P53 Overexpression as an Indicator of Overall Survival and Response to Treatment in Osteosarcomas

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The p53 gene located at chromosome 17p13 is found to be altered (allelic loss or other mutation) in multiple human cancers, including osteosarcomas. The mutated gene produces a protein with a prolonged half-life thus rendering it detectable by conventional immunohistochemistry. We examined the correlation between p53 expression and clinical prognosis as well as response to therapy. Twenty-one patients with previously untreated and histologically verified highly malignant osteosarcoma were used for this study. Biopsy material taken both prior to the start of COSS 91 protocol and at the time of surgery (ten weeks later) was examined for alterations in p53 protein expression and drug resistance. Two patients who had strong (+++) p53 protein expression and three others who became positive during the chemotherapy had significantly worse prognosis (all of them died within one year) than those who showed no p53 expression both at biopsy and after chemotherapy (all 11 patients are alive, average follow-up time: 3.5 years). All patients who showed any kind of positive p53 protein expression on initial biopsy were non-responders to chemotherapy. In contrast, 69% (9 out of 13) of those who exhibited no p53 expression on initial biopsy were responders or intermediate responders to chemotherapy. We concluded that p53 expression may be a useful prognostic factor in osteosarcomas. The direct correlation between p53 positive expression and resistance to therapy can help in identifying patients who are in need of a more vigorous or different chemotherapeutical protocol. (Pathology Oncology Research Vol 3, No 1, 13–19, 1997)

Key words: osteosarcoma, p53 overexpression

Introduction

In recent years, many genes have been discovered to play a part in the development of cancer. Mutations at chromosome 17p13 are probably the most common gene lesions in human cancers.13,16,24 This is the location of the p53 gene which appears to act both as a tumor suppressor gene and an oncogene, depending on the circumstances. The p53 function is lost in transformed normal cells when both normal (wild type) alleles of the tumor suppressor gene are lost or inactivated.4 The presence of one wild type copy of the gene is sufficient to interfere with onco-

Received: Feb 22, 1997, accepted: March 23, 1997
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Table 1. P53 protein expression in stage IIA and IIB osteosarcomas

<table>
<thead>
<tr>
<th>Biopsy material</th>
<th>Surgical specimen</th>
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<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>+++++</td>
<td>+++++</td>
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</tbody>
</table>

IIA  11 | 11 | 3 | 9 | 5 | 1 |

IIB  11 | 1 | 3 | 2 | 9 | 5 | 1 | 2 |

cell growth at the G1 phase. P53 plays a more important role in the G1-S checkpoint control of stressed cells (where p53 levels are increased) than of normal cells. It facilitates DNA repair in cells exposed to DNA damaging agents, by terminating the replication and allowing time for repair. In addition, there is a clear correlation between p53 gene dosage and resistance to radiation induced apoptosis. Cells containing only one copy of the p53 gene are slightly more resistant than cells that are homozygous for p53 gene and p53 deficient mice with absence of both active copies are the most resistant. This implies that cells with one somatically mutated p53 allele may have growth advantage in the presence of DNA damaging agents over cells with two intact alleles. Furthermore, p53 can act as a transcription factor. Genes regulated by p53 are important in cell cycle progression, monitoring of gene amplification events and in the commitment of some cells to apoptosis. Mutant p53 proteins are incapable of activating transcription from a template and in fact they block this activity of wt p53 by complexing with it.

Loss of alleles at the p53 gene site (17p13) is seen in more than 75% of osteosarcomas (OS), suggesting that this alteration may contribute to the development of OS. OS is the most frequent bone tumor in children and young adults, representing 8% of all childhood malignancies. In regards to its etiology, genetic factors are especially important in children. For example, 40% of patients with retinoblastoma have a hereditary predisposition to other cancers. These patients with a mutation in chromosome 13q14 have a 500 times greater risk of developing OS than the normal population.

