, 13) in contrast to other detoxifying systems. Besides, the lysosomal system has basic functions in cell catabolism, as well as in transport and synthesis of macromolecules and in the unspecific immune response. Therefore, damage of the lysosomal system impairs the normal functions of hepatocytes resulting in metabolic disorders, cell transformation or in cell death.

The investigation of seasonal effects on the lysosomal system clearly demonstrated the participation of the lysosomal system in the synthesis of the yolk-precursor protein vitellogenin in the flounder liver during gonadal growth. Biochemical data on RNA, DNA and glycogen in flounder liver during yolk-precursor protein synthesis (8, 17) fit well to morphological alterations observed in this study, described and discussed in detail elsewhere. The lysosomal system in fish liver appears to be more stable against the physiological changes during reproduction than it is documented for that of the marine mussel (14). Though the contamination effects on the lysosomal system are not superimposed by the demands on the lysosomal system during vitellogenesis, the monitoring of fish populations during identical physiological phases (feeding- or reproduction period) is recommended.

The results of the test battery for lysosomal perturbations applied during this study reflected a clear gradient from the Elbe estuary in the direction of the northern Wadden Sea stations. This gradient coincided with the results of the chemical analyses of heavy metals and organochlorines in flounder liver measured during the present study as indicator substances for the contamination situation in the Wadden Sea area (U. Harms / K. Soffker, unpublished data; ICES C.M.1990/E29, 9).

In fish hepatocytes, only few and small lysosomes can be identified under normal conditions, whereas the hepatopancreas of marine mussels
and snails for example generally possess a well-developed lysosomal system based on the function of endocytosis of nutrients. In contrast to marine invertebrates a clear "two step response" of the lysosomal system could be detected in flounder liver. The activation of the lysosomal system was reflected by an increase in number and size of lysosomes which accumulate foreign compounds and lipids in the attacked liver. This first step represents an adaptive and protective response to injury.

Decreased membrane stability led to release of degrading enzymes into the cytoplasm and nucleoplasm, reflecting the overloading or damage of the lysosomal digestive and detoxifying system. This second step of lysosomal response indicated the injury of this central cell compartment, resulting in severe liver lesions. Megalocytic hepatitis and caryomegaly, were accompanied by such liver degenerations as necrosis, fibrosis and steatosis and have to be regarded as putative preneoplastic lesions in fish liver (6,7,16). The significant negative correlation between the lysosomal stability and the extension of liver lesion indicates that the lysosomal stability test clearly reflects the overcharge and breakdown of the detoxifying capacity of liver (11). These results are additionally confirmed by observations during the present study that the preneoplastic changes observed in younger flounder (17-25 cm) progressed towards benign and malignant liver nodules, as cholangioma and cholangiocarcinoma, hamangioendothelioma and angiosarcoma, such as liver cell adenoma and hepatocellular carcinoma in female flounder over 30 cm total length.

Based on this results the measurement of lysosomal perturbations in fish liver can be recommended as a integrative biological warning system for the biological effects monitoring responding to different classes of pollutants. Increased lysosomal activity represents an adaptive response to a sublethal toxic cell injury, comparable to the increased activity of the MFO system under specific contamination conditions. But additionally, lysosomal membrane stability in fish liver sensitively reflects the damage and breakdown of the adaptive detoxifying capacity of liver cells leading to damage of cell function and irreversible pathological alteration. Therefore, the 'lysosomal labilisation test' could be used as a sensitive and highly practicable tool for the future monitoring of fish populations.

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REFERENCES


**Fig. 1.** Frequency distribution of contaminant concentrations in muscle tissue of flounders from Station 1 and 2. Open bars = summer samples of Station 1 and 2; hatched bars = spring samples at Station 2 superimposed for comparison. x-axis: relative frequency (left) and total number (right) of individuals analysed.

**Fig. 2.** Mean concentrations of contaminants in flounder liver (µg/kg wet weight) caught in the Elbe estuary (habitat) in comparison to the mean contaminant levels of non-regenerating (nr), initially regenerating (ir) and complete regenerating livers (cr) during 40 days of contaminant-free maintenance.
PHASES OF VITELLOGENESIS

Fig. 3

Lysoosomal activity

Fig. 4

Lysoosomal activity

Lysoosomal estrogens

Lysoosomal stability

Liver histopathology

Stations
Fig 5: Liver lesion of flounder caught in the German Wadden Sea in relation to lysosomal membrane stability measured in identical individuals. N=75

