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Introduction

Inflammatory bowel disease (IBD) refers to two diseases: Crohn’s disease (CD) and ulcerative colitis (UC), which are generally considered to be two distinct forms of IBD. The conventional gold standard for diagnosis and stratification of IBD had been based on a combination of established clinical, endoscopic, histopathologic, and radiologic criteria [28]. The disease is characterized by cycles of clinical exacerbation and remission, with periods of improvement followed by relapse. The first clinical signs of disease typically begin between adolescence and the third decade of life, but in 15% to 25% of cases, the disease starts in childhood [21], and the incidence of IBD in the pediatric age group had increased substantially over the past 2 decades [3, 34].

CD is a nonspecific granulomatous inflammatory disease affecting lower end of the ileum and often involving the colon and other parts of the intestinal tract and is characterized by segmental and transmural inflammation, fistulas, oedema and granulomas in whole intestinal wall. UC is restricted to the large intestine and is associated with continuous mucosal inflammation, including crypt abscesses and ulcers [38]. UC and CD present some overlapping clinical features, and it is not possible to differentiate between the two diseases in 10%–15% of cases. Disease in these patients is classified as indeterminate colitis [39].

Recent evidence suggests that IBD may represent multiple inflammatory intestinal disorders, with CD and UC representing the extremes of this spectrum [49]. It has been proposed that the pattern of expression of certain serum immune antibodies reflects immune responses that could be related to disease phenotype. Thus, serological markers may help to cluster the IBD patients into more homogenous subgroups.

Serological markers in diagnosis of inflammatory bowel diseases

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Abstract

Correct diagnosis of inflammatory bowel disease (IBD), especially the differentiation between Crohn’s disease (CD) and ulcerative colitis (UC), is highly important toward treatment and prognosis. Serological markers are noninvasive diagnostic tools that could be of value in differentiating CD from UC, and in the identification of subgroups in IBD as well as in objective assessments of early diagnosis, prognosis evaluation and surveillance. This review summarizes typical IBD biomarkers, such as pANCA, ASCA, others, like pancreatic, anti-microbial and anti-goblet cells antibodies, and new anti-glycan antibodies: ACCA, ALCA and AMCA.

Key words: ANCA, ASCA, Crohn’s disease, inflammatory bowel disease, ulcerative colitis
Serum immune markers in IBD

There are several immune markers which have been reported and some of them are currently used for diagnosis and management of IBD in the clinical laboratories [1, 40]. Serologic tests detect the presence of abnormal antibodies directed against self or non-self proteins shown in Table 1. The most practical and commonly used serological antibody markers are anti-neutrophil cytoplasmic autoantibody (ANCA) and anti-Saccharomyces cerevisiae antibody (ASCA).

Table 1
Serum antibodies in IBD

| Deoxyribonuclease (DNase) – sensitive anti-neutrophil cytoplasmic autoantibody (pANCA) |
| Anti-Saccharomyces cerevisiae antibody (ASCA) |
| Pancreatic antibody (PAB) |
| Goblet cell antibody (GAB) |
| Anti-outer membrane porin from Escherichia coli antibody (anti-OmpC) |
| Antibody to Pseudomonas fluorescens (anti-I2) |
| Antibody to anaerobic coccoid rods |
| Anti-flagellin (C Bir1) antibodies |
| Anti-glycan antibodies (ALCA, ACCA, AMCA) |

ANCA and association with IBD

ANCA are the autoantibodies directed against the intracellular components of neutrophils [4]. The two main fluorescence patterns of ANCA are observed in human granulocyte smears allowing for recognition cytoplasmic (c) ANCA and perinuclear (p) ANCA (Fig. 1). In contrast to the cANCA characteristic of Wegener’s granulomatosis, which are directed against the specific antigen – the enzyme proteinase 3 within the cytoplasmic granules, in IBD patients predominate pANCA, which exhibit perinuclear staining pattern [4, 40]. Billing et al. have been provided evidence that pANCA antigen associated with IBD is nuclear in location and differs from other types of pANCA found in patients with inflammatory vasculitis [5]. pANCA pattern observed in IBD is DNase-sensitive one and has been called atypical pANCA. The pANCA staining is lost after DNase digestion of the substrate cells. It appears that the pANCA antigens of IBD patients located in nuclei may be a complex epitope associated with the histone H-1 [12], high mobility group nuclear protein (HMG-1, HMG-2) and a 50 kD nuclear envelope protein [48]. In contrast, pANCA found in vasculitis react with myeloperoxidase, elastase, lactoferrin, cathepsin G [4]. As the antigens to which pANCA in IBD react have not been definitively determined, the optimal technique for diagnosis of IBD patients is an indirect immunofluorescence (IIF) method with the use of human granulocyte smears.

Still the role of pANCA in immunopathogenesis of the IBD is not known. However, it has been presented that IBD associated pANCA cross-react with antigens of microbial agents which could be involved in the development of inflammatory response. Cohavy et al. identified a novel mycobacterial histone H1 homologue (HuB) to be an antigenic target of patients’ pANCA [6], whereas Wei et al. reported that pANCA reacted with a 100 kDa protein – an outer membrane protein of Bacteroides caccae isolated from the gut of IBD patient [54].

pANCA are present in the sera of 45% to 83% of both adult and pediatric patients with UC, and 2% to 28% of patients with CD [11, 37, 52]. In CD, expression of pANCA identifies a subgroup of CD characterized as „ulcerative colitis-like” phenotype with clinical features of left-sided colitis [50]. It was presented that pANCA positive CD patients did not respond that well to anti-TNF monoclonal antibody therapy in comparison with the majority of CD patient [40]. However Esters and co-workers did not confirm such correlation [13].

High levels of pANCA in CD patients were also associated with later age of onset and a relative decreased occurrence of fibrostenosis and penetrating disease [11, 17, 22, 56].

The pANCA expression allows the separation of UC patients with higher probability of more aggressive disease, left-sided UC which is more resistant to treatment than the usual case, requiring surgery early in the course of the disease, developing pouchitis in following ileal pouch-anal anastomosis [22, 40, 56].

ASCA in IBD patients

Levels of serum antibodies against multiple strains of Saccharomyces cerevisiae (baker’s and brewer’s yeast) were found to be significantly elevated in the serum of CD patients [35-37]. It is well established that ASCA recognize manno-
se sequences (oligomannoses), which is a major antigenic components of yeast cell walls and other microorganisms. As the antigen for ASCA antibodies is known both immunoenzymatic (ELISA) and IIF techniques could be used in their determination in IBD patients. Fig. 2 presents positive ASCA antibodies detected by IIF.

Reported ASCA prevalence is 50–70% in patients with CD, 6–14% in patients with UC, and 0–5% in control subjects [35-37, 49, 51]. Most studies present that specificity of ASCA in IBD patients is high (~90%), but recently ASCA have been detected in 61–64% of adult patients with untreated celiac disease [9, 18], and in 26% of pediatric celiac patients [8]. ASCA positivity was also observed in patients with Behcet’s disease, primary biliary cirrhosis, autoimmune hepatitis. The role of ASCA in IBD pathogenesis is not known, but ASCA positive patients in both IgG and IgA classes present more aggressive type of CD [7, 22, 25]. The presence of ASCA is strongly associated with small bowel location of CD (lower prevalence or absent of colonic disease), younger age of onset, fibrostenosing and penetrating disease, multiple small bowel surgeries [1, 7, 19, 36, 41, 51, 53].

Recently antibodies against chemically synthesized two major oligomannose epitopes: mannotriose α1-3 Man α1-2 Man α1-2 Man (M3) and mannotetraose α1-3 Man α1-2 Man α1-2 Man α1-2 Man (M4) have been described [29, 45]. It was presented that while the specificity of M3 and M4 for CD was quite similar to that of ASCA (89% vs 93%), the sensitivity was lower (38% vs 55%). Interestingly, 11% of ASCA-negative CD patients were M3/M4 positive (5% for M3, 4% for M4, and 2% for both), suggesting a previously unrecognized new subset of anti-mannose antibodies are present in patients with CD.

ASCA /ANCA in diagnosis and monitoring of IBD

It was presented that the combined measurement of pANCA and ASCA could help in the differential diagnosis of CD and UC [40, 49]. Studies performed both with adult and pediatric subjects reported that the combination of a positive pANCA and a negative ASCA was highly specific (95-100%) for UC, whereas the combination of a negative pANCA and a positive ASCA was highly specific (95–100%) for CD [32, 36, 41]. Retrospective studies present that patients who are pANCA positive and ASCA negative are 19 times more likely to have UC, whereas patients who are ASCA positive and pANCA negative are 16 times more likely to have CD [52]. However, prospective analysis in patients with indeterminate colitis reported lower positive predictive values for ASCA/pANCA markers. After a mean 1 year of follow-up a definitive diagnosis after was reached in 32% of patients. ASCA+/pANCA– predicted CD in 80% of patients, whereas ASCA–/pANCA+ was predictive for UC in 64% [24].

All studies agree that the sensitivity of the combined pANCA plus ASCA test is still too low (around 50–60%) to be useful as general screening tool.

In contrast with systemic vasculitides, most studies do not support a relationship between the presence of ANCA/ASCA and IBD activity. The presence of ASCA in CD is stable over time and independent on disease duration and treatment. Also ANCA is not useful for follow-up of disease activity and prediction of relapses [35, 41, 52].

Antibodies to intestinal microbiota

Chronic intestinal inflammation, as seen in IBD, results from an aberrant mucosal immune response to the intestinal microbiota, and antibodies against several microbial specific antigens in patients with CD and UC have been described [26]. As loss of tolerance to normal commensal bacteria has been implicated as an initial step in the inflammatory cascade in IBD patients, serological responses to bacterial components may represent another family of serologic markers that are associated with the disease [55]. The main antibodies to intestinal microbiota found in IBD patients react with:

- OmpC – an outer membrane porin antigen purified from *Escherichia coli*,
- I2 peptide – a fragment of bacterial DNA that has been cloned from lamina propria mononuclear cells in active CD, and this sequence is associated with *Pseudomonas fluorescence*,
- gram-positive anaerobic coccoid rods,
- Cirb1 – the flagellin isolated from commensal bacterial which could contribute to the pathogenesis of experimental mouse IBD.

Anti-OmpC antibodies occur in 55% of patients who are seropositive to ASCA and in 24% with positive pANCA tests [26]. Anti-I2 antibodies were reported in 54% of CD patients, and less commonly in UC (10%), other enteric inflammatory control subjects (19%) and normal control subjects (4%) [47]. In CD antibodies against coccoid rods were found in 52% [30]. Lodes et al. reported that about 50% of CD patients presented serum reactivity to bacterial flagellin – CBir1, whereas such responses was found in 6% of UC patients and 8% of control subjects [31].
CD patients who are positive in multiple anti-microbial antibodies have increased risk of having more complicated disease. CD patients positive with three anti-microbial markers (anti-OmpC, anti-CBir, and anti-12) are more likely to have small bowel surgery than those who were negative (72% vs 23%) [33, 46]. No similar association of serotype was found with disease phenotype of UC.

**Other antibodies associated with IBD**

Pancreatic antibodies (PAB) detected by an indirect immunofluorescence test with human pancreas substrate are specific marker for CD [42, 43]. PAB occur in 27% to 39% of CD patients, compared with less than 5% in UC. However, recently the study from Belgium presented a much higher – 23% prevalence of PAB in patients with UC [23]. The specific antigen reacting with the pancreatic antibodies has not been yet identified [16].

Goblet cell antibodies (GAB) have been described in up to 40% of patients with IBD [20]. Folwaczny et al. reported the presence of GAB in 33% of patients with UC and in 30% of patients with CD [15]. Recent study presented that in contrast to PAB which were highly specific for CD, GAB were not useful in diagnosis both UC and CD in Chinese and Caucasian patients [27]. In this population of IBD patients they were positive in less than 2%.

Such discrepancies could be the result of which GAB are detected. As the antigen against which GAB are directed still is not known, the presence of GAB in patients’ sera is determined by IIF with the use of animal intestine. Recently, Ardesjo et al. analyzed GAB reactivity using human intestinal specimens [2]. They presented that 84% of sera of IBD patients reacted with goblet cells localized in the appendix and the reactivity of IBD sera was weak at the base of the crypts and gradually increased towards the lumen.

**New anti-glycan antibodies: ACCA, ALCA and AMCA**

New serological biomarkers in IBD, identified since 2007, include so called anti-glycan antibodies: anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), anti-mannobioside IgG (AMCA) [10, 14, 44].

Mannobioside (AMCA) is a dimer of 1,3 linked mannose, and is a component of mannan from pathogenic fungi and yeast. Laminaribioside (ALCA) is the building block of laminarin, a polysaccharide of the β-1-3-glucan family and is found in the cell walls of fungi, yeast, and algae. Chitobioside (ACCA) is a component of chitin, found in the insect cuticle and cell walls of infectious pathogens such as bacteria and yeast [29].

The initial study was showed that ACCA, ALCA, and AMCA exhibited the highest discriminative capability between CD and UC [10]. In CD patients that were positive with one of the 3 markers, the sensitivity and specificity for diagnosis of CD were 77.4% and 90.6%, respectively. In patients with 2 or 3 of these antibodies, the specificity increased to 99.1%. Higher levels of ALCA and AMCA were significantly associated with small intestinal disease. A study presented by Ferrante et al. involved a larger cohort, including 1225 IBD patients (913 CD, 272 UC, and 40 IC), 200 ethnically matched healthy controls, and 113 patients with non-IBD intestinal inflammation [14]. All anti-glycan antibodies were specific for CD (80.5%-93%), but the sensitivity was lower as compared with ASCA (ASCA = 56.4%; ALCA = 17.7%; ACCA = 20.7%; AMCA = 28.1%). However, fifty percent of CD patients who were ASCA negative, were positive for at least one of the anti-glycan markers, suggesting usefulness of novel serological markers in CD diagnosis.