Prior to effective chemotherapy the prognosis of OS patients was very poor; the 2-years overall survival ranged 5% to 20%. However, with current treatment schedules the 5-year disease free survival and overall survival rates have increased to about 60 to 80%. In these days the most common cause of death among OS patients is failure to respond to chemotherapy. The reason is that tumors become multidrug resistant. To achieve a better therapeutic success rate it is important to select those patients with a worse prognostic outlook and those with a higher risk for multidrug resistance, in order to start a modified and more aggressive therapy regimen for them.

In this retrospective immunohistochemical study we examined the expression of p53 protein and the loss of heterozygosity of p53 gene in 21 OS patients. The aim was to evaluate the correlation between p53 expression and prognosis as well as the correlation between p53 expression and multidrug resistance.

Materials and Methods

Patients

Biopsy material was obtained from 21 patients (11 males, 10 females; age: between 15 and 24 years, with an average of 20 years) with previously untreated highly malignant OS verified by histology. The location of the primary tumor in descending order of frequency was the distal femur 43% (n = 9), proximal tibia 33% (7), proximal humerus 19% (4) and sacrum 4% (1). The occurrence rate of the different histologic variants of osteosarcoma was as follows: 12 osteoblastic (61%), 3 mixed cell (14%), 2 fibroblastic (10%), and 1 periosteal, teleangiectatic and small cell (5%, respectively). According to the Enneking surgical staging system 17 tumors (81%) were in stage IIB and 4 (19%) in stage IIA.

Treatment

After establishing diagnosis the COSS 91 protocol was started. This consisted of 9 weeks of neoadjuvant chemotherapy, surgery on the 10th week and 14 more weeks of chemotherapy.

Table 2. Correlation between p53 expression and response to chemotherapy

<table>
<thead>
<tr>
<th>Case</th>
<th>p53 expression</th>
<th>response to chemotherapy</th>
<th>survival after chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++</td>
<td>NR</td>
<td>10 mo, died</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
<td>NR</td>
<td>1 yr, died</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>NR</td>
<td>1.5 yr, died</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>R</td>
<td>3 yr</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>IR</td>
<td>3.2 yr</td>
</tr>
<tr>
<td>6</td>
<td>++</td>
<td>NR</td>
<td>2.3 yr #</td>
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<tr>
<td>7</td>
<td>-</td>
<td>IR</td>
<td>3.5 yr</td>
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<tr>
<td>8</td>
<td>-</td>
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<td>R</td>
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<tr>
<td>10</td>
<td>-</td>
<td>NR</td>
<td>1 yr, died</td>
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<tr>
<td>11</td>
<td>-</td>
<td>no preop chemoth 4 yr</td>
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</tr>
<tr>
<td>12</td>
<td>-</td>
<td>NR</td>
<td>2.5 yr</td>
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<tr>
<td>13</td>
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<td>no preop chemoth 2.5 yr</td>
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<td>14</td>
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<td>R</td>
<td>2 yr</td>
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<tr>
<td>15</td>
<td>++</td>
<td>NR</td>
<td>1.3 yr, died</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>NR</td>
<td>5 yr</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>IR</td>
<td>1 yr, died</td>
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<tr>
<td>18</td>
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<td>7 yr</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>IR</td>
<td>2.5 yr</td>
</tr>
</tbody>
</table>

# died from other reason
NR: non-responder; IR: intermediar responder; R: responder

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weeks of adjuvant chemotherapy using a combination of Ifosfamid (3 g/m²), Adriamycin (30 mg/m²), Cisplatin (120 mg/m²), and high-dose Methotrexate (12 g/m²).15

