Serbian Biochemical Society
First Conference

"Towards co-operation and integrated research"

Proceedings

Faculty of Chemistry
Belgrade 2011.

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Edited by: Mihajlo Spasić
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Cover and logo design: Aleksandra Nikolić - Kokić
Publisher: Faculty of Chemistry, Serbian Biochemical Society
Printed by: Colorgrafx, Belgrade

Supported by

Faculty of Chemistry, University of Belgrade
Institute for Biological Research "Siniša Stanković"
University of Belgrade

Serbian Biochemical Society
First Conference

University of Belgrade, Faculty of Chemistry.
15.11.2011. Belgrade, Serbia.

"Towards co-operation and integrated research"

Coinciding with the 100-year anniversary of The Biochemical Society (UK)

PROGRAM

10.00-10.10	Welcome message from the President of the Serbian Biochemical Society Prof. M. B. Spasić.
10.15-10.45	David R. Jones, PhD Inositide Laboratory, Paterson Institute for Cancer Research, University of Manchester, UK **Signalling networks in cancer.**
10.45-10.50	Discussion
10.50-11.00	Pause
11.00-11.20	Marija Gavrović-Jankulović, PhD Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia **Immediate hypersensitivity: Molecular and immunological features of allergens.**
11.20-11.40	Djordje Miljković, PhD Department of Immunology, Institute for Biological Research "Siniša Stanković" University of Belgrade, Belgrade, Serbia **Neuroinflammation: From rat to man.**
11.40-12.00	Ivan Spasojević, PhD Institute for Multidisciplinary Research, University of Belgrade, Belgrade, Serbia **Iron chelation: Way to hell and back.**
12.00-12.15	Discussion
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12.30-12.50	Duško Blagojević, PhD Department of Physiology, Institute for Biological Research "Siniša Stanković" University of Belgrade, Belgrade, Serbia **Molecular mechanisms of redox signalling in homeostasis, adaptations and pathology; hypothermia.**

12.50-13.10 Snežana Dragović, PhD

Department of Radioecology and Agricultural Chemistry, Institute for the Application of Nuclear Energy – INEP, University of Belgrade,Belgrade,Serbia

Biomonitoring for human and environmental health: Current status and perspectives in radioecology.

13.10-13.30 Snežana Marković, PhD

Institute of Biology and Ecology, Faculty of Science, Kragujevac, Serbia

The effects of nitric oxide donors and the other bioactive substances on the energy and oxidative-antioxidative metabolism of blood cells and cancer cell lines.

13.30-13.45 Discussion

13.45-14.20 Pause /Cocktail

14.20-14.40 Karmen Stankov, MD, PhD

Clinical center of Vojvodina, Medical Faculty Novi Sad, Serbia

Mitochondrial redox metabolism in oxyphilic thyroid cancer

14.40-15.00 Jelena Bašić, MD, PhD

Institute of Biochemistry, Medical Faculty, University of Niš, Serbia.

Matrix metalloproteinase-9, TNF alpha- and TNF receptor gene polymorphisms in juvenile idiopathic arthritis

15.00 Discussion and CONCLUDING REMARKS

Foreword

Dear colleagues,

It is my great pleasure to warmly welcome you to the first conference entitled "Towards co-operation and integrated research" organized by the Serbian Biochemical Society.

The first Biochemical Society in the world was established in the UK in 1911. The Biochemistry Section of the Serbian Chemical Society was established in 1967. The Biochemical Society of the Socialist Republic of Serbia was active from 1976 to 1991. In 1997 the Yugoslav Biochemical Society was registered (Niketic 2010). Today's Serbian Biochemical Society was recently registered on the 5th of July 2011. Prof. Israel Pecht, the FEBS president, announced in a letter dated the 25th of August 2011 that the FEBS Council had accepted that the Serbian Biochemical Society should replace the Yugoslav Biochemical Society thereby becoming a full FEBS member.

The Serbian Biochemical Society clearly has deep roots, but this is officially its first conference. Its aim is to stimulate collaborative research work for many years to come. This year, 2011, coincides with the centennial anniversary of the UK Biochemical Society. Therefore, we have invited one lecturer from the UK and eight from Serbia to present their research interests. Their presentations are published in the conference proceedings. This first conference serves as an initiative to organize future annual scientific conferences in Serbia in order to present high quality work in fast moving research fields and to improve scientific interactions in the field of biochemistry and other disciplines of life sciences. I express my gratitude to the members of the governing board of the Serbian Biochemical Society who suggested the lecturers and to all of them who accepted their invitation.

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Vesna Niketić: BIOHEMIJSKO DRUŠTVO SRBIJE. Knjiga: SRPSKA HEMIJSKA NAUČNO-STRUČNA DRUŠTVA: Prilog istorijskoj gra|i. Urednik: akademik Paula Putanov. Izdavač: SRPSKA AKADEMIJA NAUKA I UMETNOSTI – OGRANAK AKADEMIJE (COBISS.SR-ID 258339335), Novi Sad 2010, str. 203-232.

Prof. Mihajlo B. Spasic
President of the
Serbian Biochemical Society

Signalling Networks in Cancer

David R. Jones*

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Abstract

Cancer is a complex disease. It requires multiple components in multiple regulatory systems to fail before the disease manifests. Cancer heterogeneity is clearly apparent as over 200 types of cancers have been documented. In the UK approximately 25% of people die from cancer. Cancers of the lung, colorectum, breast and prostate account for 47% of cancer deaths. 76% of cancer deaths occur in people aged ≥65 years old. Six common traits "hallmarks" of cancer have been identified: (1) cancer cells stimulate their own growth; (2) they resist inhibitory signals that might otherwise stop their growth; (3) they resist their own programmed cell death (apoptosis); (4) they stimulate the growth of blood vessels to supply nutrients to tumors (angiogenesis); (5) they can multiply forever; and (6) they invade local tissue and spread to distant sites (metastasis). In short, the hallmarks are distinctive and complementary capabilities that enable tumour growth and metastatic dissemination. These have provided us with a solid foundation for our current understanding of the biology of cancer. Recent advances in technology over the last two decades have identified four more hallmarks: (7) abnormal metabolic pathways; (8) evading the immune system; (9) chromosome abnormalities and unstable DNA; and (10) inflammation. Unfortunately, due to time constraints the focus of my lecture will only consider one of these hallmarks; how cancer cells have re-programmed their metabolic pathways.

Alterations in cell metabolism associated with cancer may be selected by cancer cells to meet the distinct metabolic needs of high cell proliferation rates. Unlike metabolism in differentiated cells, which is geared to efficient ATP generation, the metabolism in cancer cells must be adapted to facillitate the accumulation of biomass. Cancer cells divert a larger fraction of their nutrient metabolism to pathways other than mitochondrial respiration regardless of oxygen availability. The reprogramming of metabolic pathways in cancer cells is under the control of both oncogenes and tumor suppressors, many of which are downstream of signal transduction pathways involving phosphoinositide metabolism. In order to integrate a vast array of incoming signalling information into downstream cellular responses, a number of effector proteins rely on a master switch; the evolutionary conserved target of rapamycin (TOR) which is present in all eukaryotes. The mammlian TOR (mTOR) is a serine/threonine protein kinase which has been the focus of a multitude of research studies. Understanding more about phosphoinositide metabolism, mTOR regulation and cancer cell metabolomics together with help from metabolic imaging technology and computation of metabolic networks will enable us to design new therapeutic approaches to target cancer-specific metabolic pathways.

Proceedings

Immediate hypersensitivity: Molecular and immunological features of allergens

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Allergens are proteins or glycoproteins which in atopic individuals induce IgE immune response. One of the major challenges in the field of molecular allergeology it to predict the allergenic potential of a protein. Nowadays more that thousand proteins with IgE binding potential have been identified from various allergen sources, and for certain number of them the crystal structure have been determined. Still it is not quite clear which intrinsic properties of a protein makes it an allergen. In order to understand molecular mechanisms and to design successful therapeutic approaches for allergy treatment, molecular and immunological features of allergens have to be resolved.

Introduction

The immune system is involved in the defense of the human body from foreign antigens. When the immune system exerts inappropriate response that induces tissue damage, a condition is marked as hypersensitivity. Typical examples of hypersensitivity include antibody-mediated response against self antigens, and immune complex deposition in the kidney, joints and skin[1]. Nowadays the most common form of hypersensitivity is allergic response, which is termed Type I or immediate hypersensitivity.
The hallmark of Type I hypersensitivity is the production of IgE antibodies against foreign antigens, which are commonly present in the environment (pollen grains, dust mite, animal dander). Individuals who produce specific IgE antibodies to otherwise harmless environmental antigens are genetically predisposed or atopic. Manifestations of allergic diseases include anaphylaxis, seasonal hay fever, atopic dermatitis and allergic asthma.

Natural allergen sources

Although the first records on allergic reactions dates forms ancient times, immunoglobuline E was discovered in 1967 in the USA by Kimishige and Teruko Ishizaka and in Sweden by Bennich and Johansson. Diagnosis of Type I allergy is based on the detection of allergen-specific IgE antibodies or the elicitation of immediate symptoms by provocation testing (e.g.skin testing, nasal, oral or bronchial provocation)[2].

Diagnosis is currently performed with extracts obtained from natural biological sources. Allergen extracts represent complex mixtures of allergens, enzymes, other proteins, and various small metabolites derived from natural allergen sources. Such extracts are used in a variety of applications, including *in vivo* (diagnostic skin testing) and *in vitro* (serological) tests, as well as allergen specific immunotherapy.
Allergenic source materials include pollens, animal dander, fungal spores, house dust mite fecal particles, insect venoms, and foods. Allergen extracts are prepared by aqueous extraction of allergenic source materials obtained from natural biological material. The production process is intended to be compatible with the physiological conditions to which the allergen is exposed upon contact with the human body.
Current diagnosis of allergy only permits the identification of a given allergen source, but not the molecular entities involved in the pathogenesis of the disease.
In order to reveal the precise molecular nature of allergens and to provide more specific tools for diagnosis and possibly therapy of Type I allergy, extensive research on isolation and characterization of allergens have been conducted.

Allergens

Antigens which induce IgE – mediated immune response are designed as allergens. Allergens are heterogeneous group of proteins/glycoproteins derived from various biological sources, although IgE antibodies to nonpeptidic epitopes are known. For example, to classical haptens, such as the penicilloyl group[3], and to glycosidic side chains of nonmammalian glycoproteins[4]. A glycan-related IgE-reactivity has been demonstrated in most allergen sources, especially in plant kingdom. Several criteria can be employed for allergen classification: *i*) according to the prevalence in the allergic patients (major, intermediate, and minor), *ii*) route of sensitization (inhalatory, nutritive, and contact), *iii*) biological source (plant, animal), and *iv*) capability to induce the immune response.
Allergen exhibit the property to *sensitize* (induce the immune system to produce high-affinity IgE antibodies) and the property to *elicit* an allergic reaction (to trigger allergic symptoms in a sensitized subject by releasing biologically active mediators). It has become evident that not all allergens are capable to provoke the immune response, although they are capable to induce allergic symptoms. Based on immunological versus clinical allergenicity allergens are categorized as complete and incomplete.
The primary characterization of allergens was related to their route of exposure, because it defines the way in which the antigens are presented to the immune system.
Allergen structure has been extensively studied at different levels: primary structure (i.e., the amino acid sequence), protein fold, domain structure, and surface topography, which is considered as the most relevant for antibody binding, particularly epitope, the part of the surface that on an atomic level interacts with the antibody[5].