The relationship between novel antibodies and CD activity is controversial. Ferrante et al. reported that the presence of ALCA, ACCA, AMCA, likely the occurrence of ASCA and anti-OmpC antibodies, was significantly associated with more complicated disease behavior, including stricture, fistula and need for surgery [14]. However, a recent report by Simondi et al. found that, while the level of ASCA appeared to be associated with ileal disease and penetrating/structuring disease, the level of ALCA has a similar trend, but did not reach statistic significance [45].

**Conclusions**

Correct diagnosis of IBD, especially the differentiation between CD and UC, is highly important toward treatment and prognosis. Serological markers are noninvasive diagnostic tools that could be of value in IBD as well as in objective assessments of disease activity, early diagnosis, prognosis evaluation and surveillance.

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**References**


Functional studies of the respiratory chain abnormalities in fibroblasts of patients with various oxidative phosphorylation disorders

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Abstract

Phenotypic spectrum of OXPHOS defects expands making difficult establishing the diagnosis of mitochondrial disease in individual patients. In nearly half of the cases the highest diagnostic level is a detection of enzymatic or functional mitochondrial abnormalities. New instruments are needed to improve the diagnostic reliability, especially in patients with non-classical phenotype or not confirmed by mutation(s) identification. The aim of the studies was to assess usefulness of mitochondrial transmembrane potential measurement using Safranine O in fibroblasts of patients with possible/probable mitochondrialopathy. Five patients with OXPHOS deficit: a boy with severe isolated complex IV deficiency and SURF1 gene mutation, two children with complex I deficiency of unknown molecular background (one with progressive encephalopathy and one with Leigh disease), and two adolescent brothers with undefined OXPHOS defect in muscle and fibroblasts and progressive cardiomyopathy were investigated. Measurement of mitochondrial membrane potential in fibroblast cultures showed abnormalities confirming in this way the mitochondrial pathology in all five examined cases. Faster rate of mitochondrial membrane depolarization, comparing to the controls, after rotenone addition was observed independently from the clinical and enzymatic phenotype in all cases. The higher uptake of Safranine O was found in the patients’ fibroblasts with increased amount/number of mitochondria (as indicated by citrate synthase activity). Our studies indicate that measurement of mitochondrial membrane potential in fibroblasts using Safranine O may be useful (with some limitation) as a way to confirm the mitochondrial defect in some patients with clinical suspicion.

Key words: mitochondrial membrane potential, fibroblasts, OXPHOS disorders, mitochondrial disease

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Introduction

Recent remarkable progress in understanding the genetics and molecular physiology of oxidative phosphorylation (OXPHOS) has revealed both its intrinsic structural complexity and the causal heterogeneity of mutations affecting mitochondrial function. As the phenotypic spectrum of OXPHOS defects in humans rapidly expands and the molecular complexity steadily increases, the diagnosis in the individual patients becomes laborious and quite expensive. There is a growing need for screening tests in mitochondrial laboratories, in particular methods with reliable discerning power between pathological and physiological pattern of mitochondrial function. Establishing the final diagnosis of the mitochondrial disease is not difficult if pathogenic mutation may be easily identified, i.e. in so-called nosological classic clinical entities with mutations in mitochondrial tRNA genes (MELAS, MERRF, others) or in some relatively new mitochondrial defects associated with mutations in nuclear genes (SURF1, SCO2, nuclear structural subunits of complex I, others). Unfortunately, the search for DNA mutations responsible for the disease appears frequently unsuccessful.

It is generally accepted that the molecular background of mitochondrial disease remains unknown in the half of the patients with the suspicion of mitochondrial disorder. In such cases the highest diagnostic confirmation level is phenotypic, enzymatic and/or functional abnormalities detection. Occurrence of secondary (or non-conclusive) OXPHOS changes makes an achievement of convincing diagnosis difficult. New instruments with improved reliability are needed for the investigation of mitochondrial defects especially with non-classical clinical phenotype, and not confirmed by the mutation(s) identification.

The OXPHOS is run by a set of four multiprotein complexes, embedded in the lipid bilayer of the inner mitochondrial membrane: complex I (NADH:ubiquinone oxidoreductase, E. C. 1.6.99.3.), complex II (succinate:ubiquinone oxidoreductase, E. C. 1.3.99.1.), complex III (ubiquinol:cytochrome c oxidoreductase, E. C. 1.10.2.2.), complex IV (cytochrome c oxidase, E. C. 1.9.9.1.), and ATP synthase sometimes called as complex V (ATP synthase, E. C. 3.6.1.3.). This structural system harbors at least 85 proteins, 13 of which are encoded by the small mitochondrial genome.

Mitochondrial membrane potential (Δψ) – the result of a proton gradient formation across inner mitochondrial membrane, is generated by the mitochondrial respiratory chain. The activity of complexes I, III and IV is accompanied by the transfer of protons from the matrix across the mitochondrial inner membrane to the intermembrane compartment. The electrochemical proton gradient formed in this way consists of the electric component (Δψ) of 180–200 mV (negative inside) and the chemical component (ΔpH) of 0.5 – 1.0 pH unit (alkaline inside). This gradient among other things, is utilized for the formation of ATP by the ATP synthase. For this reason Δψ is an important parameter for mitochondrial function.

Δψ in isolated mitochondria or permeabilized cells can be determined by measuring accumulation of lipophilic (membrane-penetrating) cationic probes [e.g. nonylacetridine orange (NAO), 3,3′-dihexiloxadiocarbocyanine iodide DiOC6(3)], safranine O, rhodamine-123 (Rh123), tetracythylrhodamine methyl ester (TMRM), tetramethylrhodamine ethyl ester (TMRE) and tetraphenyl-phosphonium ion (TPP±)]. In this study we decided to use safranine O whose fluorescence was measured in a Shimadzu Spectrofluorimeter RF 5000 at 495 nm and 586 nm excitation and emission wavelengths, respectively [2]. Accumulation of safranine O inside energized mitochondria is accompanied by fluorescence quenching and hence increase of Δψ is depicted by a decrease of fluorescence.

The aim of our study was to assess if the measurement of mitochondrial transmembrane potential using Safranine O in fibroblasts of the patients with possible/probable mitochondrialopathy may improve the diagnostic abilities.

Material and Methods

Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Bioethics at the Children’s Memorial Health Institute. Informed consent was obtained from the parents before any biopsies or molecular analyses were performed.

Patients

Five patients with different clinical phenotypes and various OXPHOS abnormalities in the muscle were included in the study. The level of probability of mitochondrial disease according Nijmegen scale [5] has been determined from definite (patient 1 with SURF1 gene mutation) to possible. The overall patients characteristics is shown in the table 1 and 2. Three fibroblasts cultures of healthy volunteers were included as the reference.

Fibroblasts cultures

Human skin fibroblasts were grown from explants of skin biopsies of healthy individuals and the patient in Dubbecco’s modified Eagle’s medium with glucose (4500 mg/l), sodium pyruvate (110 mg/l) and L-glutamine (Sigma Aldrich, Ref. D6429), supplemented with 10% (v/v) fetal bovine serum (Gibco, Ref. 10106-169), and 1,2% Antibiotic, anticytotic solution (100x) (Sigma Aldrich, Ref. A5955) in an atmosphere of 5% (v/v) carbon dioxide in air in 37°C. Cells were grown in 75 cm² culture flasks and used within 5 days after reaching confluency [7].

Enzymatic studies

Spectrofotometric assays in postnuclear supernatants obtained from skeletal muscle biopsies and fibroblast cell cultures have been used for the measurement of complex I (NADH:ubiquinone oxidoreductase, rotenone sensitive), complex II (succinate:ubiquinone oxidoreductase, malonate sensitive),
Table 1

Clinical characteristics and histological findings of the patients with mitochondrial disorder

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at onset</th>
<th>Symptoms, course of the disease</th>
<th>Muscle histology and histochemistry</th>
<th>Mitochondrial disorder probability (Nijmegen scale 0-12). Final diagnosis</th>
</tr>
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<tbody>
<tr>
<td>Patient 1</td>
<td>8 mo</td>
<td>Episode of vomiting, failure to thrive and hyperventilation evoked by an infection. Hypotonia, stridor, dissociation of eye movements. Lactic acidemia. Leigh disease in brain MRI. At age of 4 yrs severe clinical condition, artificial respiration, joint contractures</td>
<td>Total COX deficit, severe lipid accumulation in muscle fibers</td>
<td>Defined (8 points) Leigh disease with COX deficit and SURF1 gene homozygous mutation</td>
</tr>
<tr>
<td>Patient 2</td>
<td>Birth</td>
<td>Floppiness from birth. Chronic myopathic changes (EMG), walking difficulties. Respirator dependency at night since 8 yr of age. Normal school performance. Transient lactic academia. Alive at the age of 13.</td>
<td>Decreased COX activity; moderate lipid accumulation in muscle fibers; reduction in SDH activity</td>
<td>Probable (6 points) Encephalomyopathy with decreased complex I activity</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3 1/2 yrs</td>
<td>Strabismus and walking difficulties appearance evoked by an episode of anxiety and crying. MRI – Leigh changes in basal ganglia. Plasma lactate 31.9 – 17.6 mg%. Alive at the age of 7; she doesn’t walk without support. Speech indistinct and slow.</td>
<td>Mild diffuse decrease of COX activity, moderate lipid accumulation in muscle fibers</td>
<td>Probable (8 points) Leigh disease with decreased complex I activity</td>
</tr>
<tr>
<td>Patient 5</td>
<td>18 yrs</td>
<td>Muscle weakness, exercise induced rhabdomyolysis, dilated cardiomyopathy, progressive circulatory insufficiency, heart transplantation (23 y). Normal lactate level.</td>
<td>Mild increase in endomysial connective tissue; mild lipid accumulation; normal COX activity; no RRF’s</td>
<td>Possible (3 points) Familial progressive cardiomyopathy</td>
</tr>
</tbody>
</table>

complex III (ubiquinone: cytochrome c oxidoreductase, antimycin sensitive), complex IV (cytochrome c oxidase), and citrate synthase (cs) enzyme activities as described previously [3]. Protein content in postnuclear supernatants was determined according to the method of Lowry et al. [4]. The ratio between the activity of individual respiratory chain complexes and cs was calculated to be independent on different amount/number of mitochondria in control and patient cells.

Monitoring of the mitochondrial membrane potential with the use of Safranine O

Functional experiments were carried out using the patient’s and controls fibroblasts cell lines. Once culture reached 80%–90% confluence, cells were washed using phosphate buffer saline (PBS-Sigma D8662) without Ca²⁺ or Mg²⁺ and trypsined with the use of porcine trypsin supplemented with 0.2g EDTA (Sigma T3924). Fibroblasts, harvested by trypsinization, were resuspended in 2 ml of measurement buffer [225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA, 1 mM MgCl₂, 1 mM P₅, 5 mM Tris-HCl (pH 7.4) ] and protein concentration in cell suspension was determined according to the Bradford’s method using Bio-Rad protein estimation kit [1]. Afterwards equal amounts of protein (cells) were transferred to the fluorimeter cuvette and missing volume to the total volume (3 ml) of the measurement medium was added. Than glutamate (5mM), malate (5mM) and safranine O (8,3mM) was added and measurement of fluorescence was initiated. Next 8 ml of 1% digitonin (to permeabilize the plasma membrane) was added enabling in this way potential-dependent accumulation of Safranine O in mitochondria. To determine the „working condition“ of individual OXPHOS complexes, one by one, inhibitors and substrates for subsequent respira-
In the following consecutive additions were applied: 8 ml 1% digitonin; 1,7 μM rotenone; 5 mM succinate; 1,7 μM antimycin A.

### Results and discussion

#### Clinical and enzymatic characteristics of the patients with OXPHOS disorders

The clinical characteristic and histology/histochemistry data of muscle biopsies from patients with the suspicion of mitochondrial disorder are presented in Table 1. Additionally, mitochondrial defects in these patients have been confirmed by the spectrophotometric measurement of the individual respiratory chain complexes activity in the muscle biopsies (for details see Table 2).

To correlate such defined or suspected defects in the mitochondrial respiratory chain with the alteration of mitochondrial function the present studies have been performed on the cultured skin fibroblasts obtained from these five patients. Spectrophotometric characterization of respiratory chain complexes in skin fibroblasts has been performed. Table 3 contains comparison of enzymatic activities of complex I, II, III and IV in the patients’ and the control fibroblast cell lines. Severe isolated complex IV deficiency was found in one patient (patient 1 with SURF1 gene mutation) and complex I deficiency in two others (patient 3 with progressive encephalopathy, and patient 4 with Leigh disease). Combined respiratory chain defects were detected in remaining fibroblasts of two cardiomyopathic brothers; these changes were not conclusive and differed from one to another.