<table>
<thead>
<tr>
<th>Grades of liver lesions</th>
<th>station 1</th>
<th>station 2</th>
<th>station 4</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>age groups</td>
<td>age groups</td>
<td>age groups</td>
</tr>
<tr>
<td>0 Normal liver structure</td>
<td>Oa Ob I II III</td>
<td>Oa Ob I II III</td>
<td>Oa Ob I II III</td>
</tr>
<tr>
<td></td>
<td>85 100 12 71 10.7</td>
<td>81 68 66.6 62.5 n.e.</td>
<td></td>
</tr>
<tr>
<td>1. Normal structure, cellular oedema</td>
<td>15 7</td>
<td>28 22.2 7.1 14</td>
<td>9 32 16.8 25</td>
</tr>
<tr>
<td>2. Single dark staining shrunken hepatocytes, perisinusoidal lipid accumulation</td>
<td>7</td>
<td>21.4 11.1 14.2 10.9</td>
<td>16.8 12.5</td>
</tr>
<tr>
<td>3. Cord structure dissolved in large areas, network of dark cells, accompanied by specific lesions a-f for stat. 1 and a-c for stat. 2</td>
<td>45.6 43 28.5 37.5</td>
<td>24.4 66.7 57.1 32.1</td>
<td></td>
</tr>
<tr>
<td>4. Complete disintegration of the parenchymal structure accompanied by specific lesions a-g for stat. 1 and a-g for stat. 2</td>
<td>64.6 43 71.7 62.6</td>
<td>14.2 14.5 32.1</td>
<td></td>
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</tbody>
</table>

TABLE I
FREQUENCY (%) OF LIVER ABNORMALITIES IN FLOUNDER CAUGHT AT DIFFERENTLY CONTaminATED AREAS OF THE ELBE ESTUARY (STATIONS 1 AND 2) AND IN A REFERENCE AREA (STATION 3) AGE GROUPS: Oa. 3 cm, Ob 9 cm, I. 14 cm, II. 19 cm, III. 25 cm TOTAL LENGTH: N = 150
<table>
<thead>
<tr>
<th>Organellar cell types</th>
<th>Station 1</th>
<th>Station 2</th>
<th>Station 2*</th>
<th>Station 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm and nuclei</td>
<td>Hepatocytomegaly, network of dark shrunken cells with condensed cytoplasm, pychosis, caryorrhexis</td>
<td>Basophilic cells, single dark shrunken cells around sinusoids and bile ducts, occasional pychosis, indented nuclei</td>
<td>Decrease of shrunken hepatocytes, occasional pychosis</td>
<td>Occasional dark staining cells</td>
</tr>
<tr>
<td>Lipid</td>
<td>Extremely large lipid droplets of low osmophilia, penetrated by smooth membranes</td>
<td>Small ome of lipid droplets</td>
<td>Large lipid droplets, degraded in fingerprint-like membrane configurations</td>
<td>Single small lipid droplets of moderate osmophilia</td>
</tr>
<tr>
<td>Glycogen</td>
<td>Drastic diminution, monoparticular glycogen</td>
<td>Small fields of β-glycogen</td>
<td>Single rosettes, β-glycogen</td>
<td>Large fields of α-glycogen</td>
</tr>
<tr>
<td>Rough ER</td>
<td>Degranulation, aggregates of free polyosomes, disorganisation into sinusoidal and circular profiles</td>
<td>Increase in RER, densely occupying the cytoplasm, free polyosomes</td>
<td>Reorganisation of RER in parallel stacks</td>
<td>Parallel stacks of RER around nuclei and mitochondria</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>Tubular SER at the periphery of the smooth stacks of RER still present</td>
<td>Tubular SER in the transcytotic area in the Golgi region</td>
<td>Proliferation of tubular SER</td>
<td>–</td>
</tr>
<tr>
<td>Membrane configurations</td>
<td>Extensive proliferation of unorganised tube-like smooth membrane systems in association to lipid droplets</td>
<td>Single myelin-like membrane arrays in mitochondria</td>
<td>Fingerprint-like membrane configurations in lipid droplets</td>
<td>–</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Dark swelling, irreversible degeneration into dark flocculent bodies, smooth vesicles</td>
<td>Clear reversible swelling, myelin-like degradation</td>
<td>Regeneration by elongation and budding with subsequent division</td>
<td>–</td>
</tr>
<tr>
<td>Golgi complexes</td>
<td>Atrophy, vacuolation</td>
<td>Increase in size and number of Golgi complexes indicating high stimulation</td>
<td>Partial atrophy, single active Golgi complexes</td>
<td>–</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>Few primary lysosomes, increased transformation into lipophagic granules</td>
<td>Extensive formation of lysosomes at the Golgi complexes, large number of secondary lysosomes and residual bodies</td>
<td>Formation of primary lysosomes</td>
<td>Single autolysosomes</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>Significantly enlarged microtubules (up to 34 nm) in RER lumen</td>
<td>No change of microtubules (15 nm)</td>
<td>Single enlarged microtubules (14 nm) in RER lumen, which transform into ISER</td>
<td>–</td>
</tr>
<tr>
<td>Space of Disse</td>
<td>Microvilli regression, increase of collagen fibres</td>
<td>Dense microvilli border</td>
<td>Slight microvilli regression</td>
<td>–</td>
</tr>
<tr>
<td>Sinusoidal endothelium</td>
<td>Atrophy, necrosis</td>
<td>Extensive proliferation of phagocytotic endothelial cells to MMCs</td>
<td>Atrophy</td>
<td>–</td>
</tr>
<tr>
<td>Bile ducts and canaliculi</td>
<td>Necrosis, microvillous regression</td>
<td>Unaffected</td>
<td>Partial bile duct necrosis</td>
<td>–</td>
</tr>
</tbody>
</table>