Allergen nomenclature

Rapid advances achieved in the late eighties on the allergen characterization and sequence determination by biochemical and molecular biologic approaches gather a wealth of novel data on allergen structure. Therefore, allergen nomenclature has been proposed according to

the accepted taxonomic name of allergen source as follows: the first three letters of the genus, space, the first letter of the species, space, and an Arabic number. The numbers are assigned to the allergens in the order of their identification, and the same number is generally used to designate homologous allergens or related species. As an example Der p 1 refers to the first house dust mite allergen identified from *Drematophagoides pteronyssinus*. An allergen from a single species may consist of several very similar molecules. These similar molecules are designed as isoallergens when they share the following common biochemical properties: 1) similar molecular size, 2) identical biological function, if known, and 3) ≥67% identity of amino acid sequence. Allergens from different species of the same or different genera, which share the above-mentioned common biochemical properties, are considered to belong to the same group and the sequence identity requirement can be less than 67%, as is the case for allergens of the same species[6]. Complementary DNA cloning of allergens often shows nucleotide mutations, which are either silent or can lead to single or multiple amino acid substitutions. Therefore members of an allergen group that have 67% or greater amino acid sequence identity are designed as isoallergens. Each isoallergen may have multiple forms of closely similar sequences, which are designed as variants.

Knowledge on the genomic structure can provide an understanding of how the different polymorphic forms are generated by differential splicing and/or exon exchange.

The nomenclature for recombinant and synthetic peptides of allergenic interest has been based on the nomenclature for naturally occurring allergens. An allergen that is prepared by recombinant (r) or chemical synthetic (s) means should be differentiated from a natural (n) allergen by the addition of the prefix of r or s followed by a suffix of the amino acid residue positions given in parentheses. Natural allergens may contain posttranslational modifications which include glycosylation, acylation, methylation, and other changes.

The official website for the systematic allergen nomenclature approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee is http://www.allergen.org/. The WHO/IUIS Allergen Nomenclature Sub-committee is responsible for maintaining and developing a unique, unambiguous and systematic nomenclature for allergenic proteins. The committee maintains an allergen database that contains approved and officially recognized allergens.

The most common sources of inhalant allergens

Inhalant allergens are the primary causal agents in hay fever, rhinitis, asthma and atopic dermatitis. Pollen grains, mite faecal particles, particles of fungal hyphae or spores and animal dander are among the best characterized sources of inhalant allergens.

Pollen grains from three (birch, olive, mountain cedar), grass (orchard grass, ryegrass, timothy) and weed (short ragweed, mugwort) are among the most common cause of allergies worldwide and airborne allergens are the major cause of allergic rhinitis, affecting approximately 20% of the population of western European countries.[7,8] Timothy grass (*Phleum paratense*) pollen extract is the most well characterized allergen extracts[9]. Besides 9 identified and characterized allergen groups: Phl p 1 (beta-expansin), Phl p 2 (Grass group II/III), Phl p 4(berberine bridge enzyme), Phl p 5, Phl p 6, Phl p 7 (calcium binding protein), Phl p 11 (Ole e 1-related protein), Phl p 12 (profilin), Phl p 13 (polygalacturonase), it contains also nonallergenic proteins and glycoproteins as well as other components

(carbohydrates, nucleic acids, etc). The major timothy grass pollen allergens are Phl p 1 (beta-expansin), Phl p 4 (berberine bridge enzyme), and Phl p 5 (unknown function). Homologous proteins have been identified in other Pooidae grass species (*Poa pratensis*, *Anthoxantum odoratum*, *Dactylis glomerata*, *Lolium perenne* and *Phleum pratense*). Group 1 grass pollen allergens are recognized by more than 95% of patients with grass pollen allergy and hence constitute the major allergenic components of monocot pollens[10,11]. They represent glycoproteins with a molecular mass of ~32 kDa and occur as cross-reactive allergens in most grass and corn species. Group 1 allergens are localized in the cytoplasm of the pollen grain but are released rapidly when pollen is hydrated, as occurs on humid mucosal surface[12]. There is a high amino acid sequence homology among the allergens from the same allergen group. By employment of the recombinant DNA technology the gene of Phl p 1 was expressed in *Escherichia coli* as recombinant allergen. To identify IgE binding epitopes on Phl p 1 allergen the expression cDNA library was constructed from the randomly fragmented Phl p 1 cDNA and recombinant Phl p 1 fragments were generated by polymerase chain reaction (PCR). Sera from grass pollen allergic patients were used to define rPhl p 1 fragments containing respective IgE epitopes[10]. Another important grass allergen group is group 4, with the first reported allergen from rye grass (*Lolium perenne*)[13] and later from other grass species[14,15]. N-terminal blockage and resistance to trypsin has hampered the elucidation of the primary structure and cloning of group 4 allergens. By employment of the recombinant DNA technology it become possible cloning, expression and immunological characterization of full-length timothy grass pollen allergen Phl p 4, a berberine bridge enzyme-like protein[16]. Recombinantly produced Phl p 4 shared IgE epitopes with natural Phl p 4, suggesting its application in allergy diagnosis.

The pollen of common ragweed (*Ambrosia artemisiifolia*) is a major cause of hay fever and associated asthma in Northern America. During the past few decades, ragweed has started to spread in many parts of central Europe, where it has become a serious health problem in the sensitized population. Several initiatives have formed to prevent further spread in e.g. France, Austria or southern Germany. The Compositae (or Asteraceae) family is one of the largest families of flowering plants, but only a few are important allergenic sources. These include Ambrosia (ragweed), Artemisia (mugwort), Helianthus (sunflower), and Parthenium (feverfew)[17]. It was demonstrated that sera of mugwort allergic patients show considerable cross-reactivity with ragweed pollen extracts[18,19]. IgE-binding to mugwort allergens in immunoblots was inhibited effectively by ragweed pollen extract[18], indicating close homology of the allergens in ragweed and mugwort pollen. Six groups of allergens have been identified in ragweed pollen. Most patients were classified as ragweed allergic if they reacted with the pectate lyases of the Amb a 1/2 group. The homologous pectate lyase Art v 6 in mugwort has been reported to play only a minor role in allergic disease. Amb a 6 (lipid transfer protein), Amb a 8 (profilin), Amb a 9 and Amb a 10 (both calcium-binding proteins) are small proteins belonging to the group of well known cross-reactive pan-allergens.

Pollen of trees belonging to the order Fagales (birch, alder, hazel, hornbeam, and oak) are important elicitors of allergic symptoms in the northern parts of Europe, North America, East Asia, Northwest Africa, and certain parts of Australia[20,21]. The prevalence of allergy to pollens of trees belonging to the Fagales order has been estimated to be 20% of the allergic individuals in Europe[22]. Birch was identified as the most relevant allergen source among the Fagales trees for 2 reasons: (1) population studies indicated that most of the patients allergic

to tree pollen are sensitized primarily to birch pollen and less to other Fagales pollens[23,24], and (2) birch pollen contained most of the IgE epitopes present in pollen of other Fagales species.[23] Bet v 1, a 17-kd protein, has been identified as the major birch pollen allergen that shares epitopes with the major pollen allergens of trees belonging to the Fagales order and plant-derived food.[24] Bet v l represents a target for IgE antibodies of more than 95% of patients allergic to birch pollen, and almost 60% of them are exclusively sensitized to Bet v 1[25]. Another well characterized birch pollen allergen, Bet v 2 (birch profilin), is recognized by 10% to 20% of patients allergic to tree pollen and represents an important cross-reactive plant allergen that is present in tree, weed, and grass pollens, as well as plant-derived food[26].

The three-dimensional structure of the major birch pollen allergen, the acidic protein Bet v 1 (from the birch, Betula verrucosa), was determined both in the crystalline state by X-ray diffraction and in solution by nuclear magnetic resonance (NMR) spectroscopy. This was the first experimentally determined structure of a clinically important inhalant major allergen, estimated to cause allergy in 5–10 million individuals worldwide. The structure shows three regions on the molecular surface predicted to harbor cross-reactive B-cell epitopes which provide a structural basis for the allergic symptoms that birch pollen allergic patients show when they encounter pollens from related trees such as hazel, alder and hornbeam[27].

House dust mites (HDM) represent a major cause of aeroallergens contributing to the increasing incidence of type I hypersensitivity disease worldwide[28,29]. More than 50% of allergic patients and up to 80% of asthmatic children are sensitized to mite allergens[30].

The predominant species of HDM of the genus *Dermatophagoides*, are a major cause of immediate hypersensitivity throughout the world[31,32]. Mites are divided into *Pyroglyphidae*, referred to as house dust mites, and *Glycyphagidae*, referred to as storage mites[33,34,35]. *Pyroglyphidae* mites have been established as the main source of house dust allergy with *Dermatophagoides pteronyssinus* (European HDM) and *Dermatophagoides farina* (American HDM) identified as the most important sources of aeroallergens in temperate climates [32, 36].

HDM allergen extracts are made by aqueous extraction of a variable mixture of whole mite bodies, nymphs, faecal pellets, eggs and spent culture media[37,38,39]. The protein extract obtained is a very complex mixture of more than 30 proteins or glycoproteins, with up to 21 denominated allergen groups, shown to exhibit versatile biological functions[40,41]. The major IgE-binding components of HDM extract have been reported for groups 1, 2, 3, 9, 11, 14, and 15, of which the most important are groups 1 and 2.

Food allergens

According to the clinical appearance, the pattern of allergens, and the underlying immunological mechanisms, two forms of food allergy can be distinguished. In class 1 food allergy the sensitization process occurs in the gastrointestinal tract. One special feature of the allergens eliciting this manifestation is their particular resistance to gastric digestion. This kind of food allergy is rare in adults. The most important allergens are cow's milk, hen's egg, and legumes. Class 2 food allergy is mainly seen in adults and develops as a consequence of an allergic sensitization to inhalant allergens. The immunological basis for these food

allergies is IgE cross-reactivity, which can be clinically manifested or irrelevant[42]. The allergens eliciting these adverse reactions do not display obvious physicochemical characteristics. According to their behavior during the digestion process, they can cause symptoms ranging from the oral allergy syndrome (OAS) to anaphylactic shock. About 3% of adults and 6%-8% of children have clinically proven true allergic reactions to food.
Plant food allergens belong to a limited number of protein families and are characterized by a number of biochemical and physicochemical properties, many of which are also shared by food allergens of animal origin. These include thermal stability and resistance to proteolysis, which are enhanced by an ability to bind ligands, such as metal ions, lipids, or steroids. Other types of lipid interaction, including membranes or other lipid structures, represent another feature that might promote the allergenic properties of certain food proteins. A structural feature clearly related to stability is intramolecular disulfide bonds alongside posttranslational modifications, such as N-glycosylation[43].
Molecular biology and biochemical techniques have significantly advanced the knowledge of allergens derived from plant foods[43]. Many of the known plant food allergens are homogeneous to pathogenesis-related proteins (PRs), proteins that are induced by pathogenesis, wounding, or certain environmental stresses. PRs have been classified into 14 families. Examples of allergens homologous to PRs include chitinases (PR-3 family) from avocado, banana, and chestnut; antifungal proteins such as the thaumatin-like proteins (PR-5) from cherry, apple and kiwi; proteins homologous to the major birch pollen allergen Bet v 1 (PR-10) from vegetables and fruits; and lipid transfer proteins (PR-14) from fruits and cereals. Allergens other than PR homologues belongs to other well-known protein families such as inhibitors of α-amylases and trypsin from cereal seeds, profilins from fruits and vegetables, seed storage proteins from nuts and mustard seeds, and proteases from fruits[42].