### Mitochondrial membrane potential as a function of the respiratory chain activity

Measuring of mitochondrial membrane potential using Safiranine O may be used for general assessment of the cell mitochondrial functioning as shown in the Figure 1. Malate and glutamate are substrates for NADH production (to supply Complex I). In this case $\Delta \psi$ is built by complexes I-III-IV. Addition of rotenone, inhibitor of complex I, stops electron flow and causes decrease of $\Delta \psi$. Than, addition of succinate, substrate for complex II, causes a re-polarization of inner mitochondrial membrane due to proton pumping activity of complexes III and IV. Afterwards, addition of antimycin, inhibitor of complex III, again stops the respiratory chain activity and $\Delta \psi$ building. Addition of ascorbate and TMPD (measured only for 3T3L1 cell line, causes a $\Delta \psi$ building due to complexes IV activity only. Ascorbic acid maintains TMPD (an artificial electron donor) in the reduced state. Reduced TMPD donates electrons to cytochrome c. Addition of KCN (1,5mM), inhibitor of complex IV stops the last active respiratory chain complex and $\Delta \psi$ building what results complete depolarization of mitochondria. Unfortunately, in human fibroblasts the difference in the fluorescence level before and after addition of KCN was to small to estimate possible complex IV dysfunction. The ability to sustain a proton gradient by complex IV only is very poor comparing to another cell lines e.g. hepatocytes or mioblasts. As presented on Figure 1, „the proton pumping” activity of complex IV in the presence of artificial substrates (133 μM ascorbate; 100 μM of TMPD) is very low, what suggest that in fibroblasts this parameter can not be used in our comparative studies.

### Table 2

<table>
<thead>
<tr>
<th>Respiratory chain complex activities</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase (CS)</td>
<td>334.9</td>
<td>130.6</td>
<td>183.5</td>
<td>121.6</td>
<td>203.5</td>
<td>123.3±26.8, 96.5–150.1</td>
</tr>
<tr>
<td>Complex I (%CS)</td>
<td>20.9</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
<td>&lt;3.0</td>
<td>10.2</td>
<td>13.1±4.9, 8.2–18.0</td>
</tr>
<tr>
<td>Complex II (%CS)</td>
<td>9.5</td>
<td>14.4</td>
<td>12.1</td>
<td>7.9</td>
<td>6.4</td>
<td>10.0±2.5, 7.5–12.5</td>
</tr>
<tr>
<td>Complex II+III (%CS)</td>
<td>3.8</td>
<td>5.6</td>
<td>6.3</td>
<td>3.6</td>
<td>7.2</td>
<td>7.6±1.9, 5.7–9.5</td>
</tr>
<tr>
<td>Complex III (%CS)</td>
<td>114.3</td>
<td>66.1</td>
<td>146.6</td>
<td>54.1</td>
<td>144.9</td>
<td>69.2±24.3, 44.9–93.5</td>
</tr>
<tr>
<td>Complex IV (%CS)</td>
<td>&lt;3.0</td>
<td>25.7</td>
<td>3.9</td>
<td>3.6</td>
<td>13.0</td>
<td>26.4±7.4, 29.4–46.2</td>
</tr>
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</table>

Complex I (NADH-ubiquinone oxidoreductase, E.C.1.6.99.3 )
Complex II (succinate-ubiquinone oxidoreductase, E.C.1.3.99.1 )
Complex II+III (succinate-cytochrome c oxidoreductase )
Complex III (ubiquinone-cytochrome c oxidoreductase, E.C.1.10.2.2 )
Complex IV (cytochrome c oxidoreductase, E.C.1.9.9.1 )
As was expected, mitochondrial membrane potential measured with the use of Safranine O in fibroblasts of all five patients showed some abnormalities. Individual results for the patients fibroblasts in comparison to the control cell lines are shown in Figures 2–4. Presented data seemed to confirm the presence of a mitochondrial dysfunction/defect in these patients earlier found by the clinical and enzymatic studies.

### Table 3
Activities of respiratory chain complexes in fibroblast cultures of the control cases (K1-3) and the patients with mitochondrial disorders (P1-P5)

<table>
<thead>
<tr>
<th>Complex</th>
<th>K1</th>
<th>K2</th>
<th>K3</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>Reference values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase (CS)</td>
<td>96.6</td>
<td>ND</td>
<td>86.3</td>
<td>121.3</td>
<td>58.2</td>
<td>77.1</td>
<td>103.0</td>
<td>69.4</td>
<td>87.0±28.1</td>
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<td>58.9–115.1</td>
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<tr>
<td>Complex I (%CS)</td>
<td>9.8</td>
<td>ND</td>
<td>ND</td>
<td>4.7</td>
<td>3.9</td>
<td>5.8</td>
<td>8.7</td>
<td>&lt;3.0</td>
<td>20.9±12.2</td>
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<td>8.7–33.1</td>
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<tr>
<td>Complex II (%CS)</td>
<td>14.3</td>
<td>ND</td>
<td>11.9</td>
<td>6.8</td>
<td>4.6</td>
<td>20.2</td>
<td>7.9</td>
<td>6.4</td>
<td>10.6±3.7</td>
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<td>6.9–14.3</td>
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<tr>
<td>Complex II+III (%CS)</td>
<td>7.8</td>
<td>ND</td>
<td>5.5</td>
<td>4.8</td>
<td>3.4</td>
<td>9.2</td>
<td>3.6</td>
<td>2.8</td>
<td>7.2±2.3</td>
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<td>4.9–9.5</td>
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<tr>
<td>Complex III (%CS)</td>
<td>159.4</td>
<td>ND</td>
<td>234.9</td>
<td>42.9</td>
<td>96.6</td>
<td>120.3</td>
<td>67.1</td>
<td>364.0</td>
<td>78.2±13.3</td>
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<td>64.9–91.5</td>
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<tr>
<td>Complex IV (%CS)</td>
<td>15.5</td>
<td>ND</td>
<td>9.0</td>
<td>&lt;3.0</td>
<td>12.0</td>
<td>26.8</td>
<td>9.9</td>
<td>5.6</td>
<td>37.8±8.4</td>
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<td>29.4–46.2</td>
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**Fig. 1** Measurement of mitochondrial membrane potential with Safranine O in 3T3L1 cells. A) The basic incubation medium was supplemented with 5 mM glutamate 5 mM malate and 8.3 mM Safranine O. Each trace was started by addition of 8 ml 1% digitonin per 3.0 ml of the final volume. Other additions, where indicated, were: 1.7 μM rotenone; 5 mM succinate; 1.7 μM antimycin A, 133 μM ascorbate; 100 μM of TMPD and 1.5 mM of KCN. B) The participation of respiratory chain complexes in the proton gradient formation (DY). (I–II–IV) – DY generated by the complexes I, III and IV; (II–IV) – DY generated by the complexes III and IV; (IV) – DY generated by the complexes IV only

### Table 3

<table>
<thead>
<tr>
<th>Complex</th>
<th>K1</th>
<th>K2</th>
<th>K3</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>Reference values</th>
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<tr>
<td>Citrate synthase (CS)</td>
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<td>86.3</td>
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<td>58.2</td>
<td>77.1</td>
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<td>69.4</td>
<td>87.0±28.1</td>
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<td>58.9–115.1</td>
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<tr>
<td>Complex I (%CS)</td>
<td>9.8</td>
<td>ND</td>
<td>ND</td>
<td>4.7</td>
<td>3.9</td>
<td>5.8</td>
<td>8.7</td>
<td>&lt;3.0</td>
<td>20.9±12.2</td>
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<td>8.7–33.1</td>
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<tr>
<td>Complex II (%CS)</td>
<td>14.3</td>
<td>ND</td>
<td>11.9</td>
<td>6.8</td>
<td>4.6</td>
<td>20.2</td>
<td>7.9</td>
<td>6.4</td>
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<td>6.9–14.3</td>
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<tr>
<td>Complex II+III (%CS)</td>
<td>7.8</td>
<td>ND</td>
<td>5.5</td>
<td>4.8</td>
<td>3.4</td>
<td>9.2</td>
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<td>2.8</td>
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<td>Complex III (%CS)</td>
<td>159.4</td>
<td>ND</td>
<td>234.9</td>
<td>42.9</td>
<td>96.6</td>
<td>120.3</td>
<td>67.1</td>
<td>364.0</td>
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<td>64.9–91.5</td>
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<tr>
<td>Complex IV (%CS)</td>
<td>15.5</td>
<td>ND</td>
<td>9.0</td>
<td>&lt;3.0</td>
<td>12.0</td>
<td>26.8</td>
<td>9.9</td>
<td>5.6</td>
<td>37.8±8.4</td>
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<td>29.4–46.2</td>
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</table>

**Fig. 2** Measurement of mitochondrial membrane potential with Safranine O in the control and Patient 1 with defined complex IV deficiency due to SURF1 mutation. The basic incubation medium was supplemented with 5 mM glutamate 5 mM malate and Safranine O 8.3 mM. Each trace was started by addition of 8 ml 1% digitonin per 3.0 ml of the final volume. Other additions, where indicated, were: 1.7 μM rotenone; 5 mM succinate; 1.7 μM antimycin A
Briefly, Patient 1, only in the studied group with defined complex IV deficiency due to \textit{SURF1} mutation, showed remarkable abnormalities in mitochondrial membrane potential and deep depolarization of the membrane after rotenone, in comparison with the control (P1, Figures 2 and 5). Dysfunction of the whole respiratory chain following the isolated deficit of cytochrome oxidase due to the assembly Surf1 protein deficit was showed earlier by us [6] and others [8].

Isolated deficiency of complex I in the muscle characterized next two patients included in the study (P2 and P3). Although complex I deficit was confirmed only in one of the fibroblast lines (of P2), the abnormal mitochondrial potential was found in both (Figure 3). This indicates that a basic mitochondrial defect may be expressed in the fibroblasts of both unrelated complex I deficient patients.

Results obtained in the remaining two patients, the brothers with very similar phenotype of severe progressive cardiomyopathy are difficult to interpret. The mitochondrial disease in these two patients was suspected depending on rather weak evidences (association of progressive cardiomyopathy and skeletal myopathy, COX deficiency in the muscle found only in one brother) with low level of the probability (“mitochondrial disease possible”). The results of the present study are also not consistent in the siblings. Enzymatic assessment of fibroblasts showed deficiency of the complex I in one sibling and deficiency of the complex IV in second one. Mitochondrial membrane potential measured for these two brothers is presented in the Figure 4.
Interpretation of the mitochondrial membrane potential data in patient’s fibroblasts

Surprisingly, as is visible on Figures 2-4 we observed higher loading of Safranine O in patient’s mitochondria, what can raise to a wrong interpretation e.g. as a presence of higher mitochondrial membrane potential (better condition of mitochondrial respiratory chain) in these cells. Only when the data were compared with the activity of citrate synthase in these fibroblasts (as a marker of mitochondrial mass/number in the cell) (see Table 3) showed that in all cases the higher loading of Safranine O can be explained by the higher amount/number of mitochondria in patient’s fibroblasts (probably as a compensatory effect).

For this reason we decided to found another more convincing parameter to describe abnormalities in the functionality of mitochondrial respiratory chain. Such parameter can be the rate of depolarization after rotenone addition and further ability of Complexes II, III and IV to rebuilt the DY after succinate addition (see individual Figures 2-4 and Figure 5 containing calculated overall data). The curves (Fig. 2-4) differ in all cases especially showing the faster membrane depolarization after rotenone independently from the patients clinical and enzymatic phenotype but indicating in which cell line there are abnormalities in the proper function of the respiratory chain (see Figure 5). Interestingly, concerning patients 4 and 5 a remarkable abnormality of the mitochondrial potential and the post-rotenone membrane depolarization was found only in one of the brothers (Fig. 4, Fig. 5). Further search for a rare pathogenic mtDNA mutation in this family seems to be reasonable. A tissue heteroplasmy of mtDNA might explain some discrepancies in the mitochondrial study results in these brothers. However the presence of a primary mitochondrial pathology in this family seems still uncertain.

Conclusions

In summary, our study indicate that the measuring of mitochondrial membrane potential in fibroblasts using Safranine O may be useful in confirmation of the occurrence of a mitochondrial defect in some patients with clinical suspicion. However the technique has a number of limitations, and is not recommended for the practical use. For this reason another method based on JC-1 fluorescent dye, which is faster (no need to permeabilize plasma membrane), more reproducible and needs smaller amount of the material should be tested in the future. However, presented results gave a collection of important information to improve further investigations of the OXPHOS functionality in mitochondrial disorders and understanding their pathomechanism or basic pathology.

Acknowledgment

Research was supported by Internal Project of The Children’s Memorial Health Institute Grant CMHI 146/06 (principal investigator – dr. E. Karczmarewicz). This research was also partly granted by a research grant from the Polish State Committee for Scientific Research under grant N301 092 32/3407 for MRW, ML and JD. This work was also supported by the Polish Mitochondrial Network (mitoNET.pl).

References

Physical activity is a significant factor stimulating a man’s development, fitness and health. In the population of developmental age, it is a basis of the organism’s functional integration, especially, with regard to the skeletomuscular, circulatory and respiratory systems [2, 8, 10, 13].

Nowadays, decreased physical activity is observed in numerous social groups, beginning at the developmental age. At each age low physical activity is a known causative factor of numerous chronic non-infectious diseases e.g., cardiovascular diseases, diabetes type 2, neoplasms, and osteoporosis. Therefore, more and more significance has been attached to reduction of risk factors contributing to these diseases by means, among the others, increased physical activity starting in the youngest age groups [1, 8].

Creating active life style and a proper diet is vital in childhood and early youth, because then people get themselves into healthy life style habits that contribute to general health and fitness in adulthood. It is important to adjust forms of physical activity to individual needs and capabilities and to choose the exercises bringing pleasure.