Immunohistochemistry

The p53 antigen was detected using the streptavidin-biotin-alkaline phosphatase immunostaining method. The paraffin sections were routinely deparaffinized and pretreated, as described by Cattoretti et al.2 by microwaving at 750 W in 10 mM citrate buffer, pH 6.0. After incubation for 20 min at room temperature with 1% BSA to block the non-specific binding of the reagents, a monoclonal mouse anti-human p53 antibody (DAKO-clone DO-7) was applied at a dilution of 1:25 at 4°C overnight. This antibody reacts with both wild and mutant types of p53 protein. Slides were washed three times in Tris buffer (pH 7.6) and incubated with biotinylated anti-mouse antibody (Amersham) for 30 min at room temperature and washed twice with Tris buffer. Last, they were incubated for 45 min at room temperature with the streptavidin-biotin-alkaline phosphatase. The enzymatic activity was detected by incubation with its substrate (New fuchsin, DAKO). Endogenous alkaline phosphatase (AP) was inhibited by the addition of levamisol (DAKO) to substrate. Specimens were slightly counterstained with Mayer's haematoxylin, and mounted with glycerol gelatin. Sections of ovarian carcinoma known to contain mutant P53 protein were used as a positive control. The same slides after omitting the primary antibody, were used as a negative control. In addition, 4 testes removed for non-neoplastic disease and 10 uninvolved normal tissues, not adjacent to tumor location, were used as additional control. All controls gave satisfactory results. Three slides of each tumor were evaluated by two of the authors without knowledge of the clinical data, and the average value was considered.

P53 scoring

Since the number of cells labelled may be more meaningful than the intensity of staining per se 11 and since the latter differed from one slide to another and sometimes, within different areas of the same slide, we were encouraged to exclude the intensity of staining from the interpretation of our results. The extent of staining was evaluated as the percentage of positively stained versus total number of tumor cells in five adjacent high power fields at a magnification of (x 400) as recently described by Lipponen et al.18 First the entire section was screened carefully and counts were performed in representative areas, i.e. regions with the maximum fraction of positively stained cells, well fixed and free of background. Stromal components were avoided by comparing the section with the hematoxylin and eosin-stained counterpart. Tumors according to their extent of staining were classified as: (−) no obvious positive staining; (+) 5% or less of tumor cells are positive; (++ 5-50%) of tumor cells are positive; (+++) more than 50% of tumor cells are positive.2 The change in p53 protein expression was determined both in the initial biopsy specimen as well as in the surgical biopsy specimen.

In an attempt to evaluate the effectiveness of the preoperative chemotherapy and thereby multidrug resistance, patients were also divided into three groups according to the modified Salzer-Kuntschik method22 where the percent of surviving tumor cells was determined in the surgical biopsy specimen. The three groups were: responder (living tumor cells less than 10%); intermediate responder (living tumor cells 10-50%); and non-responder (living tumor cells more than 50%). Therefore, non-responders were designated as multidrug resistant neoplasms.

Results

Alteration in p53 protein expression was observed in 29% (6/21) of initial biopsies and 43% (9/21) of surgical specimens. There were no p53 positive biopsy materials among the stage IIA tumors (Table 1). Among the stage IIB tumors 35% (6/17) showed positive p53 expression. In the surgical specimens only one case was p53 positive (+) among the stage IIA tumors. In contrast, 47% (8/17) of the stage IIB tumors were p53 positive. Table 2 shows the p53 protein expression, measure of response to chemotherapy and survival time for each of the 21 cases.

Correlation between p53 expression and survival time

When biopsies showed positive p53 protein expression a shorter mean survival time was perceived (Table 2). Both ++ cases had metastases and local recurrences and died within 1 year. One patient with ++ in both specimens, survived only 1.5 years after having metastases and local recurrences. Two other biopsies were ++. One of them became + by the time of surgery and also survived only 1.5 years. The other turned negative by the time of surgery, but died 2 years later of a different cause. Therefore, the ++ cases had a longer survival time than the +++ cases. Only one case was + in both specimens and despite a non-responder status, the patient is still living after 7 years. Essentially, all initial biopsies that showed ++ or +++ p53 overexpression were non-responsive to chemotherapy and all the patients have died.