Allergens homologous to PR-2 proteins

Plant β-1,3-glucanases are proteins and glycoproteins with a molecular mass in the range of 25 to 35 kDa that belong to PR-2 family of proteins. Most are endoglucanases with the potential to partially degrade fungal cell walls. Pathogen-induced acidic enzymes are mostly extracellular, whereas basic counterpart occurs in vacuoles. A basic β-1,3-glucanases isolated from the latex of the tropical rubber tree *Havea brasiliensis* has been characterized as Hev b 2. The association between *Havea* latex allergy and hypersensitivity to foods, such as avocado, banana, chestnut, fig, and kiwi has been termed latex-fruit syndrome. Phylogenetically conserved, homologous proteins present in these fruits and vegetables are responsible for the cross-reactivity. Molecular and immunological characterization of Mus a 5, β-1,3-glucanases from banana revealed that at least two protein isoforms exist, and the allergen is capable to activate basophils from banana allergic patients and in this regard to induce clinical symptoms of allergy[44].

Allergens homologous to PR-3 proteins

Chitinases are abundant proteins found in a wide variety of seed- producing plants. They are part of plant's defense system and belong to PR-3 protein family. Chitinases are proteins of 25 to 35 kDa. Latex prohavein (Hev b 6) belongs to the group of chitin-binding proteins with

a havein domain. Two major IgE- binding proteins of 32 and 34 kDa from banana were identified as class I chitinases with a havein-like domain further explaining the cross-sensitization between *Havea* latex and fruits[45].

Allergens homologous to PR-5 proteins

The family of PR-5 proteins comprises unique proteins with diverse functions including antifungal activity. Because of the sequence homology between PR-5 proteins and thaumatin, an intensely sweet-tasting protein isolated form the fruits of the West African shrub *Thaumatococcus daniellii*, members of this family of proteins are referred to as thaumatin-like proteins. Mal d 2, a 31-kDa major apple allergen whose amino-terminal sequence shared 46% of identity with PR-5 proteins was the first thaumatin-like protein described as allergen[46]. In sweet cherry (*Prunus avium*) a 23 kDa thaumatin-like protein was identified as a major allergen, designed as Pru av 2, and its cDNA was cloned[47]. An important kiwifruit allergen is Act d 2, a thaumatin-like protein from this allergen source[48]. The sequence similarity between Act d 2 and other PR-5 proteins was 78% to banana Mus a 4, 72% to bell pepper Cap a 1, 59% to the mountain cedar Jun a 3, 56% to *Cupressus arizonica* pollen Cup a 3, 56% to Japanese cedar Cry j 3, 38% to cherry Pru av 2, and 34% to apple Mal d 2.

Allergen homologous to PR-10 proteins

The most frequent clinical syndrome caused by cross-reactive IgE antibodies is the OAS, an association of food allergies to fruit, nuts, and vegetables in patients with pollen allergy. The molecular basis of cross-reactivity is related to Bet v 1, a member of the PR-10 family, and its homologs which form a ubiquitous group of proteins that have been identified in a wide range of flowering plants, such as apple (Mal d 1), cherry (Pru av 1), apricot (Pru ar 1), pear (Pyr c 1), celery (Api g 1), carrot (Dau c 1), peach (Pru p 3), apple (Mal d 3), soybean (Gly m 1).

Allergens homologous to PR-14 proteins

Plant lipid transfer proteins (LTPs), named for their ability to transfer phospholipids from liposomes to mitochondria, form a family of about 9 kDa widely distributed throughout the plant kingdom. Most of these proteins contain 8 conserved cysteines forming 4 disulfide bridges, which makes them highly resistant to harsh temperature and pH changes. IgE-reactive LTPs are identified in peach (Pru p 3), apple (Mal d 3), soybean (Gly m 1), kiwifruit (Act d 11).

Other plant food allergens with known biological function

Inhibitors of proteases and α-amylases are found in plant storage organs such as seeds or tubers. The best characterized allergens of cereal flours have molecular masses of 12 to 15 kDa, possess inhibitory activities against heterologous α-amylase or trypsin, and belong to a single protein family present in many cereals. Also Act d 4, cysteine protease inhibitor, with a molecular mass of about 12 kDa showed a capacity to cross-link IgE receptors on effector cells[49].

Profilin, the protein of actin cytoskeleton, was first identified as an allergen in birch pollen and designed as Bet v 2. Profilins are recognized as ubiquitous cross-reactive plant allergens. IgE of food - tree pollen allergic individuals cross-reacted with Bet v 2 homologous proteins from apple, pear, carrot, celery, and patoto[50].

The major allergens of yellow mustard (*Sinapsis alba*), Sin a 1, and oriental mustard (*Brassica juncea*), Bra j 1, which can elicit allergic reactions when ingested by sensitized atopic subjects, are found in the 2S albumin fraction. The 2S albumins are also found in the seeds of the Brazil nut (*Bertholletia excelsa*).

Allergy to peanut (*Arachis hypogea*) is a significant health problem because of the high frequency of systemic reactions. It is the most common cause of fatal and near-fatal food-induced anaphylaxis. Three of the major allergens, Ara h 1, Ara h 2, and Ara h 3, are abundant proteins in the peanut.

The class of thiol proteases includes several proteolytic plant enzymes such as papain from papaya, ficin from fig, bromelain from pineapple, and actinidin from kiwi. Actinidin, a thiol protease of the kiwi (*Actinidia deliciosa*) was identified as its major allergen and designed as Act d 1.

Conclusion

Biochemical and molecular biology techniques have significantly advanced the knowledge of allergens from various allergen sources. Several batabases collect data from literature on a daily base and updated with novel results. Seven hundred seven allergens have been classified into 134 protein allergen families. Classifying allergens by structure revealed that 5% of 3012 Structural Classification of Proteins families contained allergens. The biochemical functions of allergens most frequently found were limited to hydrolysis of proteins, polysaccharides, and lipids; binding of metal ions and lipids; storage; and cytoskeleton association. Rather small number of protein families that contain allergens and the narrow functional distribution of most allergens confinm the existence of still undefined factors that render proteins allergenic. The question what makes an antigen an allergen? still waiths for the answer.

Acknowledgements

This work was supported by the Ministry of Education and Science of the Republic of Serbia (Project No. 172049).

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Neuroinflammation: From Rat to Man

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Experimental autoimmune encephalomyelitis (EAE) is a well established model of multiple sclerosis (MS). Inflammation of the CNS and demyelination are typical features of MS, while (auto)immune response is considered to have a key role in its pathogenesis. Still, it is assumed that MS is not a single entity, as there are various clinical and pathologycal subtypes of the disease. Accordingly, there are numerous EAE variants developed in different animal species, predominantly in mice and rats. Every EAE variant has some specific clinical and/or pathological feature and such a diversity of EAE has been allowing us to investigate various aspects of MS pathogenesis. Indeed, many cellular and molecular factors participating in neuroinflammation have been discovered through EAE studies. Here, our research on EAE in Albino Oxford and Dark Agouti rats will be presented from its very beginning some thirty years ago with a special emphasis on our recent findings regarding role of CXCL12, nitric oxide and NK cells in neuroinflammation.

Experimental autoimmune encephalomyelitis. What is it good for?

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) which is induced in susceptible animals, including rodents and primates. EAE can be induced in experimental animals by immunization with CNS-specific antigens, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), CNS tissue homogenate, etc. (active EAE). Alternatively, the disease can be evoked by transfer of encephalitogenic $CD4^+$ T cells, obtained from draining lymph nodes of animals immunized for active EAE induction, into syngeneic animals (transfer or passive EAE)[1]. Current concepts suggest two steps in the autoimmune pathogenesis of active EAE. First, dendritic cells (DC) located at the site of injection ingest the inoculated CNS antigens and migrate into the lymph nodes draining the sites of immunization. Alternatively, CNS antigens are transported via the soluble routes into the lymph nodes where they are ingested by resident DCs. Within the lymph nodes, DCs present the antigenic peptides in the context of major histocompatibility complex (MHC) class II molecules to naive autoreactive $CD4^+$ T cells. These cells then differentiate into interferon (IFN)-γ- and/or interleukin (IL)-17-producing effector T cells, namely T helper (Th) cells,

and even more specifically Th1 and Th17 cells, respectively[2]. Second, Th cells migrate from the secondary lymphatic organs into the CNS where they are reactivated after interacting with MHC class II^+ CNS resident phagocytes[3,4]. The stimulated effector T cells locally release proinflammatory cytokines and thus initiate an inflammatory reaction, which may finally result in demyelination and axonal degeneration[5,6]. Inflammation, demyelination and axonal loss manifests in various neurological deficits, usually an ascending progressive paralysis in a caudal to rostral direction starting with tail paralysis and eventually leading to forelimb paralysis and/or a moribund state in severe cases.

Since it shares many clinical, histological, immunologic, and genetic features with human demyelinating diseases, including multiple sclerosis (MS), EAE has been widely used to gain important insight into MS pathogenesis and to validate new targets for MS therapy[7,8]. It is also extensively studied as a prototype of organ-specific autoimmune disease in general, since it enables discerning of the mechanisms for maintaining and breaking self-tolerance. MS is an inflammatory disease of the CNS. The most prominent feature of the disease is demyelination which is the major cause for neurological symptoms expressed in the patients. Axonal loss and neurodegeneration are becoming increasingly appreciated as pathological hallmarks of MS, as well[9]. MS prevalence varies between 2 and 150 per 100,000 depending on the country and ethnicity. There are at least four subtypes of MS regarding clinical expression: relapsing-remitting, primary progressive, secondary progressive and relapsing progressive. Also, there are four defined pathological subtypes of the disease: macrophage mediated, antibody mediated, distal oligodendrogliopathy and primary oligodendrocyte damage with secondary demyelination[10]. Without going further into details, which can be found in the review papers cited herein, we can say that MS is not a single ailment, but rather a common name for a group of diseases that, although different in specific inductive and effector pathogenic mechanisms converge at the point of inflammation and demyelination. Thus, it is relevant to have variants of the EAE model which provide information of importance for understanding pathogenic specificities of MS forms. Importantly, depending on the species and strain used in experiments, on encephalitogenic emulsion applied to these animals, as well as on the way the immunization is performed there is a divergence in clinical manifestation of EAE, roughly corresponding to the major clinical subtypes of MS[7,11]. Actually, from numerous strains of mice and rats in which EAE can be induced, every strain or even substrain has specificities regarding immunization protocol that leads to EAE induction. These specificities include myelin proteins and/or peptides, intensity of costimulation with adjuvants, number of injections, etc. Also, every strain has some pathogenic and clinical feature that makes it unique to other strains and species. Therefore, investigation of one particular EAE submodel will give us useful information regarding some aspects of pathogenesis of one clinical subtype of MS. As EAE is mostly studied in inbred strains of animals, we can think of one animal strain as of one human being. Unlike humans and natural populations of animals where every individual is genetically unique, with an exception of identical twins, all animals of an inbred strain are genetically identical to each other. Of paramount interest are those strains of mice and rats that show exceptional susceptibility to EAE induction, as well as those strains that are (almost) completely resistant to the disease induction. Having in mind correlation to humans, an extremely susceptible strain corresponds to a MS patient and a persistently resistant strain corresponds to a healthy person. Thus, through comparative investigation of such strains it is possible to identify

genes, molecules, mechanisms, etc. that define susceptibility and/or resistance to the autoimmune CNS disorder. In our laboratory, we are investigating such strains of rats, namely Dark Agouti (DA) rats and Albino Oxford (AO) rats.