The aim of the study was to evaluate the level of physical activity among the 18-year-old pupils of secondary schools in Bialystok. The study was carried out among 1631 pupils, aged 18 years in secondary schools of Bialystok during the school year 2007/2008, using the author’s questionnaire. Girls constituted 64.5% (1051 people), whereas boys 35.5% (580 people). A total of 19.1% of pupils avoided physical education classes, including 13.1% of girls and 6.0% of boys. In their free time, after classes or at the weekends 26% of the study pupils took part in these classes for most days of the week. The study pupils pointed the following factors hindering physical activity, with the possibility of multiplied answer: time shortage (73.2%), unwillingness (29.3%), no facilities (13.2%) and equipment for exercises (10.7%) of all factors making physical activity difficult. Insufficient physical activity was reported among the 18-year-olds of Bialystok city. The youth were not interested enough and unwilling to take part in extra-curricular physical activities.

Key words: secondary school pupils, physical activity, free time

Introduction

Physical activity is a significant factor stimulating a man’s development, fitness and health. In the population of developmental age, it is a basis of the organism’s functional integration, especially, with regard to the skeletomuscular, circulatory and respiratory systems [2, 8, 10, 13].

Nowadays, decreased physical activity is observed in numerous social groups, beginning at the developmental age. At each age low physical activity is a known causative factor of numerous chronic non-infectious diseases e.g., cardiovascular diseases, diabetes type 2, neoplasms, and osteoporosis. Therefore, more and more significance has been attached to reduction of risk factors contributing to these diseases by means, among the others, increased physical activity starting in the youngest age groups [1, 8].

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Methods

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The questions included in the questionnaire referred to: socio-demographic characteristics of the study group, participation in physical education classes at school and in extra-curricular physical activities.

The study was accepted by the Educational Authorities of Bialystok headmasters/mistresses of particular secondary schools included in the project. The Bioethical Commis-
sion of the Medical University of Bialystok gave consent to perform the study.

Before entering the questionnaire study, each adult pupil confirmed consent to the study in the especially prepared form. In the study pupils, anthropometric measurements referring to height and body mass were carried out to calculate BMI index. The BMI values of the 18-year-olds were referred to centile charts of the Institute of Mother and Child, regarding the gender. [12] – Tab. 1.

The pilot study was followed by the proper study to check its organization and course and in the case of a questionnaire to check whether the youth understands the questions. The pilot study was carried out among the pupils of two 3-grade – forms of the secondary school.

Table 1

| Centile distribution of BMI in the 18-year-olds regarding the gender [10] |
|------------------|---|---|---|---|---|---|---|---|---|
|                  | 3 | 5 | 10 | 15 | 25 | 50 | 75 | 85 | 90 | 95 | 97 |
| Centile for girls|   |   |    |    |    |    |    |    |    |    |    |
| 17.3             | 18.0 | 18.6 | 19.1 | 19.5 | 20.8 | 22.3 | 23.7 | 24.2 | 26.1 | 27.0 |
| Centile for boys |   |   |    |    |    |    |    |    |    |    |    |
| 17.9             | 18.3 | 18.7 | 19.5 | 20.2 | 21.5 | 23.1 | 24.5 | 25.2 | 26.3 | 27.0 |

Results

BMI values within the norm of centile 5 and 85 were found in 78.3% of the examined. Eating disorders were established in 21.7% of the study youth, underweight (BMI below 5 centiles) in 6.8% of girls and 1.5% of boys, overweight (BMI > 85 centiles) in 7.1% of girls and 6.3% of boys, of whom 1.3% of girls and 2.7% of boys had obesity (Tab. 2).

Analyzing the results obtained, the attention should be turned to insufficient physical activity of the study pupils. A total of 53.5% of pupils systematically participated in physical education classes and 34.4% of pupils exercised irregularly. A total of 12.1% of pupils had a long-term sick leave. Of the study pupils participating in physical education classes, 73.5% of the young people had a positive attitude towards classes and 26.5% did not accept these classes (Fig. 1).

Table 2

| BMI values in the study group |
|-------------------------------|---|---|---|---|---|---|---|---|
|                              | <5 centiles | 5–85 centiles | 85–95 centiles | ≥ 95 centiles |
| N %                           | N %          | N %           | N %           | N %          |
| Total                         | 135 8.3     | 1277 78.3     | 153 9.4       | 66 4.0       |
| Girls                         | 111 6.8     | 824 50.5      | 94 5.8        | 22 1.3       |
| Boys                          | 24 1.5      | 453 27.8      | 58 3.6        | 44 2.7       |

Behavior connected with physical activity was obviously determined by a gender of the questioned pupils. Boys took part in obligatory sports activities more eagerly than girls. Similar differences referred to the percentages of boys and girls joining sports organizations (36.1% of boys and 19.6% of girls). Only 9.1% of the study 18-year-olds took part in sports activities at schools, and 16.4% joined various sport clubs, whereas 80.4% of girls and 63.9% of boys did not belong to any sport clubs (Fig. 2).

The pupils that took part in extracurricular physical activities once a week constituted 20.3%, twice a week – 19.3%, three times a week – 17.9%, four times a week – 9.3% and 17.5% of the questioned exercised on most days of the week, i.e., 5 and more days.

A significantly higher level of physical activity was characteristic of boys of whom 24% exercised in their free time for most days of the week, whereas 13.9% of girls were interested in exercising on most week days.

Only 11.8% of the study girls and 30.2% of the study boys exercised for 90 minutes and more during extra-curri-
circular physical education classes; 20.6% of the girls and 29.9% of the boys for 60 minutes; 23.8% of the girls and 15.9% of the boys for 30 minutes. A significant percentage of the study pupils i.e., 43.8% of girls and 24.0% of boys devoted no extra time on physical activity apart from physical education classes (Fig. 3).

A total of 29.1% of the study pupils were engaged once a week in intensive physical activity meaning great effort connected with accelerated breathing and an increase in the heart beat 24.7% – 2–4 times a week, 13.5% – 5 times and more. Boys constituted most of the questioned, declaring physical activity for most days of the week (8.9% of the study group). Only 4.6% of the school girls were engaged in this type of activity. Of the study school youth, 32.8% did not exercise intensively, (25.1% of girls and 7.7% of boys). A total of 89.9% of pupils were engaged in moderate effort, meaning slightly accelerated breathing and heart beat; 34.0% of them – once a week, 36.5% – 2–3 times a week and 19.4% – more frequently.

Team games, jogging, cycling, gym and aerobics were reported as the most frequently chosen activities (the questioned could point to more than one answer). The pupils without features of overweight and obesity (BMI below 85 centiles) preferred forms of physical activity requiring more energy i.e., jogging, dancing, whereas overweight and obese pupils more willingly undertook walks, training, gymnastics and martial arts.

Most of the questioned spent free time in a passive way (the young people indicated usual forms of spending time with a possibility of a multiplied answer) in front of the computer screen (72.9%), in front of TV (54.3%), listening to music (82.8%), learning (39.1%), or taking part in extra-curricular classes (72.6%). The study pupils more frequently took part in static extra-curricular classes, such as language courses (39.9%), individual classes (39.7%), and artistic classes (9.9%). A total of 26.1% of the questioned took part in sports activities on a daily basis, whereas as many as 27.4% were not engaged in any extra-curricular activities.

Discussion

Physical activity should be an essential element of a man’s everyday life. The Polish youth is not active enough and spends their free time in a passive way. Spontaneous willingness to be active, so typical of younger children, declines with age, especially, between the age of 13 and 18. The level of physical activity diminishes most at the age of maturation [6].

The data about insufficient physical activity come from HBSC (Health Behavior in School-aged Children) from 2001/02, indicating that in the European Union countries, including Poland, about 2/3 of young people do not show a recommended level of physical activity [6].

Participation in the classes of physical education plays a significant role in creating positive attitudes of young people towards regular physical activity. In our study, 19.1% of pupils avoided physical education classes, including 13.1% of girls and 6.0% of boys. Similarly, Jodkowska’s et al. studies indicate that young people at puberty avoid physical education classes (20% of the study pupils did not exercise at school) [11]. Łoś-Rycharska et al. proved that of pupils at Bydgoszcz schools, about 90% systematically took part in physical education classes, whereas 38.3% of the stu-
According to Charzewksa et al. to reduce the risk of obesity, diabetes and cardiovascular diseases, children and young people should spend 90 minutes daily doing physical exercises [5]. According to our studies, 23.9% of pupils did exercises only for 60 minutes, whereas only 18.4% exercised for 90 minutes and more.

Development of new informative and communicative technologies using computers and the Internet must have contributed to low activity in children and young people. They are attractive to young people and competitive compared to existing forms of physical activity.

Our study showed that more than a half of boys (51.5%) spent from 2 to 3 hours daily in front of the computer screen. Girls (42.5%) usually used the computer for an hour. However, 26.1% of boys and 12.6% of girls used the computer for 4 hours daily. Every day 47.0% of boys and 46.1% of girls watched TV for an hour, whereas 6.3% and 4.8% for 4 hours daily, respectively.

Chabros et al. proved that activities not requiring physical activity, including watching TV, video, using the computer took pupils on average 4.5 hours daily [4]. Bialokoz-Kalinowska et al. observed that mean time spent at the computer monitor was 15 hours per week [3]. Łoś-Rycharska et al. demonstrated that children and young people spent more than 3 hours daily watching TV, working or playing the computer [15].

Summing up, physical activity should be a part of everyday life, especially, the effort that can be a part of everyone’s daily activities such as vigorous march, gardening etc. It is important to spend free time in an active way, especially, in the population at the developmental age. Thus, the propagation of physical activity is so significant in the times of fast development of techniques.

**Conclusions**

1. Physical activity of secondary school pupils aged 18 is insufficient both in boys and girls.
2. The percentage of the 18-year-olds from secondary schools attending physical education classes is very low.
3. Young people, especially, girls are not interested in sports activities after school.

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**References**

Respiratory chain complexes in healthy and cardiomyopathic hearts

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Abstract

Dysfunction of mitochondrial oxidative phosphorylation (OXPHOS) in heart muscle is postulated to play a major role in heart failure development. Abnormalities in ATP production and mitochondrial ATP synthase (complex V) activity, increased respiratory stress (ROS) with free radicals overproduction as well as apoptotic mechanism stimulation were found in humans and animal models with non-mitochondrial cardiomyopathies. Different secondary changes of respiratory chain complexes I, II, III and IV were reported in heart failure but the way of their pathological or compensatory influence not established. Also frequency of the primary mitochondrial cardiomyopathies is not known, especially in adults. The aim of the study was to assess the respiratory chain activity in 28 cardiomyopathic patients qualified for heart transplantation due to end-stage heart failure and 7 patients with hypertrophic cardiomyopathy undergone myomectomy. Own laboratory reference values were prepared using 9 healthy donors hearts. The whole study group demonstrated a number of significant changes in respiratory chain activity. There were decrease in citric synthase activity and complex III activity, and increase in complex II activity. Activities of complex I and IV did not differ significantly from the reference values. In five out of 36 patients the results might indicate a probability of mitochondrial disease (complex I or complex IV deficit). The further proteomic investigations did not reveal any primary pathological changes. Conclusion. 1/ The study confirms the general presence of OXPHOS dysfunction in hearts of the patients with end-stage heart failure and severe cardiomyopathies. New research techniques should be applied to uncover its mechanism and role. 2/ The primary mitochondrial cytopathy should be carefully excluded in the patient with cardiomyopathy of unknown etiology.

Key words: heart, respiratory chain complexes, spectrophotometry, healthy, cardiomyopathy, heart transplantation, myomectomy, mitochondrial disease

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Introduction

Possible contribution of mitochondria in the development of various types of cardiomyopathies (CMPs) seems almost certain due to mitochondrial role in energy production, free radicals generation and apoptosis [1, 3, 8]. The significance of mitochondrial dysfunction in different types of CMP is still not fully recognized and elucidated [22, 27, 32, 34, 36]. Empirical data (usually individual case reports) indicate that CMP both in adults and children may be associated with pathogenic mutation of genes located in mitochondrial DNA (mtDNA) [19, 26, 30, 35] as well as primary deficit of respiratory chain complex I, III, IV, or V [9, 11, 29, 33]. Heart involvement may be part of multiorgan clinical dysfunction (MELAS, MERRF) or appear as an isolated finding. Mitochondrial cardiomyopathy as a salient clinical feature may be observed for example in mutations of mtDNA gene coding for leucine tRNA at the position 3302-3303 [4, 31]; in complex IV deficit associated with SCO2 gene mutation [14]; in Barth syndrome [37] and in several other mitochondrial disorders [6, 21]. Recognition of these primary cardiomiopathies may be accidental and their prevalence particularly in adults is unknown. On the other hand the role of mitochondrial oxidative phosphorylation (OXPHOS) dysfunction in non-mitochondrial CMPs has been suspected for a long time and extensively investigated [5, 10, 12].

The results frequently appear controversial and still must be considered inconclusive.

Heart muscle mitochondria may be studied using well established techniques applied to skeletal muscle investigation. Spectrophotometric assessment of respiratory chain complexes activity in tissue sample homogenate and histochemical assessment of oxidative enzymes including cytochrome c oxidase are among most widely used techniques. However the reliable data concerning the range of normal and pathological values have not been available for a long time. Before heart transplantation era OXPHOS assessment was possible only in small endomyocardial biopsy samples limiting the range of investigation methods. Development of heart transplantation changed the situation since explanted hearts as well as hearts of potential donors became available for investigation. Yet, in medical literature there are still scarce data concerning normal OXPHOS measurements [5, 20], its practical usefulness is anyway limited by the necessity of establishing own norms in each mitochondrial laboratory.