15 cases showed no p53 expression on initial biopsy, 4 cases became p53 positive (+) after chemotherapy. Three of these cases died within 1 year, however the remaining p53 negative patients are alive after a mean follow-up time of 3.5 years.
Table 3. Correlation between p53 expression and response to chemotherapy

<table>
<thead>
<tr>
<th>p53 expression in biopsy material</th>
<th>response to chemotherapy (according to morphology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++++</td>
<td>non-responder 0 0</td>
</tr>
<tr>
<td>+++</td>
<td>intermediate responder 0 0</td>
</tr>
<tr>
<td>++</td>
<td>responder 0 4 4</td>
</tr>
<tr>
<td>+</td>
<td>responder 5 5 4</td>
</tr>
</tbody>
</table>

Correlation between p53 expression and response to treatment

The sensitivity to treatment is summarized on Table 3. Only 19 of the 21 cases have information about the response to chemotherapy because 2 patients did not receive preoperative chemotherapy. As seen both in Table 2 and Table 3, all the p53 positive cases fell into the non-responsive category. This demonstrates a positive correlation between p53 overexpression and multidrug resistance.

Among the patients who showed no p53 overexpression, 31% were responders, 38% were intermediate responders and 31% were non-responders. None of the responders turned p53 positive after receiving chemotherapy and all survived, with the mean follow up time of 3.1 years. In the intermediate responder group 40% turned positive (+) after chemotherapy and all but one patient survived.

In the non responder group 2 of the 4 patients who were initially p53 negative, became p53 positive (+) after chemotherapy, in addition, only these patients died, both within one year. Furthermore, the fact that 3 out of the 4 that turned p53 positive died within a year shows that tumors that gain p53 expression during or after neoadjuvant chemotherapy tend to show a worse prognosis than those that remain p53 negative.

Discussion

Undifferentiated OSs were shown to commonly display c-myc amplification, p53 and RB1 mutations and autocrine growth-factor production. Of these, p53 was altered in five of six OS cell lines. The data available on the p53 overexpression, response to chemotherapy and length of survival in OS patients are rather contradictory.

Nishikawa et al.13 analyzed p53 protein expression in 35 resected OSs. Abnormality in p53 protein expression was found in 29%. While these results showed no correlation with any clinicopathological features, the staining pattern of p53 protein seemed to correlate with aggressive growth and metastatic potential. Diffusely stained tumors had a worse prognosis than those stained focally. In addition, maximum overexpression was detected in cells in the S phase, indicating that the nuclear accumulation of p53 protein is a potentially useful prognostic factor for OS. Ueda et al.14 found p53 positivity in more than half of the tumor cells in the majority of 18 OSs. They concluded that point mutation of the p53 gene is frequently involved in the development of OS. They found however no correlation between p53 protein expression and any clinical or pathological factors.

In our study, P53 protein overexpression was detected in 29% of initial biopsies and in 43% of surgical specimens taken after nine weeks of preoperative chemotherapy. The increase in p53 expression after neoadjuvant therapy may be due to the mutagenic affects of the cytotoxic drugs used.

While there were no p53 positive biopsy materials among the stage IIA tumors, 35% of the stage IIB tumors showed positive p53 expression. In the surgical specimens only 25% of the stage IIA tumors were p53 positive compared to 47% of the stage IIB tumors. Intensive positive reactions (++) and (+++) were found only in stage IIB tumors. This may imply a correlation between the tumor size and p53 expression frequency and intensity. The number of spontaneous mutations and the primary drug resistance of the tumor may increase with the size and progression of the tumor.

P53 positive (++) and (+++) expression was associated with a less mean survival time than that of p53 negative cases and the ++ cases had a longer survival time than the +++ cases. However, all the patients, except one, that showed any kind of p53 expression on initial biopsy have died. In addition, 3 out of the 4 p53 negative cases which turned positive after preoperative chemotherapy also died. Lastly, the fact that neither p53 positive cases responded to preoperative chemotherapy indicates a direct correlation between p53 expression and multidrug resistance. In addition, 69% of the biopsies that demonstrated no p53 expression fell into the responder or intermediate responder category; this represents a majority. Furthermore, a gain of p53 expression during the time of neoadjuvant chemotherapy is associated with a worse prognosis. Therefore, we feel that p53 expression may be useful as a prognostic tool of OS.