Before going into details about these two rat strains and our related research, I would like to make a short discourse and to address criticism that has been increasingly present regarding validity of EAE as MS model, and that is exemplified in the recent paper of Handel and colleagues[12]. First, in most studies of EAE mice and rats are used, and these are phylogenetically distant from humans, unlike, for instance, simians which are underrepresented in the research. Second, CNS lesions that appear in most of EAE models are limited to spinal cord and optic nerve, unlike in MS where lesions may occur throughout the CNS. Further, while demyelination is a major feature of MS, it occurs in EAE models rarely. Moreover, while in most EAE models $CD4^+$ cells are the major culprits in various forms of MS various immune cells have pathogenic predominance. Also, while relapsing-remitting course is dominant in MS, acute monophasic course is characteristic of the most of EAE variants. Finally, MS is a disease of unknown aetiology, for which there is a broad consensus that it is induced in genetically (or even epigenetically) susceptible individuals by unknown environmental factor(s). On the other hand, EAE is induced in controlled fashion (often in pathogen-free conditions) and no cases of spontaneous EAE have been reported so far in experimental animals. It may be that humans are the single species that develop MS "naturally" or that we have not intensively scanned natural population of experimental animals in a search for spontaneous EAE. Actually, there are so called "spontaneous" EAE forms, but these animals are heavily genetically manipulated in order to develop EAE without induction. These are mice that are transgenic for TCR for MBP[13], TCR for PLP[14], TCR for MOG[15] or TCR and antibody for MOG[16-18]. Having this in mind, as well as other discrepancies between EAE and MS we have to carefully interpret data obtained in EAE. This is especially important in attempts of transferring knowledge obtained in EAE into therapeutic designs for MS. Numerous agents that have been shown beneficial in EAE are inefficient or even detrimental in MS[19]. Still, we have to admit that EAE is as far as we can get close to MS at the moment. Numerous molecular and cellular mechanisms of MS pathogenesis have been discovered in experimental animals[8,11,19]. Thus, while attempting to make some new useful models for dissecting MS pathogenesis, we stick to EAE and try to use as much as we can of it, although being aware of its limitations.

Neuroimmunological Rat Pack

The first discovery that standard immunization with encephalitogen and adjuvant did not produce neurological signs and histological lesions in Brown Norway (BN) rats was reported almost 40 years ago[20]. In the course of studying EAE, number of rat strains that are more or less resistant to the induction of EAE emerged, including BN, AO, PVG, Fischer rats, as well as rat strains that are highly susceptible to the disease induction, including Lewis (Lew) and DA rats. For exploration of the resistance to EAE induction in most of the experiments conducted so far, BN and PVG rats were compared to Lew rats, while AO rats were compared to DA rats. Here it has to be emphasized that EAE susceptible/resistant phenotype is not "all-or-nothing" phenomenon because it is profoundly influenced by antigen and

adjuvant used to induce the disease. More details regarding susceptible and resistant strains of rats and can be found in our review paper[21], and here the focus will be on AO and DA rats.

Initial paper of Mostarica Stojkovic and colleagues in which it was shown that rats of inbred AO strain are highly resistant to EAE induction was published some 30 years ago[22]. This work is the beginning of the series of investigations aiming to elucidate mechanisms that protect AO rats from CNS autoimmunity, as well as of reasons for high susceptibility of DA rats to EAE induction. AO and DA rats are at the opposite poles of susceptibility to EAE induction. We can claim this as we have shown that AO rats do not develop EAE in conditions under which other relatively resistant rat strains, such as Fischer 344, BN or PVG rats do[23-26]. Also, we have demonstrated that DA rats develop the clinical disease after immunization with spinal cord homogenate (SCH) even in the absence of any adjuvant[27], which is a rare exception from the rule that EAE induction requires application of an adjuvant. Therefore, in our research, our basic approach has been to compare AO and DA rats at multiple levels, including clinical, cellular and molecular.

During the course of the research, the resistance of AO rats has been ascribed to multiple cellular and molecular mechanisms acting both at the periphery as well as within the CNS. Following the timeline, it was shown that lymphocytes derived from AO rats produced lower amounts of IL-2[28]and IFN-γ[29] when compared with rats of DA strain. The analysis of T cell subsets in the peripheral blood, draining lymph node (DLN) and spinal cord (SC) lesions after the induction of EAE demonstrated that significantly higher number of $CD4^+$ cells was generated in the DLN, in response to both nervous tissue antigens and CFA in susceptible DA rats, compared to AO rats. In the peripheral blood of DA rats, the percentage as well as absolute number of $CD4^+$ cells increased in the preclinical phase of EAE, but declined as the disease developed. The percentage of $CD8^+$ cells decreased in both these phases of EAE. In resistant AO rats, however, there were no significant changes in the T lymphocyte subset percentages after EAE induction[30]. Genetic basis of this strain difference in susceptibility to EAE and in production of proinflammatory cytokines was analysed in (DA x AO) F1 hybrids and backcross generation rats[31]. Results of these experiments have shown that autosomal dominant gene(s) control(s) both susceptibility to EAE and proinflammatory cytokine production. High production of IL-2 and IFN-γ and susceptibility to EAE appear to be dominant traits[32]. Further, both $CD8^+$ and $RT6^+$ T lymphocytes were significantly more numerous in AO rats. As RT6-expressing T cells in the rat may have a regulatory role[33] it has been important that depletion of $RT6^+$ cells by treatment with anti RT6 antibody increased both proinflammatory cytokine production and DLN cells proliferative response to MBP in vitro in AO rats[32]. However, elimination of either $CD8^+$ cells[34] or $RT6^+$ cells[33] did not overcome resistance to the induction of EAE in AO strain of rats.

We have recently conducted a detailed investigation of the difference between AO and DA rats at the level of the DLN. As the result we detected marked difference between AO and DA rats in their DLN cell number and their capability to express various cytokines after encephalitogenic immunization. Namely, DA rats DLN had markedly higher numbers of cells than AO rats, and these cells expressed much higher levels of cytokines known to be important for the development of EAE, including IFN-γ, IL-17, IL-6, IL-12, IL-23, but not of an immunoregulatory cytokine TGF-β[26,35]. Strikingly, AO rats did not express detectable

levels of IL-6 in DLN[35]. These results strongly imply that the reason for the resistance of AO rats to active induction of EAE is a consequence of some regulatory mechanism active at the level of DLN. As such a mechanism could be anti-proliferative action of NO, an investigation of the role of NO in AO resistance has been conducted. As a result, *ex vivo* iNOS gene expression and NO production were similar in AO and DA rats after encephalitogenic immunization, while AO rats were resistant to immunization even when they were treated with iNOS inhibitor aminoguanidine[26], thus suggesting that NO is not crucial factor for the resistance of AO rats to EAE induction.

As we have shown that upon immunization the number of cells increased more vigorously in DLN of DA rats than in AO rats, suggesting a higher proliferation rate and/or a more efficient recruitment of immune cells, accompanied by a larger production of pro-inflammatory cytokines[26, 35], we can assume that fewer cells arising in the lymph nodes correlate to fewer cells infiltrating the CNS. Indeed, a clear difference in the number of infiltrating cells was observed between the two strains when the number of cells infiltrating the SC was examined at the time when DA rats had severe neurological deficits while AO rats were free of symptoms[36]. Thus, lower numbers of cells infiltrating the CNS as a consequence of a limited cell proliferation at the periphery could well explain the observed resistance of AO rats towards EAE. However, more than 20 years ago Sedgwick and colleagues showed that a small number of encephalitogenic cells was sufficient to induce severe EAE in Lewis rats[37]. Therefore, the relatively low numbers of the cells infiltrating the SC of AO rats following immunization with SCH might not necessarily be the limiting factor for the induction of the disease. Such an assumption is further supported by the previous observation that highly encephalitogenic (DAxAO) F1 T cell line did not induce any clinical symptoms in AO recipients, while it provoked a severe paralytic disease in F1 hybrids and DA rats[38]. Thus, in this experimental setting equal numbers of encephalitogenic F1 cells are pathogenic in DA and F1 rats, but not in AO rats, which implies that other factors than mere numbers are decisive for their encephalitogenic potential. Importantly, mononuclear infiltrations of similar degree and distribution were observed in AO and DA recipients of F1 T cells, thus suggesting that regulatory mechanism working within the CNS make AO rats resistant to the induction of EAE.

Therefore, we focused our research on the influence of the CNS on the encephalitogenic cells. First, we performed a phenotypic analysis of cells isolated from SC of immunized rats. At the peak of the disease in DA rats, many immune cells were observed, mostly $CD4^+$ T cells, but also macrophages/microglia and others, while the infiltration in the SC of AO rats was poor[36]. As $CD4^+$ T cells are well known to represent the major culprits in the pathogenesis of EAE[2,8], we further examined $CD4^+$ T cells in CNS lesions of AO and DA rats. We observed a substantial accumulation of $CD4^+$ T cells at the peak of the disease in both rat strains, but this increase was much more pronounced in DA rats. Nonetheless, $CD4^+$ T cells isolated from the SC of AO rats were less activated and there were more naïve T cells. Also there was a clear difference in the ratio of naïve and activated $CD4^+$ T cells in the CNS vs. peripheral blood between two strains[36]. This implied that the CNS milieu in AO rats exerts a downregulatory effect on potentially encephalitogenic $CD4^+$ T cells. In line with this, the expression of IFN-γ and IL 17 was also significantly lower in AO rats when determined in T cells immediately after isolation from the SC. However, if cells infiltrating

the SC were removed from the CNS milieu and propagated *in vitro* there was no difference in the percentage of cells expressing these cytokines, thus indicating that the CNS tissue of AO rats limits the expression of these cytokines in the infiltrating cells. Finally, we observed that AO SCC are more prone to apoptosis, which confirmed previous findings reported for passively transferred encephalitogenic $CD4^+$ T cells in AO and DA rats[39]. Thus, we assumed that factors in the CNS contribute to the limited autoimmune response and therefore to the resistance of AO rats to EAE induction.

Twelfth player or much more

The influence of the CNS tissue on the composition and activation state of the infiltrates could be based on at least two levels: i) the function of blood-brain-barrier could result in a limited infiltration rate of intruding cells; and ii) regulatory mechanisms of the CNS parenchyma could change the activation levels of infiltrating cells. Entrance of immune cells into the CNS is highly regulated through various interactions of infiltrating cells and CNS-resident cells, including interactions between chemokines and their receptors[40]. We were particularly interested in the interaction of CXCL12 and its receptor CXCR4, as it has been previously shown that CXCL12 could be a key chemokine regulating the entrance of lymphocytes into the CNS. CXCL12 is a highly efficient and potent chemoattractant for T cells that express CXCR4 or CXCR7, and it also acts as a co-stimulator of T cell activation[41]. Moreover, it induces the adhesion of T cells to ICAM-1 and VCAM-1 by up-regulating the binding activity of LFA-1 and VLA-4 on T cells, respectively, and modulates the $\alpha 4$-$\beta 7$ integrin-mediated lymphocyte adhesion to MAdcam1 and fibronectin[42-44]. Therefore, CXCL12 seems to be essential for attracting activated, $CXCR4^+$ T cells to the sites of inflammation.