Material

The study group consisted of 28 heart explants and 7 myomectomy patient hearts.

End-stage heart failure being a basis of qualification for transplantation was the main inclusion criterion in the heart explant subgroup. All the patients suffered from cardiomyopathy (dilated – DCM, idiopathic or ischemic), they were in NYHA III/IV class and had EF < 40. The age of the patients ranged from 14 to 65, average 52 years. Only one child was included. There were 4 females and 24 males. In the heart explant subgroup the material was collected in the period of 2001–2005. The maximum storage period was 6 years.

The myomectomy study subgroup consisted of the heart tissues excised during surgical treatment from the left ventricle in 7 patients, two children and 5 adults (2 females, 4 males). All patients demonstrated severe hypertrophic cardiomyopathy (HCM) which fulfilled a standard clinical indications for myomectomy. These heart samples were obtained in the period of 2005–2007. Biochemical analysis was performed within 24 months from the surgery.

Hearts obtained from 10 healthy, adults organ donors not suitable for transplantation served as the reference group. Only donor patients with maintained ventilatory and hemodynamic requirements were included. From each heart three samples were obtained (left ventricle, right ventricle, intraventricular septum) and immediately snap frozen into liquid nitrogen until biochemical analysis.

The study protocol was approved by the Bioethical Committee of Institute of Cardiology in Warsaw.

Methods

Histological, histochemical and immunohistochemical studies

Histological, histochemical and immunohistochemical methods applied in the study were performed as described earlier for frozen skeletal muscle [23]. Stains and histochemical reactions included: hematoxylin and eosin, modified Gomori trichrome, oil red O, cytochrome c oxidase, succinate dehydrogenase, NADH dehydrogenase and acid phosphatase.

Immunohistochemistry was performed with antibodies against CD3, CD4, CD8, CD68, ICAM, CD15, CD45 and desmin.

Spectrophotometric studies

Spectrophotometric assays was performed for the measurement of complex I (NADH: ubiquinone oxidoreductase, rotenone sensitive), complex II (succinate: ubiquinone oxidoreductase, malonate sensitive), complex III (ubiquinone: cytochrome c oxidoreductase, antimyceine sensitive), complex IV (cytochrome c oxidase-COX), and nitrate synthase enzyme activities in left ventricle, right ventricle and intraventricular septum heart homogenates as described previously for skeletal muscle [16, 17] with modification in volume of homogenization buffer to reach cardiac muscle aliquots protein in concentration in the range of 2–4 mg/ml. Protein was determined by the Lowry method. The ratio between the activity of individual respiratory chain complex and citric synthase (CS) was calculated to eliminate the possible effect of changes in number of mitochondria in patient cells.

Western blot analysis

Proteomic study was adopted from the methods described earlier for the skeletal muscle biopsy [18].

Protein concentrations of each sample was determined with a Bio-Rad Protein Assay. Western blotting was carried out as previously described [24]. Samples were loaded...
onto SDS-PAGE gels and after electrophoresis transferred to PVDF. After blocking, blots were probed with anti-OXPHOS antibodies (1:1000), anti-complex I subunits: anti-NDUFS 3 antibody (1:1000), anti-NDUFA 6 antibody (1:700), anti-GRIM 19 antibody (1:500), and anti-COX I antibody (1:1000), anti-COX IV antibody (1:500) and anti-COX Va antibody (1:500) (MitoSciences). The signal was revealed using alkaline phosphatase (AP) Conjugate Substrate Kit (Bio-Rad) after hybridization with goat anti-mouse IgG (H+L) (Bio-Rad) AP conjugated secondary antibody.

Blue Native Gel Electrophoresis and in gel activity assay of the mitochondrial fraction

Human heart biopsies were washed twice with PBS, resuspended in the buffer containing 250 mM sucrose, 1 mM EGTA, 50 mM Tris-HCl, 1 mM DTT, and protease inhibitor cocktail at pH 7.4 and gently disrupted by 15 up-and-down strokes in tight glass-glass homogenizer. The homogenate was centrifuged at 600 g for 5 min twice. The resulting supernatants were centrifuged at 10,000 g at 10 min. The pellets containing mitochondria were washed with homogenization buffer and centrifuged again at 10,000 g for 10 min. Mitochondrial pellets were solubilized with 1.5 M aminocaproic acid, 50 mM Bis-Tris at pH 7.0 and 1% dodecylmaltoside. Samples were incubated on ice for 20 min and then centrifuged 20,000 g for 15 min to remove unsolubilized material. Protein concentration in the supernatants was determined by Bradford’s method using Bio-Rad Protein Assay. Supernatants containing protein complexes were combined with 5% w/v suspension of Coomassie brilliant blue G-250 in the 1.5 M aminocaproic acid, 50 mM Bis-Tris at pH 7.0 buffer (adding 0.5 ml of Coomassie suspension to each 10 ml of supernatant). To carry out Blue Native Electrophoresis (BN PAGE) samples were separated on a big dimensional (1 mm/16 cm/20 cm) 5%–12% gradient acrylamide gel. Mitochondria isolated from rat heart were used as an internal standard to see the quality of separation and to calibrate the BN PAGE gel (in kDa). To visualize activity of individual respiratory chain complexes the gel have to be incubated at room temperature with the suitable solutions as described [18].

Data analysis

All data are shown as mean ± SD of the mean. The Student’s t-test was used for comparison differences between control and patients groups. A p value < 0.05 was considered significant.

All tests were performed using Statistica Software Version 8.0 (StatSoft Poland).

Results

Ranges of the normal reference values of complex I, II, III and IV of enzymatic respiratory chain activities in healthy human heart left ventricle, right ventricle and interventricular septum are shown in Table 1 using 95% confidence interval (95% CI).

Heart explant subgroup

Significant changes in the activity of some respiratory chain complexes were found in the study group of 28 explanted cardiomyopathic hearts (Table 2). Mean value of citric synthase activity was markedly decreased (p<0.01) being below the lowest control value in 12 out of 28 left ventricle specimens studied (Fig. 1). Protein content did not differ significantly between the reference and the study groups.

Activity of complex I did not differ significantly between the control and the whole studied group (Table 2, Fig. 2). However two transplanted left ventricle tissues (patient 14 and 15) showed abnormally low complex I activity.
Activity of complex II was significantly higher in the failing explants group in comparison with the healthy controls (Table 2). The difference was significant if activity values were expressed as percentage of citric synthase activity (p<0.05). Nineteen affected hearts showed the complex II activity value at the highest borderline or above the control (Fig. 3).

Complex III activity in the failure heart explants was generally decreased. Differences of the values expressed by protein content and control values were of high significance (Table 2, p<0.001). In 18 out of 28 affected hearts the complex III activity was below or at the lowest control level (Fig. 4). Values of the complex II+III were similarly decreased if expressed by protein content (p<0.02; Table 2).

Table 1

Activities of respiratory chain complexes in healthy donors hearts (reference values)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Mean value</th>
<th>Standard deviation (SD)</th>
<th>Confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-95%</td>
</tr>
<tr>
<td><strong>Left heart ventricle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS nmol/min/mg prot</td>
<td>8</td>
<td>504.21</td>
<td>145.19</td>
<td>382.83</td>
</tr>
<tr>
<td>Complex I %CS/nmol/min/mg prot/</td>
<td>8</td>
<td>10.65</td>
<td>5.91</td>
<td>5.71</td>
</tr>
<tr>
<td>Complex II %CS/nmol/min/mg prot/</td>
<td>8</td>
<td>9.08</td>
<td>2.51</td>
<td>6.97</td>
</tr>
<tr>
<td>Complex II+III %CS/nmol/min/mg prot/</td>
<td>8</td>
<td>4.36</td>
<td>1.33</td>
<td>3.25</td>
</tr>
<tr>
<td>Complex III %CS/nmol/min/mg prot/</td>
<td>8</td>
<td>52.46</td>
<td>9.30</td>
<td>44.69</td>
</tr>
<tr>
<td>Complex IV %CS/nmol/min/mg prot/</td>
<td>8</td>
<td>6.10</td>
<td>3.13</td>
<td>2.81</td>
</tr>
<tr>
<td><strong>Right heart ventricle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS nmol/min/mg prot</td>
<td>9</td>
<td>472.0</td>
<td>75.53</td>
<td>413.96</td>
</tr>
<tr>
<td>Complex I %CS/nmol/min/mg prot/</td>
<td>9</td>
<td>11.96</td>
<td>3.94</td>
<td>8.92</td>
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<tr>
<td>Complex II %CS/nmol/min/mg prot/</td>
<td>9</td>
<td>7.83</td>
<td>2.36</td>
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<tr>
<td>Complex II+III %CS/nmol/min/mg prot/</td>
<td>9</td>
<td>5.62</td>
<td>2.92</td>
<td>3.37</td>
</tr>
<tr>
<td>Complex III %CS/nmol/min/mg prot/</td>
<td>9</td>
<td>56.66</td>
<td>21.65</td>
<td>40.02</td>
</tr>
<tr>
<td>Complex IV %CS/nmol/min/mg prot/</td>
<td>9</td>
<td>11.2</td>
<td>2.27</td>
<td>9.46</td>
</tr>
<tr>
<td><strong>Interventricular septum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS nmol/min/mg prot</td>
<td>9</td>
<td>483.83</td>
<td>62.57</td>
<td>435.7</td>
</tr>
<tr>
<td>Complex I %CS/nmol/min/mg prot/</td>
<td>9</td>
<td>8.88</td>
<td>3.06</td>
<td>6.53</td>
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<tr>
<td>Complex II %CS/nmol/min/mg prot/</td>
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<td>8.46</td>
<td>2.43</td>
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<td>5.09</td>
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<td>3.61</td>
<td>4.10</td>
</tr>
</tbody>
</table>
Mean value of complex IV activity was comparable in both reference and study groups (Table 2). Markedly decreased activity was found in three patients with heart failure (Fig. 5, patients 1, 2 and 5).

Spectrophotometric activity assay was also performed in 10 right ventricle specimens (patients 3, 4, 10, 12, 14, 21, 22, 24, 26), including cases with very low left ventricle complex I or IV activity. Normal activity was found in the second tissue sample for four out of 5 complex I/IV deficient left ventricle, excluding generalized respiratory chain deficit. Only one patient (patient 5) had complex IV decrease in both ventricle samples.

Average values of respiratory chain parameters for left and right ventricle did not differ remarkably and were respectively: for citric synthase 426.5 ± 57.0 and 381.8 ± 60.7 nmol/min/mg protein, for complex I 11.3 ± 4.1% and 11.8 ±
2.9%, for complex II 7.5 ± 5.5% and 7.1 ± 5.6%, for complex III 42.4 ± 17.1% and 44.7 ± 11.3%, and for complex IV 5.7 ± 4.2% and 4.3 ± 2.1%.

Expression of complex I and IV subunits assessed by Western blotting in 10 left ventricle explants showing low/high spectrophotometric activity values was at the normal range in all examined cases (Fig. 6, see the same patients in Fig. 2 and 5). The data indicated that a true complex I/IV deficit may be excluded at the protein level as a pathogenic basis of cardiomyopathy in these cases.

Myomectomy subgroup
Table 3 summarizes the results obtained in the myomectomy study subgroup. Histological features of cardiomyopathy (hypertrophic) was confirmed in all cases and the features of primary mitochondrial disorder did not found.

The activity of respiratory chain complexes measured in four left ventricle homogenates by spectrophotometric method was at the same range as in the left ventricle explants subgroup.

In comparison with the reference group of healthy donor hearts the citric synthase activity was similarly lower. BN PAGE and „in gel” activity assay also shown the normal expression and activity of complex IV (Fig. 7). Activity of mitochondrial ATP synthase (complex V) examined in three specimens was normally expressed (Fig. 7).

In summary, the study did not reveal a case with primary OXPHOS defect (a mitochondrial disorder) among the.
examined myomectomised patients with hypertrophic cardiomyopathy.

Especially, the study enabled us to exclude the mitochondrial cardiomyopathy in one strongly suspected case (Table 3, patient B). The initial diagnosis of mitochondrial cardiomyopathy in this girl had been previously established indirectly depending on the low cytochrome oxidase activity in skeletal muscle [7]. In this study it was finally shown that the spectrophotometric activity of complex IV in the girl’s heart homogenate is normal (35.4 nmol/min/mg protein). Histochemistry, BN PAGE and „in gel” activity methods also confirmed normal activity and content of complex IV in the left ventricle tissue obtained during myomectomy in this patient (Fig. 7, patient B).

Discussion

The reference (own laboratory) spectrophotometric values for respiratory chain complexes and citric synthase activities were established for three regions of human heart: left ventricle, right ventricle and intraventricular septum. The spectrophotometric measurement of OXPHOS parameters in healthy donor heart showed very high value of citric synthase activity in

Fig. 5 Complex IV activity in the muscle samples from left ventricle of explanted hearts of the patients with cardiomyopathy and cardiac failure. Dashed lines show mean value and range of laboratory reference values.

Fig. 6 Western blot analysis of the presence of the individual respiratory chain subunits: (A): OXPHOS; (B): NDUFS 3, NDUFA 6; (C): GRIM19; (D): COX I; (E): COX IV; (F): COX Va.