References


Giant Mitochondria as Possible Bioindicators of Environmental Injuries in Fish Liver

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The effect of hypoxia (80 pHg) and simultaneously applied paraquat (1,1’-dimethyl-4,4-bipyridylium dichloride) was investigated on carp liver using electron microscopic methods. The appearance of giant mitochondria was the most conspicuous alteration in the liver cells. Most of the giant mitochondria were elongated and rod-shaped, often arranged side by side forming clusters beside the nucleus. Crook-like and irregular forms also occurred among giant mitochondria. The length of the giant mitochondria often was greater than the diameter of nucleus: namely 5-10 μm. The outer membrane of the giant mitochondria was well preserved, but inner membranes (crista) were usually absent, and a high density matrix filled in the inner space of mitochondria. High power magnification often revealed a regular, filamentous paracrystall arrangement in the dense material of the matrix. Swollen giant mitochondria with light matrix and tubular elements also occurred in low number. Although fine structural characteristics of carp liver giant mitochondria are not specific for inducing agents (hypoxia + paraquat treatment) the appearance of altered giant mitochondria may be a useful signal for monitoring cell damaging enviromental xenobiotics. (Pathology Oncology Research Vol 3, No 1, 20–25, 1997)

Key words: giant mitochondria, fish liver, herbicide (paraquat), electron microscopic cytopathology

Introduction

Morgan–Hughes’ discovery that some human muscle diseases are directly connected with mitochondrial deficiencies provided a new impulse for mitochondrial research. Among mitochondrial alterations, “giant mitochondria” have been frequently found in the cells of various organs. According to Tandler and Hoppel, giant mitochondria may occur in different human diseases, in experimentally induced mitochondrial deficiency, in drug-treated animals and following after alcoholism and hormone injections, as well. Most of these studies were carried out on human patients and different mammalian species, so data, on other animals are very rare. Since there do not seem to be systematic electron microscopic studies concerning fish tissues after treatment with different pesticides and herbicides, we performed several experiments to see the histopathological effects of these drugs. Among several chemical agents, only paraquat induced the formation of giant mitochondria (in carp liver, in combination with hypoxia). The fine structural characteristics of these giant mitochondria will be described in this paper.

Material and methods

Male and female carp, with weights of 1200-1500 g were used. After anaesthesia with MS 222, a cannula was introduced into the dorsal aorta. Three days after this operation the fish were put into a closed experimental box where pH, temperature and partial oxygen pressure were regulated. After adaptation for 3 days, the fish were divided into two groups. In the first group a 6h period of hypoxia was applied (80 pHg oxygen) daily for 5 days. In the second group, hypoxia was applied as above and on the first day 2.5 μg paraquat (Gramoxone with a PQ content of 25%) was injected via the cannula. For comparative purposes, a control group, not subjected to either treatment mode was also used.
Figure 1. Detail of a hepatocyte after hypoxia and paraquat treatment. N - nucleus, GM - giant mitochondria, m - mitochondria, g - glycogen, bp = bile pigment. Inset: paracrystals in the matrix of giant mitochondria ×15,000 and ×20,000.

Figure 2. The same as Fig. 1. Note a crook-like giant mitochondria (GM) beside normal mitochondria (m). ×20,000.
Figure 3. Giant mitochondria (GM) with electron transparent matrix, and tubules (t), in a hepatocyte, after hypoxia and paraquat treatment. ×20,000.

Figure 4. Giant mitochondria (GM) with dense matrix and remnants vesicular cristae (c). rEr - closely attached rough surfaced endoplasmic reticulum. ×40,000.
On the fifth day after treatment, animals were sacrificed and small pieces (about 1 mm³) were excised from the liver and kidney. Specimens were fixed in a cold fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde for 24 h. Osmium tetroxide fixation was performed for a further 2 h. After the dehydration procedures, samples were embedded in Durcupan ACM resin. The ultra-thin sections were contrasted with lead and examined by electron microscope.