Notwithstanding, CXCL12 has been reportedly associated with down-regulation of EAE[40,45,46]. It was shown that CXCL12 prevented infiltrating cells from egressing from perivascular space through the glia limitans into CNS parenchyma[45]. Furthermore, CXCL12 was reported to redirect the polarization of antigen-specific effector Th1 cells into IL-10-producing T cells[46]. Also, it was found to induce $CD4^+$ T cell apoptosis via up-regulation of the Fas-Fas ligand pathway[47]. Finally, this chemokine is also expressed in the healthy CNS where it serves as an important factor for survival and migration of neuronal and oligodendrocyte precursors, for migration of cerebellar granule cells and microglial cells, and for neurotransmission[48]. Our data indicate that CXCL12 is expressed in similar amounts in the SC of non-immunized AO and DA rats. After immunization it is increased in AO rats, but decreased in DA at the time of peak of EAE. Expression of CXCL12 in isolated microvessels was significantly higher than in total SC homogenates in AO, thus implying that the blood-brain barrier is the major source of CXCL12 expression. This notion was supported by the finding of high CXCL12 expression on blood vessel cells in SC tissue sections of AO rats at the peak of EAE in DA rats. At the same time, there was no statistically significant difference in the expression of CXCR4 at the peak of EAE in AO and DA rats, thus implying that it was not lack of CXCL12 receptor that contributed to the less prominent infiltration of CNS in AO rats. Further, we have also detected downregulation of CXCL12 expression in SC of Lew rats at the peak of transfer EAE (unpublished observation)

which shows that inhibition of CXCL12 is not limited to DA rats or active EAE. Taken together, the observed pattern of expression of CXCL12 in the SC of AO, DA and Lew rats along with data from the studies of others suggest that CXCL12 expression at the blood-brain barrier could be an important contributing factor to the effect of the target tissue on encephalitogenic cells and thus contributes to the resistance of AO rats to EAE. This was further confirmed in experiments in which an antagonist of CXCL12, AMD3100 exacerbated otherwise mild EAE in AO rats[36], thus suggesting that CXCL12 could indeed be a factor that largely contributes to the limitation of encephalitogenicity. As there are no differences in CXCL12 expression between the two strains before immunization but these differences emerge during EAE, it seems plausible to assume that immune factors, possibly extrinsic to the CNS, must stimulate CXCL12 in resistant AO rats and inhibit its expression in susceptible DA rats. Numerous immune factors might be involved in the regulation of CXCL12 expression within the CNS. Cytokines that have previously been related to the resistance/susceptibility of rat and mice strains to EAE induction, such as TNF[49], IL-4[50], TGF-β[51] and IL-10/IL-12[52], must be taken in consideration. Precise dissection of the regulation of CXCL12 expression in AO and DA rats, as well as of the influence of CXCL12 on encephalitogenic cells is the focus of ongoing work in our laboratory. We are particularly interested in the influence of NO on CXCL12 expression, as we observed negative correlation in the expression of inducible NO synthase (iNOS) gene and CXCL12 gene in SC of immunized rats. Our preliminary data show that NO inhibits CXCL12 expression, both in astrocytes and micro blood vessels isolated from the CNS.

Isn't that ironic, don't you think?

The other experimental system that we use for investigation of neuroinflammation is immunization of rats with encephalitogenic mixtures made of SCH and different adjuvants, i.e. classically used adjuvant - CFA and carbonyl iron (CI). DA rats immunized with SCH+CI, develop severe disease with early onset and strong clinical signs in comparison to SCH+CFA-immunized rats[26]. Its clinical appearance closely resembles a hyperacute EAE. Major features of a hyperacute EAE are rapid onset, severe neurologic deficits, high mortality and histologic lesions characterized by infiltration of mononuclear cells and polymorphonuclear leukocytes as well as fibrin deposition and some hemorrhages[53,54]. It corresponds to Marburg variant of MS, unremittingly progressive monophasic severe subtype of disease which results in death within a year from the onset[55]. It also might help to understand pathogenic mechanisms underlying the acute disseminated encephalomyelitis (ADEM), immune-mediated monophasic demyelinating disease of the CNS with multifocal neurologic deficits which appears 6 days to 6 weeks following vaccination and infection[56], as well as acute haemorrhagic leukoencephalopathy (AHLE), a rare, rapidly progressive disease of cerebral white matter associated with high lethality[57]. For a long time hyperacute EAE was associated with the use of *B. pertussis* in immunization[53,54]. However, it was later shown that CI used as an adjuvant in intraperitoneal immunization with spinal SCH induced severe EAE in DA rats, resembling hyperacute form[58]. In our recent work, we have demonstrated that intradermal immunization of DA rats with SCH+CI induces EAE of similar strength to hyperacute EAE[26,59]. Actually, CI was introduced as a potent adjuvant in EAE induction in Lewis rats more than 40 years ago[53,54]. Its EAE-inducing potency was further proven in its

ability to induce EAE in rat strains that were generally regarded resistant (BN rats) or semi-susceptible (PVG rats) to EAE induction[60,61], although it was inefficient in overcoming resistance of AO rats to EAE induction[26].

EAE induced in DA rats by immunization with SCH+CI is a devastating disease, with extremely higher lethality rate, earlier onset and more pronounced clinical signs in comparison to classical SCH+CFA immunization. The severity of hyperacute EAE in rats was initially ascribed to massive infiltration of neutrophils[58]. Their study, as well as a recent one[62] strongly imply that activity of neutrophils is important for EAE pathogenesis. We have also detected increased number of granulocytes in the CNS of SCH+CI immunized DA rats which further supports the claim that neutrophils contribute to the severity of EAE course. Importantly, CNS infiltration of neutrophils is also associated with Marburg variant of MS and ADEM[63,64] and clinical improvement in a patient with the fulminant form of MS coincided with high dose cyclophosphamide induced neutropenia[65]. Similarly, EAE was ameliorated if granulocytes were depleted in the effector phase of the disease[66]. We have further investigated various immune parameters in DA rats immunized with SCH+CI and SCH+CFA. The discrepancy in the clinical course was not correlated to higher numbers of $CD4^+$ T cells infiltrating CNS or their increased activation, but it was associated with more abundant NK and NKT cells in the infiltrates and to the extensive generation of NO in spinal cords. The role of NK cells in EAE pathogenesis is a matter of controversy, and both protective[67] and pathogenic[68] functions in EAE were described. Many reports demonstrate that NK cells exert anti-inflammatory function in EAE[69]. The data showing decreased number and function of NK cells in MS patients[70,71] and correlation of successful treatment with increase of these NK parameters[72,73] support the similar, downregulatory role in human disease. However, several reports demonstrate that NK cells potentiate the autoimmune damage to the CNS through cytotoxicity as well as IFN-γ production[68,74,75]. Also, NKT cells could promote neuroinflammation through stimulation of Th1 auto-immune response[76]. It remains to be elucidated whether abundance of NK and NKT cells in this model contributes to its severity or represents a consequence of blood brain barrier damage without relevance to the pathogenesis of the disease. We are currently investigating the influence of NK cell depletion in our EAE models.

Although nitric oxide and its secondary metabolite peroxyntrite have been reportedly associated with inflammatory demyelination and neurodegeneration[77-79], their contribution to the severity and outcome of EAE has not been thoroughly studied. We have previously demonstrated higher NO production and iNOS expression in DLN cells of DA rats immunized with SCH+CI in comparison to rats in which EAE was induced with SCH+CFA[26]. Further we showed that higher iNOS gene expression is evident in the CNS tissue and CNS-infiltrating cells of SCH+CI-immunized rats[36]. Also, more nitrotyrosine residues are detected in the CNS tissue of these animals, and detection of nitrotyrosine serves as a biochemical marker of peroxynitrite-induced damage. The overproduction of NO is related to generation of peroxynitrite and consequently to extensive tissue damage in the CNS autoimmunity[77,80]. Increased iNOS expression was previously linked with pertussis toxin-induced hyperacute EAE in Lewis rats[81]. Thus, it is plausible to speculate that NO and its metabolite peroxynitrite could be major effector molecules responsible for the severe symptoms of EAE induced in DA rats with SCH+CI. Our finding of high granulocytes and

NK cells content in the infiltrates of the CNS in CI-immunized animals is in line with the observed increase in iNOS expression and activity. It is well known that granulocytes produce NO and reactive oxygen species which interact with NO to generate peroxynitrite[82,83]. Also, NK cells have a limited potential to produce NO by iNOS[84] and much higher potency to induce NO generation in macrophages and other producers through release of IFN-γ. Finally, as discussed above, NO seems to have an intensive downregulatory effect on CXCL12 expression in our experimental systems. We are currently investigating the effect of NO inhibition on the course of EAE in DA rats immunized with SCH+CI.

Final remarks

Although EAE is often qualified as the model for MS, it is obvious that it does not ideally represent MS. This is true, despite numerous variants of the model that have been developed in many species and strains, despite their diversity which covers various forms of MS and despite tremendous effort put into EAE research. Nevertheless, at the time being EAE can still be used in order to get important data on MS pathogenesis. Here, rather simple systems are presented. One in which two different strains with the opposite susceptibility to EAE induction are compared, and the second in which different immunization protocols in the same rat strain are applied. Examples of usefulness of both approaches are presented. For the first, it is the precipitation of CXCL12 as a major regulatory molecule that defines resistance to CNS inflammation. For the second, it is identification of NK cells as important players in CNS autoimmunity and support to the idea that NO is one of the major effector molecules in neuroinflammation. We can also check hints that we get in one model in the other. It is NO that is identified as a major culprit in the second system and CXCL12 is identified as a protector in the first. What about if we combine these two? Our ongoing experiments show that NO strongly inhibits CXCL12 *in vitro*. Our next step is to test this hypothesis *in vivo*. But also, is it possible that CXCL12 defines not only the extent of infiltration, but also the composition of the infiltrates? What is the role of astrocytes in the regulation of infiltration through CXCL12? Astrocytes are becoming increasingly appreciated as crucial cells in neuro-immune interactions in general and for MS pathogenesis in particular[85]. They are well capable of expressing CXCL12, but also of producing NO. For this and many other reasons astrocytes are in the very scope of our research, too. Thus, to conclude, although we are aware of limitations of EAE, we are also determined to use this model further in our research as we think that there are still valuable results that could be obtained in EAE. With careful interpretation of novel data obtained in EAE we should contribute to the research of MS pathogenesis.

Acknowledgements

The author would like to thank Professor Marija Mostarica Stojković, School of Medicine, University of Belgrade for the mentorship in neuroimmunology and to thank the coworkers for the results (published and unpublished) which were presented in this paper. This work was supported by the Ministry of Education and Science of the Republic of Serbia (Project No. 173035).

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Iron chelation: way to the hell and back.

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A pronounced oxidation and misbalanced metabolism of iron usually accompanied by iron-rich deposits in specific brain regions represent hallmarks of neurodegenerative diseases. The oxidation and iron toxicity share the same mechanism - Fenton reaction, in which Fe^{2+} reacts with H_2O_2 to produce hydroxyl radical. This radical is exclusively harmful to human cells and may provoke irreversible oxidative damage and neurodegeneration. Fenton reaction is boosted in CSF under the neurodegenerative settings by biomolecules that incompletely chelate iron, making it more accessible to H_2O_2 and reducing agents. Such compounds are iron accomplices in continuing and autocatalytic production of toxic hydroxyl radical. Little is known about these biomolecules, but they may involve neurotransmitters, amino acids, peptides and proteins, and others, showing iron-binding structural properties and increased CSF level in neurodegenerative conditions. The evildoings of iron and its accomplices may be tackled by specific chelating agents or 'sacrificial' antioxidants. A deeper understanding of the metabolism of iron ligands is essential in resolving the mechanisms of different pathophysiologies and for the development of new biomarkers and therapy agents.