Legend:

Samples: 1, 2, 5, 7, 14, 15, 17, 28 (10μg of protein/lane) were separated on 10-20% SDS gel. Result of one immunoblots panel from two independent experiments is shown. HHM5, HHM10 – human heart mitochondria (Mitosciences) 5 or 10 μg protein/lane. MW molecular weight standard. V F1 – subunit of complex V, III core2 – subunit of complex III, II Ip 30kDa – subunit of complex II, COX II – subunit of complex IV, I 20 kDa – subunit of complex I.
comparison with own laboratory control obtained earlier for skeletal muscle homogenates [16] and fibroblast cultures [17]. Mean reference value established for the left ventricle tissue is approximately three times higher than observed in the control skeletal muscle (504.2 versus 123.0 nmol/min/mg protein respectively). The absolute activity of complexes I, II, III and IV in the heart (expressed by protein content) was al-

Table 3

Histological, histochemical, immunohistochemical, spectrophotometric and proteomic data of seven myomectomized heart specimens of the patients with severe hypertrophic cardiomyopathy. COX – cytochrome c oxidase activity. CS – citric synthase

<table>
<thead>
<tr>
<th>Patient, gender</th>
<th>Age of onset</th>
<th>Histological and histochemical features</th>
<th>Immunohistochemistry</th>
<th>Activity of respiratory chain complexes I-IV expressed by percentage of CS*</th>
<th>Mitochondrial proteins/complex subunits expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Male (MZ)</td>
<td>Adult</td>
<td>Cardiomyocyte hypertrophy, severe focal interstitial fibrosis. COX(+), No lipid accumulation</td>
<td>CD3(–); CD4(–); CD8(–); CD68(–); ICAM(+); CD15(+); CD45(+)</td>
<td>SC 388.5 nmol/min/mg prot, complex I 8.3 %, complex II 11.5 %, complex III 48.6 %, complex IV 8.8 %</td>
<td>Normal expression and activity of complex IV and mitochondrial ATP synthase (see Figure 7)</td>
</tr>
<tr>
<td>B. Female (WA)</td>
<td>Child /16 y/</td>
<td>Severe cardiomyocyte hypertrophy and endocardial fibrosis. Focal mononuclear inflammatory infiltrates. COX(+), No lipid accumulation</td>
<td>CD3(+); CD4(–); CD8(–); CD68(–); CD15(+); CD45(+)</td>
<td>SC 404.7 nmol/min/mg prot, complex I 5.5 %, complex II 15.3 %, complex III 55.7 %, complex IV 8.7 IV %</td>
<td>Normal expression and activity of complex IV and mitochondrial ATP synthase (see Figure 7)</td>
</tr>
<tr>
<td>C. Male (JJ)</td>
<td>Adult</td>
<td>Mild cardiomyocyte hypertrophy and interstitial fibrosis. Endocardial fibrosis and thickening. COX(+), No lipid accumulation</td>
<td>CD3(–); CD4(–); CD8(–); CD68(–); CD15(–); CD45(–)</td>
<td>SC 344 nmol/min/mg prot, complex I 6.3 %, complex II 7.1 %, complex III 67.8 %, complex IV 11.1 %</td>
<td>Normal expression and activity of complex IV and mitochondrial ATP synthase (see Figure 7)</td>
</tr>
<tr>
<td>D. Female (HF)</td>
<td>Adult</td>
<td>Severe interstitial fibrosis with focal scarring. Cardiomyocyte hypertrophy. COX(+), No lipid accumulation</td>
<td>CD3(+); CD4(–); CD8(–); CD68(–); CD15(–); CD45(–)</td>
<td>SC 390.8 nmol/min/mg prot, complex I 8.1 %, complex II 10.3 %, complex III 56.9 %, complex IV 4.9 %</td>
<td>ND</td>
</tr>
<tr>
<td>E. Male (BJ)</td>
<td>Adult</td>
<td>Mild cardiomyocyte hypertrophy. Focal interstitial fibrosis. COX(+), No lipid accumulation</td>
<td>CD3(–); CD4(–); CD8(–); CD68(–); CD15(–); CD45(–).</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F. Male (MJ)</td>
<td>Child /13 y/</td>
<td>Marked cardiomyocyte hypertrophy, altered myocardial architecture. COX(+), No lipid accumulation</td>
<td>CD3(–); CD4(–); CD8(–); CD68(–); CD15(–); CD45(–).</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G. Female (ZK)</td>
<td>Adult</td>
<td>Cardiomyocyte hypertrophy, marked interstitial fibrosis. COX and lipids not assessed</td>
<td>CD3(–); CD4(–); CD8(–); CD68(–); CD15(–); CD45(–).</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Reference values see table 1.

Fig. 7 Blue native polyacrylamide gel electrophoresis and in gel activity assay. Legend:
BN PAGE of human heart mitochondria (patients A, B and C) and rat heart mitochondria (M). In gel activity assay for the evaluation of complex IV (panel 1) and V (panel 2) activity.
so higher although the relative activity value (expressed by percentage of citric synthase activity) appeared comparable between the left ventricle and the skeletal muscles homogenates. Similar data were published earlier [15, 20], and may present relatively higher amount of mitochondrial mass in the heart. The reference values established for the other heart localizations (right ventricle, intraventricular septum) were in proportion to the left ventricle reference.

The histological, histochemical, biochemical and proteomic studies undertaken to search for a primary mitochondrial defect as the cause of cardiomyopathy were negative in all patients included in the study. Any patient with a primary OXPHOS defect or a mitochondrial cardiomyopathy was not identified among 29 transplanted and 7 myomectomized patients. Initial suspicion of a primary deficiency of complex I or IV in five transplanted patients and in one myomectomized patient were not confirmed in the study by further assessment of the complex subunits content and/or activity in the heart tissue. The results seemed to confirm that the primary mitochondrial pathology contribution is rare in isolated cardiomyopathies, especially in adult patients.

Similar approach was undertaken earlier by a few authors and gave similar results.

Jarretta et all in 2000 [15] studied mitochondrial respiratory activities in explants of 17 adult patients with dilated cardiomyopathy and 17 healthy donor hearts. Lower citric synthase activity and complex III (and complexes I+III activity) depression were found by the spectrophotometric method. Due to a suspicion of the primary deficit of complex III cytochrome b gene was sequenced in two of the patients. The search for pathogenic mutation(s) was negative.

On the other hand, screening the whole mitochondrial genome in a group of 45 idiopathic DCM patients revealed increased number of novel mtDNA point mutations (encoding mitochondrial complex I and IV subunits or tRNA genes) in comparison with the group of 62 ischemic DCM subjects. Some mutations occurred in highly conserved aminoacids region and were heteroplasmic which strongly suggested their pathogeneity. The mutation in the D-loop region (c. 16189T>C) associated with susceptibility to DCM was present in 15.6% of patients and in 9.7% of controls [26]. The authors concluded that mtDNA mutations altering OXPHOS function can be remarkably relevant for pathogenesis of dilated cardiomyopathy.

In this study significant changes of OXPHOS function were found in the heart tissue of patients with end-stage heart failure. The marked decrease in citric synthase activity, and in complex III and II+III activities were observed. Oppositely, the activity of complex II was higher then in the control. Similar mitochondrial dysfunction was already described [15, 19] and is not fully understood.

Introducing a new methodological approach by assessment not single complex activities but the active supercomplexes of respiratory chain (so called respirosome, i.e. complex I/complex III dimmer/complex IV) will possibly help to elucidate the importance and mechanism of OXPHOS activity decreasing in the end-stage heart failure [25].

Recently, a switch in energetic metabolism by activating the fetal gene expression program (with glycolytic pathways activation and beta-oxidation pathways suppression) was described in the end-stage heart failure experimental animals [12]. The switch leads to mtDNA depletion, to decline of respiratory chain enzyme activities and decreased ATP production. The lowering the mitochondrial biogenesis and function in severe heart failure (probably compensatory to avoid the consumption of large oxygen amount) may explain our findings of decrease in citric synthase activity and complex III activity. The phenomenon unlikely benefits the energy homeostasis and therefore likely aggravates the disease [12].

Finally, it must be emphasized that in each case of cardiomyopathy possible primary mitochondrial defect should be searched for by enzymatic and molecular studies, bearing in mind that secondary as well as compensatory mitochondrial dysfunction may be present. It is important that in a stage of severe heart failure mitochondrial pathogenesis is investigated due to specific problems concerned with indications for heart transplantation [2, 28], and possible maternal inheritance. It seems that the issue of mitochondrial cardiomyopathies is better known and understood in children than in adult patients [13, 29].

Tissue sample processing methods and reference values of respiratory chain activity presented in this study make it possible to implement mitochondrial diagnostics in each cardiomyopathic patient undergoing cardiosurgical intervention which allow to obtain myocardium for investigation.

Acknowledgment

The paper was supported by Internal Projects of The Children’s Memorial Health Institute Grant CMHI 182/06 (principal investigator – Ewa Pronicka), 161/06 (principal investigator – Maciej Pronicki) and 148/06 (principal investigator – Elżbieta Karczmarewicz). The research was also partly granted by the Polish Mitochondrial Network (mitoNET.pl).

References


Liver pathology in chronic hepatitis C virus infection associated with extrahepatic manifestations

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Abstract
We analyzed microscopic changes in the liver from ten patients infected by hepatitis C virus. All patients developed chronic hepatitis and extrahepatic manifestations as cryoglobulinemia, polyneuropathy, purpura cutanea, Sjögren syndrome and nephritic syndrome. The most severe microscopic changes in the liver were connected with cryoglobulinemia and cutaneous manifestations together with Sjögren syndrome and represented intensive inflammatory lymphoid infiltrates in the lobules, portal spaces with prominent interface hepatitis and septal fibrosis with architectural distortion.

Key words: HCV, extrahepatic manifestations, chronic hepatitis, vasculitis

Introduction
Hepatitis C virus (HCV) infection is the second most common viral infection of the liver with a global prevalence of 3% (about 180 million people, but many are unaware of the infection) leading to chronic hepatitis, cirrhosis and hepatocellular carcinoma. Because of its hepatotropic, lymphotropic features and replication in hepatocytes, lymphocytes and macrophages, HCV can induce systemic disease with extrahepatic manifestations [4]. The prevalence of extrahepatic manifestations is low in pediatric population, but about 50% of adult HCV-positive patients during the course of the disease develop at least one extrahepatic manifestation [7]. Among the best reported are cryoglobulinemia, peripheral neuropathy, glomerulonephritis, thrombocytopenic purpura, lichen planus, corneal ulcer, Sjögren syndrome, porphyria cutanea tarda and necrotizing cutaneous vasculitis. [5, 8].

Patients, material and methods
Total number of 10 patients (8 females, 2 males) aged 31 to 69 years (mean age 51.6) with chronic hepatitis C virus (HCV) infection confirmed by serological and virological (HCV-RNA) criteria, underwent percutaneous liver biopsy (Tab. 1). Extrahepatic manifestations of the HCV infection occurred in all patients: cryoglobulinemia in 8 patients, purpura cutanea in 5 patients, Sjögren syndrome in 2 patients, polyneuropathy in 3 cases, nephrotic syndrome in 3 cases. The majority of patients (9 cases) presented more than one feature of extrahepatic manifestations, the rest (1 patient) had...
membranous nephropathy. Patients with alcohol abuse, diabetes and metabolic syndrome were excluded from the study.

All biopsy specimens were fixed in buffered formalin and embedded in paraffin blocks. Four micrometer thick sections, displaying at least 10 portal spaces were routinely stained by Hematoxylin and Eosin, Periodic Acid-Schiff method with and without diastase, Gomori Silver impregnation and Azan or Masson Trichrome Stain. Histological interpretation was performed using internationally accepted criteria [1] and reviewed retrospectively (two independent pathologists). All histological features were finally scored using the four degree scale for the grade (inflammatory activity) and stage (fibrosis) of the disease. The grade was assessed as: 0 – portal inflammation only, without piecemeal necrosis, without lobular inflammation and necrosis; 1 – minimal portal inflammation, minimal, patchy piecemeal necrosis, occasional spotty necrosis; 2 – mild portal inflammation, mild piecemeal necrosis involving some or all portal tracts, little hepatocellular damage; 3 – moderate portal inflammation, moderate piecemeal necrosis involving all portal tracts, moderate lobular inflammation with noticeable hepatocellular change; 4 – severe portal inflammation severe piecemeal necrosis, may have bridging necrosis, severe lobular inflammation with prominent diffuse hepatocellular damage. The stage was assessed as: 0 – no fibrosis, normal connective tissue; 1 – portal fibrosis, fibrous portal expansion; 2 – perportal fibrosis, periportal or rare portal-portal septa; 3 – septal fibrosis, fibrous septa with architectural distortion; 4 – cirrhosis.

### Results

The necroinflammatory activity, described as a grade of the disease, was severe in 20%, moderate in 30% and mild in 50% of the examined material. We found lymph follicle formations in portal tracts and inflammatory infiltrates near blood vessel walls (venulitis) in all patients. Hepatocyte steatosis was observed in 4 patients. Liver fibrosis, described as a stage of the disease was characterized by numerous fibrous portal septa and architectural distortion in 20%, while portal fibrosis and portal-portal septa occurred in 60% of biopsy specimens and mild or no fibrosis in 20% of them. Lymph follicle formations were present in all biopsy specimens. They were found in the portal tracts (at least 3 per 10 portal tracts), always involved blood vessels. Lymphoid cells infiltrated also the walls of venous vessels in all portal tracts (Fig. 1).