Discussion

Oceans, lakes and jungles are the most stable self-regulatory ecosystems. These have become dangerously unstable in the last decades as a consequence of the accumulation and spread of anthropogenic chemical pollutants. Water soluble xenobiotics reach rivers and lakes, bind to epithelial cells of fish by bronchial, oral and dermal absorption, and penetrate into the tissues trans- and paracellularly. Chemicals from epithelial cells can diffuse into the blood because cell membranes are permeable to most of xenobiotics. Blood circulation transports the xenobiotics into the organs where they may become concentrated. Accumulated chemicals can act as toxic agents and, in combination with unfavourable environmental circumstances (high temperature, low oxygen content, eutrophication), lead to catastrophic fish kills in many cases. In Hungary, 20-30 severe fish kills occur annually as a consequence of water pollution. Classical toxicological, as well scientist applied histopathological, biochemical and physiological methods have been used to determine the "reasons" for these fish kills. In the course of biochemical studies, it has been established that all pesticides cause organospecific tissue necrosis as demonstrated by increased level of lactate dehydrogenase activity in blood plasma. Paraquat significantly increased transaminase and lactate dehydrogenase activity in the sera. All these data suggest that paraquat damages the liver and kidney, reduces O₂ uptake by injuring gill epithelium and can cause functional defects in the nervous- and other tissues inhibiting acetylcholin esterase activity.

Our tandem electron microscopic studies have shown that paraquat alone did not produce significant cytopathological effects in the liver. However extensive degenerative alterations were found in the exocrine pancreas. Hypoxia (80 pHg) and simultaneously applied paraquat caused several cytopathological alterations also in the liver but the appearance of giant mitochondria was the most conspicuous phenomenon in the parenchyma cells. What is the reason and significance of this structural alteration? A large amount of data suggests that formation of giant mitochondria can be induced with many different chemical substances therefore "structural changes in mitochondria are not specific markers for underlying biochemical defects."

However, it is known that paraquat
1. decreases the NADH concentration in the cells and mitochondria, inhibits the synthesis of fatty acids and compound lipids;
2. injures the cell membrane by lipid peroxidation which results in the loss of cell integrity;
3. uncouples mitochondrial electron transfer arresting energy supply to cells.

The above mentioned effects of paraquat may lead to the formation of giant mitochondria in liver cells after hypoxia.
Figure 5. Rarely, irregular giant mitochondria (IGM) also occurred in the hepatocytes, after hypoxia and paraquat treatment. Paracrystals (pc) are accumulated in the dense mitochondrial matrix. ×20,000.

Figure 6. Cytoplasm detail, from an untreated carp liver cell. N=nucleus. Most of mitochondria (m), are damaged. Myelin figure (my), collapsed Golgi apparatus (G) can be seen in the cells. Bc= bile canalicules. ×15,000.
and paraquat treatment. Considering this conclusion, we may suppose that the appearance of giant mitochondria may represent those biochemical alterations in the cells which are closely related to the decrease in NADH concentration, membrane injuries by lipid peroxidation and uncoupling of mitochondrial electron transfer. Assuming that giant mitochondria represent a sensitive cellular, the presence of cell demaging agents in the aquatic environment several questions await for further research. First, among these is to clarify the exact mechanism of the formation of giant mitochondria.

Our present observation, that formation of giant mitochondria can be induced by certain chemical substances, supports the "old" theory: some drugs (as paraquat) inhibit division of mitochondria. At the same time, protein synthesis seems to be unaltered, thus the increased amount of protein simply distends the outer mitochondrial membrane. The protein nature of inner mitochondrial cristalloids was proven long time ago. A recently published human case report indicates that the disorders of mitochondrial oxidative phosphorylation and morphology; i.e. giant mitochondria, accompany cardiomyopathy. This suggests that studies of giant mitochondria can help in the identification of human and animal diseases as well. Our unpublished data on cytopathological alterations occurring in the livers of randomly caught carp indicate the importance of systematic histopathological studies on fish in polluted freshwater lakes such as Lake Balaton, Hungary.

References