Introduction

Iron-related oxidation is implicated in the progression of neurodegenerative conditions, such as: Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Friedreich's ataxia (FA), Huntington's disease (HD), prion diseases (e.g. Creutzfeldt-Jakob disease (CJD)) and others[1-4]. An increased production of free radicals and a large number of positive markers of lipid and protein oxidation have been detected in the cerebrospinal fluid (CSF) and brain tissue of patients suffering from neurodegenerative diseases[2]. Iron is involved in the main route of production of hydroxyl radical ($^{\bullet}OH$), the most reactive and dangerous oxidising agent in the central nervous system (CNS)[2]. This reactive oxygen species (ROS) is extremely harmful to human cells and represents a toxin by definition - "poisonous substance produced within living cells or organisms". In biological milieu, $^{\bullet}OH$ lives for only about 10^{-9} s, the time in which it provokes oxidative damage to cells, which may lead to dysfunction and uncontrolled cell death - neurodegeneration[5]. Due to its reactivity, no enzyme is capable of providing protection from $^{\bullet}OH$. Low regenerative capacity and specific cellular geometry in combination with high oxygen consumption result in neurons being particularly susceptible to oxidative damage exerted by $^{\bullet}OH$[6]. The main site

of $^{\bullet}OH$ production in neurodegenerative conditions is the extracellular compartment - cerebrospinal fluid (CSF) and extracellular fluid, which are interconnected and poor in antioxidants, but the production is strongly related to intracellular processes[7]. Intracellular ROS metabolism starts with superoxide radical anion ($^{\bullet}O_2^-$), which is continuously produced in mitochondria (around 2% of O_2 involved in respiration is reduced to $^{\bullet}O_2^-$ under physiological setup)[5]. Superoxide is relatively non-toxic species, showing specific targets and acting as signalling molecule, and most of it does not leave the cell[7]. In mitochondria, $^{\bullet}O_2^-$ is dismutated to hydrogen peroxide (H_2O_2) by MnSOD (superoxide dismutase). It is noteworthy that $^{\bullet}O_2^-$ may be produced by NADPH oxidase on the surface of innate immune system cells and released directly into the CSF. Inflammation is a common characteristic of neurodegenerative conditions, because the innate immune system can be activated by the products of neurodegeneration. However, CSF is rich in CuZnSOD[8], so most of $^{\bullet}O_2^-$ in the CSF is dismutated to H_2O_2. Hydrogen peroxide is even less reactive and more involved in signalling pathways in comparison to $^{\bullet}O_2^-$, but unlike other ROS it readily crosses cellular membranes[9]. This feature, which defines the role of H_2O_2 and its redox destiny (sometimes our destiny also), results in H_2O_2 leakage from CNS cells into CSF where, even under physiological conditions, its concentration may reach up to 1mM[10], being further increased in neurodegeneration due to the dysfunction of mitochondria and increased activity of the innate immune system[11]. In the CSF, H_2O_2 may react with ferrous iron (Fe^{2+}) in a mechanism known as Fenton reaction, to produce the notorious $^{\bullet}OH$ ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$). Another product of the Fenton reaction is ferric iron (Fe^{3+}), which is generally inactive under physiological conditions due to the formation of insoluble complexes with phosphate and hydroxyl ions[12]. Alternatively, Fe^{3+} may be reduced back to Fe^{2+} by reducing agents that are present in CSF, the most important being ascorbate (Asc) which shows physiological concentrations above 150 μM[13]. It is important to note that under physiological conditions, iron pro-oxidative activity in the CSF is limited by low availability and Fe^{3+} solubility, and buffered by chelating proteins, such as transferrin, lactotransferrin, albumin, and ceruloplasmin[11]. However, it seems that low levels of Fenton reaction are required in healthy CSF, most likely being responsible for the degradation of excess of H_2O_2 and some neurotransmitters. The fact that we do need some Fenton activity of iron in the CSF for normal function of CNS was first discovered accidently, when a group of patients with rheumatoid disease who were give high doses of iron-chelating drug desferrioxamine, lost consciousness for 2-3 days and showed EEG abnormalities. The chemical analysis of CSF from these patients showed significant decrease in the physiological redox activity of iron, which provided an explanation for the observed side-effects[14].

Inappropriately chelated iron in neurodegeneration

Uncontrolled cell death and disrupted blood-brain barrier in neurodegenerative conditions result in misbalanced metabolism of iron and the formation of iron deposits, which can be visualised in specific brain regions by the means of MRI or autopsies[15,16]. However, Fe^{3+} deposits are not sufficient to explain pronounced $^{\bullet}OH$ production in neurodegeneration, since the total level of iron dissolved in CSF is not increased in patients suffering from such diseases[8,17,18]. It is noteworthy, that there have been numerous attempts to attribute neurodegenerative processes to the blunt increase of iron level in the CSF, which ended in disappointment and disbelief that iron may have any prominent role. However, it seems to be

forgotten that CSF milieu is drastically altered in neurodegeneration, showing super- or subphysiological levels of different iron ligands that may affect iron redox activity. Hence, the main question of neurodegeneration is not whether there is more iron in CNS, but is the iron more redox active (or according to the words of Euripides: *"Ten soldiers wisely led will beat a hundred without a head"*). Iron typically can coordinate six ligands in an octahedral arrangement, so molecules capable of binding to all of the six sites (*e.g.* transferrin), may completely inactivate iron (Figure 1A). By contrast, bidentate or tridentate chelators that bind only to 2 or 3 of the available iron chelation sites, increase the availability of iron to the oxidation by H_2O_2 and to the reduction by Asc[2]. Some molecules preferentially bind to Fe^{2+} or Fe^{3+} and may release the unpreffered form (Figure 1B), but some ligands bind iron in both redox states. Generally, the harder ligands that favour Fe^{3+} involve oxygen in iron binding whereas softer ligands that bind Fe^{2+} involve nitrogen and sulphur[19]. So, biomolecules that are capable of binding iron in both redox states most likely comprise O and N and/or S in their structure in a configuration allowing the formation of two or three coordinate bonds (Figure 1C). Such ligands are particularly dangerous, as they catalyse redox cycling of iron and thereby promote $^{\bullet}OH$ generation via a chain-reaction (Figure 1D). It is important to note that iron may be recruited from the deposits by chelates or by reducing agents (Asc). Presented mechanism is a sublimation of two schemes that we have published previously[20,21].

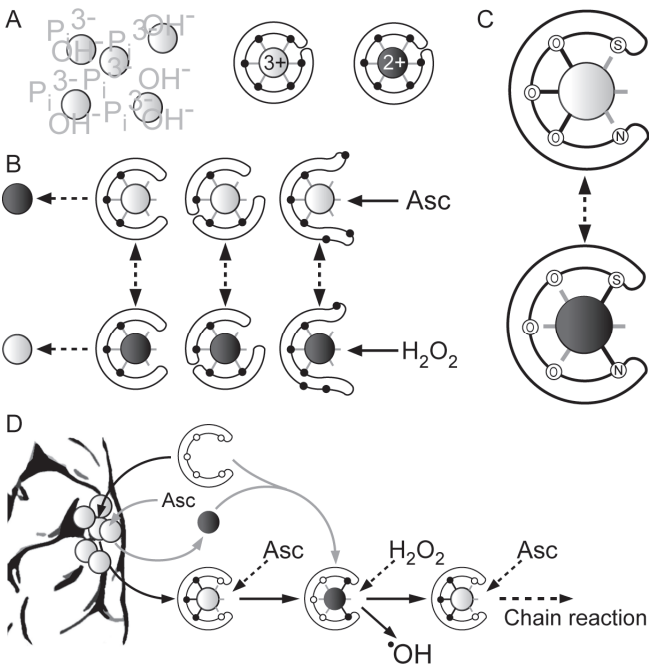

Fig. 1 Different types of iron chelates. Panel A: Completely chelated iron; Panel B: The biochemistry of incompletely chelated iron; Panel C: Proposed structure of inappropriately chelated iron complex; Panel D: $^{\bullet}OH$-generating chain reaction in CSF.

Iron chelates acting in a pro-oxidative manner have been labelled by D.B. Kell at the University of Manchester, as inappropriately chelated or poorly liganded iron, and have been placed in the centre of mechanisms of pathogenesis of various human diseases (not only neurodegenerative)[2,12]. We have proposed the term "weakly chelated iron"[11], but I will stick here with the earlier suggested idioms. The fact that poorly liganded iron is responsible for iron oxidative activity and toxicity is implied by a number of studies showing dysregulation of iron metabolism and *in vitro* cytotoxic effects of CSF obtained from patients with neurodegenerative diseases[22-24]. In addition, cells of the nervous system exposed to neurodegneration seem to make an attempt to fight the formation of poorly liganded iron by increasing lactotransferrin production[25]. My research group was the first to show explicitly that iron is not self-sufficient to provoke oxidation involved in neurodegenerative processes, but that it has accomplices in the CSF. Using human CSF and EPR spin trapping spectroscopy, which represents the best method for redox research and the only one capable of discriminating between different free radicals[5], we have shown that poorly liganded iron produces drastically more $^{\bullet}OH$ in comparison to 'free' iron (Figure 2A). EDTA was used to form inappropriately chelated iron complex, since this chelator has been recognized as a good model for low molecule weight iron ligands present in the biological milieu[11]. In the further research, we have focused on the pathogenesis of ALS. A pronounced level of oxidation in the CSF of ALS patients has been documented by an increased level of ascorbyl radical (Figure 2B)[20], which represents a supreme endogenous marker of oxidative stress[5]. The presence of inappropriately chelated iron in ALS CSF was asserted by increased production of $^{\bullet}OH$ following H_2O_2 supplementation (in order to mimic *in vivo* redox conditions) in comparison to control CSF samples (Figure 2C). In close, this means that even at normal iron levels in the CSF (< 1 μM), iron may exert excessive oxidative/toxic effects, if biomolecules capable of forming inappropriately chelated iron complexes, are present at high enough concentrations.

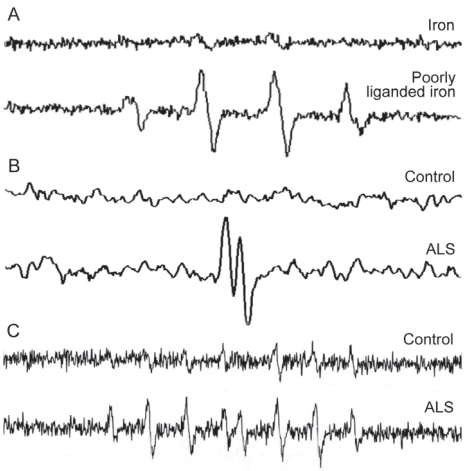

Fig. 2 Pro-oxidative activity of poorly liganded iron in human CSF. Panel A: EPR spectra of $^{\bullet}OH$ adduct with spin trap BMPO (BMPO/OH); Panel B: EPR signal of endogenous ascorbyl radical; Panel C: EPR spectra of $^{\bullet}OH$ adduct with spin trap DEPMPO (DEPMPO/OH).

Before I continue on the subject of poorly liganded iron, several facts about pro-oxidative activity of iron in the CSF should be mentioned. For an example, we observed a pro-oxidative interplay between iron and copper to develop in the CSF, which should be taken into account as copper is also implicated in some neurodegenerative conditions[11]. In addition, one should have in mind that CSF is generally poor in chelating proteins that are capable of sequestering iron. The levels of 'good' chelating agents may further deteriorate in some diseases (*e.g.* CJD[26]), which may boost the formation of inappropriate iron chelates. Finally, pro-oxidative activity of iron may result in the production of a number of different free radicals and other products of oxidation in CSF. For example, we have recently shown that $^{\bullet}$OH produced via Fenton mechanism reacts with methionine to give different carbon-, sulphur- and nitrogen-centred radicals, as well as methanethiol, a potential candidate for novel gaseous signalling species[27].