Histological activity index varied from mild inflammation to severe necroinflammatory activity with the mean activity of grade 3 (moderate inflammation). The stage of the

### Table 1

Characteristics of HCV positive patients with extrahepatic manifestations

<table>
<thead>
<tr>
<th>No</th>
<th>Age</th>
<th>Sex</th>
<th>Clinical manifestations</th>
<th>Grade</th>
<th>Stage</th>
<th>Microscopical findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>F</td>
<td>Psoriasis, Sjögren syndrome, cryoglobulinaemia</td>
<td>4</td>
<td>3</td>
<td>Portal lymph follicles, portal vasculitis</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>F</td>
<td>Polyneuropathy cryoglobulinaemia</td>
<td>3</td>
<td>2</td>
<td>Portal lymph follicles, portal vasculitis</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>F</td>
<td>Membranous nephropathy</td>
<td>2</td>
<td>2</td>
<td>Portal lymph follicle, portal vasculitis</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>F</td>
<td>Polyneuropathy cryoglobulinaemia</td>
<td>2</td>
<td>0</td>
<td>Portal lymph follicles, portal vasculitis</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>F</td>
<td>Polyneuropathy, cryoglobulinaemia</td>
<td>2</td>
<td>1</td>
<td>Portal lymph follicles portal vasculitis</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>M</td>
<td>Purpura, cryoglobulinaemia, Sjögren syndrome</td>
<td>2</td>
<td>2</td>
<td>Portal lymph follicles portal vasculitis</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>F</td>
<td>Purpura, cryoglobulinaemia, Sjögren syndrome</td>
<td>4</td>
<td>3</td>
<td>Portal lymph follicles, hepatocyte steatosis, portal vasculitis</td>
</tr>
<tr>
<td>8</td>
<td>51</td>
<td>M</td>
<td>Purpura, cryoglobulinaemia</td>
<td>3</td>
<td>2</td>
<td>Portal lymph follicles, hepatocyte steatosis, portal vasculitis</td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>M</td>
<td>Cryoglobulinaemia, membranoproliferative glomerulonephritis</td>
<td>3</td>
<td>2</td>
<td>Portal lymph follicles portal vasculitis, hepatocyte steatosis</td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>F</td>
<td>Purpura, cryoglobulinaemia Membranoproliferative glomerulonephritis</td>
<td>2</td>
<td>2</td>
<td>Portal lymph follicles portal vasculitis, hepatocyte steatosis</td>
</tr>
</tbody>
</table>
Disease varied also from 0 (without fibrosis) to 3 (architectural distortion) with the mean stage of 2 (portal-portal fibrosis without cirrhosis). The most severe microscopical changes in the liver were connected with cryoglobulinemia and cutaneous manifestations together with Sjögren syndrome and represented intensive inflammatory lymphoid infiltrates in the lobules, portal spaces with prominent interface hepatitis and septal fibrosis with architectural distortion.

Discussion

Extrahepatic manifestations in HCV infected patients involve primarily the skin and joints. The most frequent immunologic abnormalities include mixed cryoglobulinemia, renal and thyroid diseases and lymphoproliferative disorders [3]. We present patients with chronic hepatitis connected with cryoglobulinemia, Sjögren Syndrome, skin, joints renal and neurological lesions.

Vascular changes and lymph follicle formations occurred in all liver biopsies. This phenomena have been also observed in other patients of HCV infection without extrahepatic involvement, but the intensity of this changes appear to be more pronounced than in other chronic hepatitis cases. Similar morphological vascular changes have been observed in transplanted liver with acute rejection as autoimmune phenomena. Vascular changes in these patients can be considered as immunological reaction to generalized HCV infection.

The most severe changes in the liver were found in two patients with Sjogren syndrome, cryoglobulinemia and cutaneous disorders. Despite these findings we did not found any clear correlation between presence of these symptoms and staging score of chronic hepatitis, because these lesions occurred also in a patient with mild changes in the liver. The prospective study of 45 consecutive HCV infected patients revealed similar histopathological changes in the liver, but also the association between older age and liver disease activity [6].

The association between the presence of serum cryoglobulins and intensity of hepatic microscopic changes was found, with no difference in patients age or duration of infection [12]. It is possible that cryoglobulinemia results in
more rapid hepatic fibrosis in HCV infected patients. In our group of ten patients we did not found this association.

Renal diseases associated with HCV infection are relatively common in adult patients but rare in children. We found them in five patients together with other extrahepatic manifestations, but in two cases as single manifestation. Interestingly they occur in the youngest patients from our whole group (female 31 and male 32 years old). The hepatic histological activity of these patients is moderate, without progression into cirrhosis. The frequent lesions associated with HCV are membranoproliferative glomerulonephritis, membranous nephropathy, focal segmental glomerulosclerosis and mesangial proliferative glomerulonephritis with IgA [10]. In our study HCV-related glomerulopathy consisted of membranoproliferative glomerulonephritis in 2 patients and membranous nephropathy in 1 patient.

Neurologic complications in HCV infected patients can involve the peripheral and central nervous system, the most frequently subacute, distal, symmetric, sensory and motor polyneuropathy in the presence or without mixed cryoglobulinemia [10]. Polyneuropathy increased significantly with age, which is the only independent predictor. Cryoglobulinemia and the intensity of fibroinflammatory changes in the liver do not influence the prevalence of polyneuropathy [11]. In our study polyneuropathy occurred in 4 patients; three of them were more than 60 years old (mean age 65) and the histological activity was mild to moderate. The fourth patient was younger (51 years old) but he had also other extrahepatic manifestations as Sjögren syndrome, nephritic syndrome and skin lesions. Interestingly, the histological activity was not very high but the vascular involvement was prominent (Fig. 1A).

Systemic autoimmune diseases are also connected with HCV infection. In a large study of 1020 patients from Europe, America, Asia and Australia (HISPAMEC Registry) systemic autoimmune symptoms in HCV infection are most commonly described in Sjögren syndrome, rheumatoid arthritis and SLE [9].

In summary, infections with HCV can involve not only the liver but also various organs. The histological activity of hepatitis does not influence the incidence of extrahepatic involvement. Vascular involvement and prominent lymph follicles in portal tracts are constant findings in patients with HCV chronic hepatitis and extrahepatic manifestations.

References

Submandibular ectopic thyroid tissue with subclinical hypothyroidism - the diagnostic dilemma

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Abstract

Ectopic thyroid lesions constitute a rarely encountered developmental anomaly of the thyroid gland. Ectopic thyroid tissue generally presents in the lingual, sublingual or in anterior midline of neck. A lateral or submandibular location of ectopic thyroid tissue is very rare. We report the case of a 16 year old female diagnosed as a submandibular ectopic thyroid with subclinical hypothyroidism. We also stress the importance of fine needle aspiration cytology over surgical excision, as a practical procedure for differentiating this lesion from other neck swellings; as this tissue might be the only normal thyroid tissue in the patient’s body.

Key words: ectopic thyroid, hypothyroidism, FNAC

Introduction

Ectopic thyroid refers to the presence of secondary thyroid tissue in the neck, distinct from the thyroid gland and without any link to it. These lesions are generally situated in the midline region as a result of abnormal median migration which results from the failure of the migration from embryologic position of thyroid tissue at the base of the tongue to the lower neck. They can be classified anatomically as lingual, sublingual, prelaryngeal and other rare sites. Ectopic thyroid tissue presenting as palpable mass, may mimic malignancy and can reveal surprising images for a cytopathologist. Such cases are an appropriate indication for fine needle aspiration cytology (FNAC) which may protect the clinician from diagnostic pitfalls arising from ectopic lesions. Any unwarranted surgical removal of the ectopic thyroid will subject the patient to lifetime thyroid replacement therapy. Most ectopic thyroid glands are asymptomatic. Therefore, an accurate prevalence cannot be estimated. However, an ectopic thyroid tissue in the lateral and submandibular area is rarely encountered[4]. We present a rare case of 16 year old female with an ectopic thyroid tissue in the submandibular region presenting with subclinical hypothyroidism.

A case report

A 16 year old female presented with a swelling in the right submandibular region since 1 1/2 years (Fig. 1). The swelling was painless and gradually increasing in size. There was no history of fever, trauma or pain while swallowing. On examination, swelling measured 3 x 3 cm globular, firm and non tender with limited mobility; non-compressible and non-transilluminant. Ultrasonography (USG) showed a hyperechoic lesion of 2.3 x 2.9 cm with a thin halo and good vascularity on right side of neck. Based on clinical and USG findings, a clinical diagnosis of benign tumor arising from submandibular gland was made and fine needle aspiration cytology was advised. Aspiration cytology was performed...
with all aseptic precautions using a 23-gauge needle. Cytology smears revealed groups, sheets and ill formed papillae of follicular epithelial cells with features of hyperplasia in a background of scanty colloid and RBC’s (Fig. 2). Some of the clusters revealed mild to moderate anisonucleosis. However, characteristic nuclear features of metastatic papillary or follicular carcinoma were not evident. A cytological diagnosis of hyperplastic ectopic thyroid was rendered on FNAC. However, possibility of metastatic deposits from carcinoma thyroid was not ruled out. A repeat ultrasonography was advised which revealed absence of thyroid tissue at the normal site. Tc-99 sodium pertechnetate thyroid scan confirmed the diagnosis of an ectopic thyroid tissue where no uptake was seen in thyroid fossa (Fig. 3). Thyroid function tests were normal but thyrotropin stimulating hormone (TSH) was raised. The patient was put on oral thyroxine and the TSH levels decreased within one month. The submandibular swelling was reduced within three months. The patient is on follow-up and is doing well till date.

**Discussion**

The thyroid gland develops from a median endodermal thyroid diverticulum which grows down in front of the neck from the region of foramen caecum of the tongue to its normal location. Ectopic thyroid as a developmental anomaly may be encountered at sites along the course of this diverticulum. Most common site for an ectopic thyroid is sublingual region which accounts for about 90% of the reported cases, other uncommon sites being mediastinum, esophagus, lung, heart, breast, duodenum, trachea, adrenal gland etc. [8].

Ectopic thyroid tissue in the lateral and submandibular area are very rare with a very few case reports of lateral thyroids in the literature. In general, these are following possible explanations for the presence of ectopic thyroid tissue in the submandibular region:

1) displacement during the course of embryonic development 2) spread of tissue during surgery on a normally located thyroid and 3) metastasis of a highly differentiated papillary thyroid carcinoma [2]. In cases of ectopic thyroid, the gland may be normal or may exhibit different pathologic pictures such as atrophy, nodular or diffuse hyperplasia and Hashimoto’s thyroiditis [1, 3, 6, 9]. In addition, few malignant cases originating in the ectopic tissue have also been reported [5, 10]. Ectopic thyroid is usually detected during puberty or pregnancy because the increased demand for thyroid hormone elevate the thyrotropin levels. This is supposed to increase the size of the ectopic thyroid tissue developing the obstructive symptoms.

Ectopic thyroid tissue in the neck may create diagnostic difficulties from a clinical and cytopathological view point. Its presentation as a painless neck swelling makes it liable to be mistaken for a lymph node, subjecting the lesion to unwarranted surgical excision. The ectopic rests of thyroid tissue may be the only existing thyroid tissue, the removal of which will subject the patient to lifetime hormone replace-
ment therapy. Fine needle aspiration cytology is an important diagnostic tool for the differential diagnosis of these lesions which can reveal unusual findings. The FNAC of a neck mass which is not related to the thyroid gland and revealing cellular component of thyroid tissue can be a diagnostic dilemma. The cytopathologist is left with two options in such cases: either metastatic thyroid malignancy or an ectopic thyroid tissue. A cytopathologist encountering a cellular component related to thyroid tissue must be particularly careful with the diagnosis. One should take a conservative approach even if the aspirate material incorporates an evidence of moderate to marked atypia. Possibility of ectopic thyroid must be taken into consideration and one must avoid a malignant diagnosis before seeing any evidence of clear cytological findings [7]. The treatment of the ectopically located thyroid lesion goes along the line of lesions in a normally located thyroid. Since the lesion was hyperplastic goiter in the present case, the conservative management sufficed and the patient responded well to the treatment. As stressed earlier, surgical removal is unwarranted unless a malignancy is proven.

We recommend that any mass in the cervical region should be treated with high index of suspicion as it could be the only normal thyroid tissue present. Any surgery contemplated on them should be preceded by adequate investigations like radioimaging and aspiration cytology, so as to avoid the problem of unnecessary irreversible hypothyroidism. A cautious diagnostic approach is recommended in such cases and unless the findings are clear, one must refrain from a diagnosis of malignancy.

References

Introduction

The neoplastic tumors of the orbital region are not frequent. The most common benign tumors include haemangioma, lipo- 
ma, schwannoma and rare inflammatory tumors. The mali-
gnant entities in the adults encompass squamous cell carcino-
ma, adenoid cystic carcinoma, adenocarcinoma and in the chil-

Dian and therapeutic difficulties in an orbital tumor in a 3-year-old boy

Introduction

The soft tissue tumor arising from the external ocular muscle in a child is reported. The treatment including chemo- and radiotherapy was ineffective and finally the orbital exenteration was necessary to achieve complete remission. We present difficulties in histopathological diagnosis and therapeutic approach in this case.