Iron accomplices

While it is clear that iron does not act alone in its toxic doings involved in neurodegenration, next to nothing is known about biomolecules that act as iron accomplices. I present here a logical set of criteria that a potential iron accomplice should meet, and that may point up the top 'suspects' for accomplices from a myriad of biomolecules:

A) Obviously, iron accomplices in neurodegneration must be present in CSF. These may involve small molecules such as, neurotransmitters and their precursors or the products of modification or degradation, amino acids and related compounds, and some other molecules, which form soluble poorly liganded iron complexes, or peptides and proteins, which may bind iron on their surface. It is noteworthy that altered protein profiles have been observed in different neurodegenerative diseases by the means of CSF proteomics. Even more, recent findings have documented the presence of nanostructures in human CSF. These are composed of proteins (including some enzymes), peptides, lipids and neurotransmitters, and have a unique composition compared to CSF supernatant, richer in omega-3 and phosphoinositide lipids, active prostanoid enzymes, and fibronectin[28]. One may speculate that the composition of nanostructures may be changed in the presence of end-products of neurodegeneration, resulting in altered iron-binding properties.

B) They should comprise O and N (or S) atoms in their structure in an arrangement which may allow iron binding.

C) Their level should be increased in the CSF in neurodegenerative diseases or they should be directly implicated in the pathogenesis. However, this criterion should be taken with reserve, since the mechanisms of neurodegenerative diseases are not fully understood, and consequently the roles of many compounds are not unambiguously established.

I have prepared a list of top 'suspects' that are potentially capable of creating inappropriately chelated iron complexes in human CSF, according to the criteria presented. Neurodegenerative disease(s) in which CSF level of specific compound is increased according to the literature, are also enlisted.

- Neurotransmitters and related compounds: Norepinephrine - AD[29]; Epinephrine - AD[30]; Dopamine thioesters - PD[31]; Serotonin - HD[32]; Tryptamine-4,5-dione - AD[32]; 7-(S-glutathionyl) tryptamine-4,5-dione - AD[33]; GABA - ALS, HD, and FA[34]; Glutamate - AD and ALS[35].

- Amino acids and related compounds: Glutamine - FA[36]; Threonine - AD[37]; Homocysteine - PD, AD, and ALS[38,39]; Tyrosine - HD[40]; 3-Nitrotyrosine - ALS, AD, and PD[41]; Dityrosine - AD[41]; Kynurenic acid - ALS[42]; Arginine - ALS and AD[37,43]; Citrulline - AD[37]; S-adenosylhomocysteine - AD[44]; Nε-(carboxymethyl)-lysine - ALS [45]; Homocarnosine - HD and FA[34,39]; Ornithine - AD[37]; α-Aminobutyric acid - AD[37]; γ-glutamylamine - HD[46].
- Peptides and proteins: Aβ-peptide - AD and PD[39,47] Tau - AD, PD, and CJD[39,47]; Phospho-Tau - AD and PD[39,47]; α-Synuclein - PD[39,48]; γ-Synuclein - PD[49].; Osteopontin - PD[50]; Substance P - ALS[51]; Mutated SOD1 - ALS[47]; Galectin-3 - ALS[52]; Actin - AD[47]; Haptoglobin - PD and HD[50,53]; Neurofilaments - ALS and PD [47]; DJ-1 - PD[50]; 14-3-3 protein - CJD[47]; Complement factor H - PD and AD[54]; Complement C3 - PD and AD[54]; Apolipoprotein A-IV - HD[53]; Prothrombin - HD[53]; Clathrin - AD[55]; S-100B - CJD and PD[39,47]; Fatty acid binding protein - CJD[56]; Ubiquitin - ALS[57]; Methionine-enkephalin - PD[39]; Nε-(γ-glutamyl)-lysine - AD and HD[47,58]; Huntingtin - HD[59].
- Other compounds: Adenosine - ALS[60]; Neuromelanin - PD[48]; 8-Hydroxyguanosine - PD and AD[61,62]; Neopterin - PD[39]; Biopterin - PD and ALS[39,60]; Ceramide - AD[47]; Choline - AD[63]; Glycerylphosphorylcholine - AD[63]; Sphingomyelin - AD[64]; N(1)-acetylspermidine - PD[65]; cAMP - AD[66]; Creatinine - FA[36].

Some of these compounds have been already shown to create redox active iron complexes and to affect redox poise in CNS. For example, 7-(S-glutathionyl) tryptamine-4,5-dione was documented to pronounce oxidative damage in AD brain[33], while its precursors, serotonin and tryptamine-4,5-dione have been speculated to create "oxo-iron complex" that provokes damage to enzymes[67]. In addition, it is known that neuromelanin primarily consists of the products of redox chemistry of dopamine, the most abundant neurotransmitter which is involved in the pathogenesis of PD[68]. Dopamine coordinates Fe^{3+}, reduces its oxidation state and subsequently causes H_2O_2 production, setting up the conditions for Fenton chemistry[69,70]. However, it should be stressed that dopamine acts as reducing agent (similarly to Asc) and not as a ligand in the formation of poorly liganded iron, but such function may be acquired by neuromelanin. Similarly, Aβ-peptide most likely acts as both, an inappropriate iron ligand and a reducing agent, which clearly represents a vicious combination in AD and some other neurodegenerative conditions[71]. It should be noted that presented list of 'suspects' is limited by available data. The metabolome of CSF in physiological and neurodegenerative conditions is far from being resolved, with an accent on proteome[45,72,73]. So there may be many more biomolecules that act as inappropriate iron ligands. In relation to this, articles with titles like "Cerebrospinal fluid from patients with dementia contains increased amounts of an unknown factor" do not come as a surprise[74].

I see two important questions that may be addressed by a critical mind regarding the subject of inappropriately chelated iron in neurodegeneration:

1. Poorly liganded iron is present in the CSF which circulates in CNS. Why are neurodegenerative processes limited to specific brain regions?

Iron-related oxidation is implicated in the progression of neurodegenerative conditions. The word "progression" implies that some intracellular events have prepared the grounds for increased pro-oxidative activity of iron, initiating a self-sustaining and self-promoting process which is intertwined with further tissue deterioration. At that point, iron deposits are already present in specific regions of dying tissue, while H_2O_2 leaks at increased rates from dysfunctional/degenerated cells. Some chelating biomolecules and reducing agents mobilise iron from deposits thus promoting the formation of inappropriately chelated iron, which reacts with localy increased amounts of H_2O_2 (see Figure 1D). When the chain reaction is

broken, Fe^{3+} returns to deposits. Further away from the affected area, H_2O_2 level is normalised by GSH peroxidise in CSF. Altogether, this creates a pro-oxidative microenvironment ('site of inflammation' may represent a proper analogy for this), which is only mildly reflected further away from the site of iron accumulation.

2. If some biomolecule is responsible for iron-related oxidative stress, it should represent an excellent biomarker for diagnosis and progression of a specific neurodegenerative disease. Why there are no such biomarkers?

Unfortunately, scientists dealing with neurodegenerative conditions are well aware of the lack of reliable biomarkers. This implies that iron pro-oxidative activity that is inherent to neurodegeneration, is not promoted by one, but by a group of compounds, the composition of which may vary between the conditions as well as between patients, but which summary effects result in pronounced pro-oxidative activity of iron.

Fighting back the poorly liganded iron

At the time, there is no really effective way to treat the underlying pathophysiology of any of the common neurodegenerative diseases. Symptomatic therapy varies tremendously from disease to disease based on the particular neurologic systems involved - *e.g.* anticholinesterase inhibitors for AD, dopaminergic drugs for PD, or riluzole that inhibits the release of glutamate for ALS. The current situation with ALS is (almost?) shameful for neuroscience community, since riluzole, which may increase survival by "staggering" 3–5 months, is the only drug on the market, in spite of large amounts of time/money invested into research in recent years. The strategies to develop therapeutic approaches that are capable of slowing down or hopefully stopping the progression of neurodegenerative diseases can be divided into three categories: (i) those targeting iron pro-oxidative/toxic effects; (ii) gene therapy; (iii) stem cell therapy. Later two are of no concern here, but should be commented briefly. They are frequently labelled in the literature as promising, but it seems that chances for these concepts ever to transpose to truly effective therapies are rather slim. Gene therapy may show to be effective in alleviating symptoms of some neurodegenerative conditions[75], but I do not see how such sophisticated approach can stop the brute force of pro-oxidative/cytotoxic activity of inappropriately chelated iron and neurodegeneration-related toxic products. We are a long way from being capable to significantly affect simple chemical reactions in CNS (like Fenton reaction) by vectors. Stem cell therapy is still far from demonstrating sufficient safety or efficacy[76]. Initial clinical trials aiming to replace lost neurons in PD and HD seem to lessen some symptoms, but otherwise show no particularly encouraging results. In some conditions, such as ALS, the application of stem cell therapy in order to replace dying motor neurones with fully functional cells seems as a waste of time, and more importantly a waste of hope in those who suffer. Finally, both gene and stem cell therapy raise important ethical and safety questions[75,76].

Therapeutic approaches aimed at preventing/diminishing pro-oxidative/toxic effects of poorly liganded iron in neurodegeneration may target: iron, ligands and/or $^{\bullet}OH$. To target iron in neurodegeneration stands for the development of compounds that completely chelate and sequester iron (Figure 1A), thus putting an end to its redox activity. Iron chelators have demonstrated therapeutic benefits in AD and PD[12]. However, chelation generally suffers from several drawbacks: bioavailability of agents, misdosing, toxicity, selectivity, and high costs (their design is highly non-trivial). The central issue of chelation therapy is to find

appropriate dose protocol for each disease and patient, as too little iron as well as too much of it can be bad. Pertinent to this, chelation therapy in the absence of iron overload raises many safety issues including chelator overdose toxicity and toxicity related to iron and other essential metal deficiencies[77]. Preliminary preclinical and clinical toxicity evidence suggest that deferoxamine and deferasirox can only be safely used for these non-iron loaded conditions for short-term treatments of a few weeks, whereas deferiprone can be used for longer term treatments of many months, but may show some side effects such as rare but severe agranulocytosis, mild neutropenia and erosive arthritis[77]. Chelation therapy is currently a blooming field, and some novel interesting strategies have been proposed, such as to devise chelators that are only activated by oxidative stress[78], or to search for chelators among drugs that have been approved for clinical use for other purposes and that are known to be safe at high doses[12].

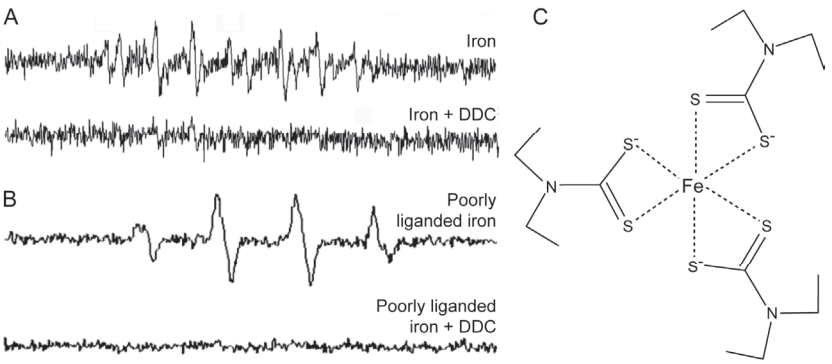

Fig. 3 The effects and structure of chelating agent DDC. Panel A: EPR spectra of DEPMPO/OH in PBS at pH = 7.4; Panel B: EPR signal of BMPO/OH in CSF; Panel C: The structure of Fe-DDC complex.