Key words: leiomyosarcoma, orbital site, soft tissue sarcoma

Diagnostic and therapeutic difficulties in an orbital tumor in a 3-year-old boy

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Abstract

The soft tissue tumor arising from the external ocular muscle in a child is reported. The treatment including chemo- and radiotherapy was ineffective and finally the orbital exenteration was necessary to achieve complete remission. We present difficulties in histopathological diagnosis and therapeutic approach in this case.

Key words: leiomyosarcoma, orbital site, soft tissue sarcoma

Diagnostic and therapeutic difficulties in an orbital tumor in a 3-year-old boy

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Report of a case

In September 1994 a 3-year-old boy presented with the divergent strabismus, flush eyelid, ptosis and recurrent purulent conjunctivitis in the right orbit. The lacrimal duct probing had been performed twice and the boy was treated several times with antibiotics and antiinflammatory drugs locally. Due to the persistent and increasing clinical symptoms, computed tomography examination (CT) was performed (Fig 2). It showed a homogenous mass in the medial part of the right orbit, measuring 15 mm in maximal diameter. The tumor extended from the posterior wall of the eyeball inside the orbit and contained the right medial rectus muscle. No bone erosion or destruction was noted.

The open biopsy of the mass was performed and revealed neoplastic tissue consistent with the embryonal rhabdomyosarcoma (RMS) of spindle cell type. The boy started chemotherapy according to the treatment protocol CWS 91 (Koscielniak, Falkowski) with no radiological response after 9 weeks. Additionally the boy was diagnosed with active type C hepatitis and he received high doses of steroids.

Lack of the therapeutic effect caused that the second surgery was performed in the Department of Ophthalmology in January 1995. The conjunctiva was incised close to the limbs in inferior nasal quadrant to assess the proper mass of tumor. The exact tumor margins were blurred and only partial tumor excision was performed. That time pathological diagnosis, confirmed in two referential centers, has been changed into low grade leiomyosarcoma.

Because of a nonradical resection the radiotherapy was introduced using 4MV photons. The total dose of 5040 Gy in 28 fractions with 1.8 Gy/fraction was delivered over six weeks. At the end of radiotherapy the residual mass with the evidence of the destruction in the middle orbital bones and the penetration to the ethmoidal cells was revealed in CT. The child underwent surgical treatment in Department of Otolaryngology. Removal of the right eyeball with soft tissues of the orbital cavity and external right ethmoidectomy were performed. Histologically neoplastic infiltration was found in soft orbital tissues with focal ethmoid invasion. Additionally 4 cycles of the complementary chemotherapy with CBDCA and DTIC were used. The next control CT and MRI examinations didn’t show any signs of the recurrence. During the 13 years period of follow-up the patient remains in complete remission.

Histologically tumor tissue was composed of spindle cells with abundant eosinophilic cytoplasm, focal paranuclear vacuoles, with centrally located cigar shaped or blunt ended nucleus with small prominent nucleoli (Fig. 1a, b) The cells were arranged in intersecting fascicles, with focal whor-
led pattern. Scattered pleomorphic were encountered. Mitoses were infrequent, moreover, neither vascular invasion nor necrosis were stated. In the tissue material from the last operation (after chemo- and radiotherapy) lympho-plasmocytic infiltration was dispersed through the neoplastic tissue and fibrosis was visible. Immunohistochemically staining for vimentin, smooth muscle actin (ASMA), desmin, myoglobin, sarcomeric actin, cytokeratins, HMB-45, S-100, MyoD1, Caldesmon H and ALK was performed. Neoplastic tissue was positive for vimentin (100%), ASMA and desmin – diffusely positive in 80% of cells (Fig 1c). Myoglobin, MyoD1 and sarcomeric actin were negative, but strongly positive in surrounding muscle. ALK, Cam5.2. and pancytokeratin AE1/AE3, HMB-45, S-100 staining was negative.

At the second operation tumor tissue was preserved for cytogenetics. The tumor specimen was processed for cytogenetics as described by Limon et al. [9], however only nonspecific structural aberrations were detected.

Discussion

We present this case because of the rarity of such type of malignancy in childhood, unusual localization and difficulties in diagnosis and treatment. Our case is one of the few head and neck LMS reported in the literature and the patient is the youngest among orbital cases [1]. LMS in children is a very rare entity, however in HIV positive children it is the second leading cancer after lymphomas [4]. Association of LMS with Epstein-Barr infection is suggested in immunosuppressed children and young adults. Opposite to the most of pediatric sarcomas, LMS shows features of slow progression [11]. Behaviour of LMS in the adults is site-dependent, and related to the maximal diameter of the tumor [10]. In children mean tumor size is low (median 2.5 cm) and usually low grade tumors are diagnosed [11].

In 20 cases reported by Sommerhausen and Fletcher 25% of LMS were located in the head and neck region [13]. Up-to-date several cases of orbital LMS in adults with hematogenous dissemination were presented. Radical treatment including orbital exenteration is postulated by some authors and the accepted prognostic indicator is the extent of tumor involvement at the presentation [8].

LMS in children still causes histological diagnostic problems. The diagnosis is difficult because its features in young patients vary in individual cases [13]. Published series are small and few, so clinical and histologic features remain poorly defined [1, 13]. LMS is often not considered in differential diagnosis of paediatric spindle cell tumors arising in nonvisceral soft tissue. In series of Sommerhausen and Fletcher [3] only 3 of 20 pediatric soft tissue cases were referred with an initial diagnosis of LMS. Differential diagnosis of LMS includes inflammatory myofibroblastic tumour, infantile myofibromatosis, leiomyoma, monophasic synovial sarcoma and spindle cell rhabdomyosarcoma [13].

In multiple kinds of sarcomas cytogenetics may provide definitive diagnosis, because many of them have different specific karyotypic abnormalities and some have consistent translocations [12]. For rhabdomyosarcoma the most common recurrent aberrations are t(2; 13) and t(1; 13) and allelic loss in chromosomal region 11p15 [5, 12]. Inflammatory myofibroblastic tumor in children often contains clonal rearrangements activating ALK receptor tyrosine kinase gene [5, 12]. LMSs are cytogenetically heterogenous and show complex karyotypic changes without any specific abnormalities [12, 14].

Orbital and other head and neck localizations are typical for RMS in childhood [3]. In our case the first histological diagnosis was spindle cell type RMS. Spindle cell RMS has better prognosis than classical embryonal RMS. The most common localization of spindle cell RMS is paratesticular region, however head and neck cases are not rare. The revision of first histological diagnosis in our patient was forced mainly due to unsuccessful chemotherapy and non-specific cytogenetic picture. Immunophenotype of presented case with expression of ASMA and desmin and negativity for myoglobin, MyoD1 and sarcomeric actin was quite typical for LMS. Lack of ALK expression together with no reaction for high doses steroid treatment (administered for hepatitis) were not consistent with the inflammatory myofibroblastic tumor.

The treatment of LMS in children is documented in few reports [3, 4, 13, 15]. Although in adults the sensitivity of this tumour to the chemo- and radiotherapy is poor, both kinds of oncological therapy of LMS in children are tried with some effects [3, 4]. In our case the lack of prominent reaction to the chemo- and radiotherapy caused the necessity of the exenteration of the right orbit, as the only way to achieve the complete remission. There are some reports about positive chemotherapy response using the protocols with DTIC, which stimulated us to use this drug in the adjuvant final chemotherapy post enucleation (Falkowski).

The main question for diagnosing pathologist is to realize that LMS can occur in such a rare site as the orbit. The problem how to treat the locally advanced leiomyosarcoma in the head and neck region without mutilation remains still open.

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References


An unusual cause of acute recurrent pancreatitis: duodenal duplication cyst diagnosed by ERCP

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Abstract

Intestinal duplications are the rare anatomic anomalies that usually present in childhood. Of these, duodenal duplications are among the uncommon. The clinical manifestations are numerous and are determined by the type, site and size of the duplication. Patients usually present with obstructive symptoms, pain, vomiting or bleeding but pancreatitis may occur as well. The etiology of intestinal duplications is unknown, although several theories have been postulated. They can be observed anywhere along the alimentary tract, and they are located most often in the ileum and least often in the duodenum. The lesions can be cystic or tubular, communicating or non-communicating, but the most common type is cystic and non-communicating. Treatment is mainly surgical and total excision, if possible, is the procedure of choice. We report a case of a pediatric patient with the duodenal duplication cyst diagnosed by endoscopic retrograde cholangiopancreatography (ERCP).

Key words: children, endoscopic retrograde cholangiopancreatography, intestinal duplications, pancreatitis

Introduction

Intestinal duplications are the rare anatomic anomalies that usually present in childhood. Of these, duodenal duplications are among the uncommon. Although usually presenting with obstructive symptoms, pain, vomiting or bleeding, pancreatitis may occur as well. They are observed in 1 of every 4500 autopsies, predominantly in white males. The small intestine is the most frequent site involved. Synchronous gastrointestinal duplications occur in as many as 15% of patients while duodenal duplications account only for 5% of all gastrointestinal duplications. Its etiology is unknown, although several theories, among which intrauterine environmental factors, such as trauma or hypoxia during a vascular accident have been postulated [1, 11]. Treatment is mainly surgical and total excision, if possible, is the procedure of choice. We report a case of a pediatric patient with the duodenal duplication cyst diagnosed by endoscopic retrograde cholangiopancreatography (ERCP).

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Case

A 8-year-old girl with acute recurrent pancreatitis, 5 years history of the acute abdominal pain and several episodes of vomiting, hospitalized in the surgical department due to the pancreatitis, was admitted to the gastroenterology department for further diagnosis. On admission physical examination reported no disorders or marks of infection. The liver enzymes were insignificantly increased. Abdominal computed tomography imaging was obtained and showed the pancreatic cyst (about 3 cm in diameter) located in the head of the pancreas with inflammatory process, biliary ducts remained without pathological changes. ERCP examination performed in general anesthesia established diagnosis of duodenal duplication. The sphincterotomy was performed. The endoscopic resection of duplication, although considered, was finally given up. The general condition of the patient remained well, although she reported periodically the mild abdominal pain and the laboratory tests showed the constant, mildly elevated serum amylase. According to these observations and non-specific symptoms she was administered protective diet and referred to liver scintigraphy. After surgical consultation, the girl was referred to the second ERCP, during which the resection of the cyst was performed. While setting endoscope into duodenum the duplication was caught using the snare and removed along with the remaining fragment beside duodenal papilla. Both pieces were restored in order to take histopathological examination which confirmed the intestinal duplication. The stent was removed out of pancreatic duct. There was no need for draining nevertheless the stent Zimmon 3 Fr 5 cm was set in order to keep the proper passage of the duct. Follow-up ERCP revealed the healing of the postoperative wound covered with normal mucosa and proper pancreatic duct. Moreover the separate ostium of biliary ducts located on the upper border of resection was identified. At present there are no indications neither for stenting nor for other procedures.

The girl was discharged from hospital in good general condition without any postoperative complications. Last follow-up examination of upper gastrointestinal tracts did not reveal any disturbing signs. Pancreatic tests were proper. The patient is under constant observation of the gastroenterology clinic.

Discussion

Duplications of the gastrointestinal system can be observed anywhere along the alimentary tract, and they are located most often in the ileum and least often in the duodenum. Duodenal duplications can be cystic or tubular, communicating or non-communicating, but the most common type is cystic and non-communicating. These are generally located at the medial border of the first and second parts of the duodenum and extend to the anterior or posterior side [3, 5, 6]. Duodenal duplication observed in our case was cystic and located in the second part of the duodenum with the opening of duodenal papilla in the lateral side of the lesion. Duplications are usually discovered during infancy and childhood. Only about 33% of the cases are reported in adults above 20 years of age. Our patient was 7 when diagnosis was established but she has been presenting with the symptoms since the age of 2.5.

A variety of clinical manifestations have been reported that are determined by the type, site and size of the duplication. Generally, patients present with a palpable mass in the abdomen, signs of intestinal obstruction, or abdominal pain. Bleeding or perforation caused by peptic ulcer, jaundice, and pancreatitis caused by biliary obstruction may also be the manifestations [4, 9]. In the reported case the most com-
Mon symptoms were pancreatitis, acute abdominal pain and vomiting episodes. Neither obstructive jaundice nor gastrointestinal bleeding were noted. Numerous congenital defects including double gallbladder, ileal and gastric duplications or vertebral abnormalities can be associated. Cancers have appeared in duplications found elsewhere in the gastrointestinal tract, but none have been reported in duodenal duplications. Those do not apply to our patient.

Accurate diagnosis of duodenal duplication is by histological examination, although radiological methods, magnetic resonance imaging (MRI) and gastroduodenoscopy are helpful. Duodenal duplication is differentiated from other cystic lesions by the „gut signature” of its wall observed by abdominal or endoscopic US. Gut signature refers to the layered pattern of the wall, with the hyperechoic inner layer representing the submucosa and the hypoechoic outer layer representing the smooth muscle. CT is valuable in identifying the type, location and the size of the duplication cyst. [2, 7, 8, 10] In our case, CT images made us think of the cyst in the head of pancreas which turned out to be neither sufficient nor correct. It was just ERCP that detected the duodenal duplication, lately confirmed by histological examination. After sphincterotomy and surgical consultation the total resection of duplication was performed during second ERCP. The procedure was successful and the patient was discharged from hospital without any complications.

In conclusion, duodenal duplication should be considered in the differential diagnosis of a patient who presents with abdominal symptoms when cystic structures neighboring the duodenum are demonstrated by radiology. In this case, ERCP was of great value not only in establishing diagnosis but also in treatment.

References