Regarding the later, my research group has conducted an investigation on the ability of diethyldithiocarbamate (DDC) to sequester iron and prevent its $^{\bullet}$OH-generating activity. DDC is the first metabolite of Disulfiram (Antabuse), which has been in use for the treatment of alcohol dependence for more than 60 years[79], and is known as generally safe even in long-term high-dose application in patients with iron-unrelated conditions[79,80]. DDC shows all the features that iron chelator compound suitable for the treatment of neurodegenerative disease should possess: strong affinity for Fe^{3+}, low molecular weight, the ability to pass blood-brain barrier, oral activity, and minimal toxicity[81]. It seems that DDC has been overseen for such application because it forms coordinate bonds with Fe^{3+} (as well as with Fe^{2+}) via sulphur atoms, while the major synthetic focus has been on the design of Fe^{3+}-selective chelators which feature oxygen donor atoms. Nevertheless, we have reported in two papers that DDC is capable not only to sequester 'free' iron under physiological pH and to inhibit Fenton reaction (Figure 3A)[82], but also to remove iron from inappropriately chelated iron complex in human CSF and to stop $^{\bullet}$OH-producing chain reaction (Figure 3B)[11]. A general absence of usual side-effects related to chelation therapy, can be explained by the fact that the complex with iron involves three DDC molecules (Figure 3C), which makes it more "dynamic" and

probably more dependent of iron concentration. Regarding this property, DDC to some extent resembles deferiprone, the least problematic amongst the three widely used chelating agents, which also binds iron in 3:1 stoichiometry[12].

Therapeutic approaches targeting biomolecules that form inappropriately chelated iron complexes may represent the future of neurodegeneration treatment. However, extensive investigations are necessary to bridge the gap between our awareness of an important role of poorly liganded iron and the knowledge of specific ligands that are involved in the pathophysiology of each of neurodegenerative diseases. When such molecules are identified, it seems that it will be comparatively straightforward to develop immuno-, pharmaco-, or gene therapy capable of decreasing their levels or modulating their metabolism in CNS. Already some trials aimed at this goal have begun and are underway, including approaches such as Aβ-based immunotherapy designed to clear both plaque deposits, and perhaps also the more pathogenic microaggregates of these proteins in AD[83]. In addition, there are some promising data obtained in experiments on animal models of AD: passive immunization with anti-Tau antibodies reduces biochemical Tau pathology and delays progression[84], passive immunization targeting phospho-Tau decrease Tau pathology and functional impairments[85], anti-homocysteic acid antibody treatment suppress cognitive impairment and attenuate brain pathological changes[86], L-3-n-butylphthalide reduces Aβ levels to improve cognitive impairment[87].

Fighting back the $^{\bullet}$OH

Hydroxyl radical is the end-product of the Fenton reaction and a direct inducer of oxidative damage, thus representing potentially important target in the treatment of neurodegenerative conditions. For this, one needs antioxidants, which by definition represent any substance that when present at low concentrations compared with those of an oxidizable biological substrate (e.g., proteins, lipids) significantly delays or prevents oxidation of those substrates[5]. Although many promising results have been obtained from *in vitro* and animal model studies, believable benefit from antioxidant application has rarely been translated into success in human clinical trials. Antioxidants seem to lose their good name, because the lucrativeness of isolation or synthesis of novel antioxidants in food/cosmetic industry/pharmacy provoked an "explosion" of antioxidant research in the past decades[5]. This resulted in countless papers on "strong" antioxidants, some of which are nothing more than 'junk' from the point of their ultimate purpose - the therapy. In order to apply a more comprehensive antioxidative approach in treating neurodegenerative (or other) diseases, one should understand some important aspects of metabolism of antioxidants and reactive species. General difficulty in antioxidative therapy is that the body acts to maintain flexible and responsive redox poise, enabling normal redox signalling and a swift genetic response to stress[88,89]. Hence, even if long-term intravenous supplementation raises blood levels of antioxidants, this may have a limited effect on CSF or intracellular levels or redox status because homeostatic mechanisms will correct for the rise above the physiological level[89]. This raises a question as to how to overcome refractory mechanisms and help the organism to fight oxidative stress against its own 'will'. The other problem is that some antioxidants, such as Asc, may act both as electron acceptors and donors, so they can oxidase/reduce iron and boost redox cycling and $^{\bullet}$OH production. Clearly, such antioxidants should not be used in the treatment of iron-related conditions such as neurodegenerative diseases, as we have documented on the example of

ALS[20]. Preferred type of compounds for such conditions are so called 'sacrificial' antioxidants, the structure of which is irreversibly altered in the reaction with free radicals to non-reactive product(s). There are three different modes of acting against $^{\bullet}OH$ radical production in the Fenton system: (i) the scavenging of $^{\bullet}OH$, following the reaction; (ii) binding of the metal in such a manner that iron participates in the Fenton reaction, but that the close proximity of the compound increases the probability of $^{\bullet}OH$ scavenging near the site of production; (iii) iron chelation[21]. Due to the extreme reactivity, $^{\bullet}OH$-provoked oxidation is diffusion limited *i.e.* it is most likely that $^{\bullet}OH$ will react with the nearest molecule whether it comes to antioxidant, membrane or an enzyme (Figure 4A). So in order to efficiently scavenge $^{\bullet}OH$, antioxidant must be placed as near as possible to the site of production, and this can be achieved by Fe^{2+} binding (Figure 4B). This 'scenario' seems to be particularly desirable in neurodegeneration, having a safe degradation of H_2O_2, which is present in excess in such conditions, as a bonus.

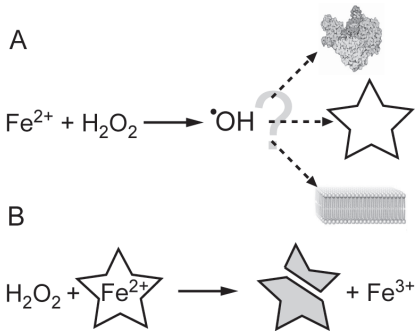

Fig. 4 Modes of $^{\bullet}OH$ scavenging: Panel A: in solution; Panel B: at the site of production, by 'sacrificial' Fe^{2+}-binding agent.

An example of molecule that meets the requests of antioxidative therapy in neurodegeneration is fructose 1,6-(bis)phosphate (F16BP). F16BP is known to enter CNS by diffusion in a dose-dependent manner[90], and to a lesser extent via active transport by dicarboxylate transporters present in brain cells[91]. F16BP does not react directly with H_2O_2, and we have shown that it is not particularly reactive towards $^{\bullet}O_2^{-}$[92]. This implies that F16BP does not significantly interfere with redox signalling pathways; hence it should not be targeted by the refractory mechanisms. In addition, the organism will not reject the 'energy supply' in the form of F16BP if presented in times of crisis, such as neurodegeneration. When exposed to Fenton system, F16BP is degraded to non-reactive products, analogously to the previously described mechanisms for fructose[93], which means that it belongs to the group of 'sacrificial' antioxidants. In a series of investigations[21,91,94], my research group (using a variety of methods) showed that: (i) F16BP binds to Fe^{2+}, most likely via ionic bonds in 2:1 stoichiometry, but not to Fe^{3+} (^{31}P NMR) (ii) F16BP does not form poorly liganded iron complexes in human CSF (EPR spin-trapping); (iii) F16BP drastically suppresses $^{\bullet}OH$ production in the Fenton system (EPR spin trapping); (iv) F16BP protects CNS cells from oxidative damage (confocal fluorescent microscopy and viability tests). Based on these results, we have proposed a mechanism of anti-Fenton activity of F16BP in the CSF (Figure 5), in which F16BP prevents the release of $^{\bullet}OH$ and forces iron back to deposits. According

to the commentary on our work, that has been published in CFG Nature Research Highlights by E. Leah, an Online Editor at the Nature Publishing Group: "...acute administration of fructose or F16BP might be therapeutically useful in limiting oxidative damage in neurological disease"[95].

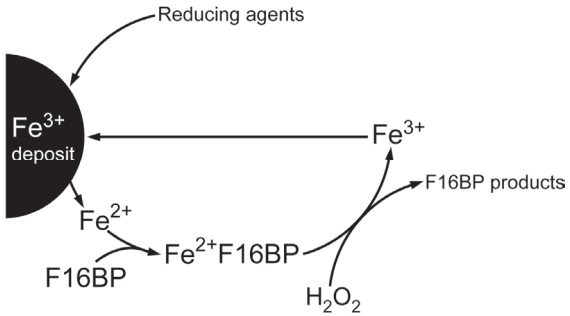

Fig. 5 The mechanism of antioxidative activity of F16BP in the Fenton reaction in CSF.

Conclusion

Inappropriately chelated iron represents a frontier concept in neurodegeneration research, integrating the roles of pronounced oxidation, misbalanced iron metabolism, and altered CSF metabolome. It provides a deep and coherent insight into the pathophysiology of different diseases and shows the way for the development of more efficient biomarkers and therapies. I have tried here not only to present the state-of-the-art on this subject, but also to raise some important questions and to suggest the guidelines for future research.

Acknowledgements

This work was funded by the Ministry for Education and Science of the Republic of Serbia, grant No. 173014 entitled: "Molecular mechanisms of redox signalling in homeostasis, adaptation and pathology".

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Molecular mechanisms of redox signalling in homeostasis, adaptations and pathology: hypothermia

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For endotherms, hypothermia is a severe stress that can lead to injuries and death. However, for ectotherms, the hypothermic state is a normal feature of their life cycle. When the ambient temperature is low, these animals establish a new thermal balance (a hypothermic steady-state) in response to changes in the environment. The hypothermic steady-state is established by the same genetic apparatus that operates in endotherms but is functionally reorganized towards stringent cellular regulation, elevated heating capacity and an increased physiological and behavioural capability to regulate thermal balance. Several regulatory levels are involved in the regulation of hypothermic balance in ectotherms, and redox signalling is one of them. Since respiration also generates feedback in the form of reactive oxygen species (ROS), the free radical equilibrium is a component of the cell's redox milieu and thermal homeostasis.

Introduction

Living organisms produce heat and the "metabolic rate" is usually defined as "the rate of heat production"[1]. The latter depends on the level of adenosine triphosphate (ATP) which is mainly produced by mitochondrial oxidative phosphorylation. However, during mitochondrial ATP synthesis, the production of partially reduced oxygen and the formation of reactive oxygen species (ROS) inevitably occur. ROS can induce and/or propagate different types of oxidative damage to molecules (e.g. oxidative modifications of proteins, the formation of lipid peroxides and DNA damage). Cells are protected from ROS by the antioxidant defence system (ADS) which is comprised of enzymes as the main components (superoxide dismutases – SODs, catalase – CAT, glutathione peroxidase – GPx, and glutathione reductase – GR), and different compounds with antioxidant activities (ascorbic acid and vitamin E as molecules with a very high antioxidant potential) that transform ROS to non-reactive molecules. However, as ROS are involved in different types of cellular regulatory systems such as oxygen sensing[2], the ADS can also be viewed as a system that balances cellular ROS levels and supports environmental and programmed adaptations to low temperatures[3]. The involvement of ROS in different regulatory processes can be directly

effected through reactions involving a redox-sensitive moiety of proteins, or indirectly through changes in the cell redox state as defined by the ratios of GSH/GSSG, NADP/NADPH and NAD/NADH redox couples[4]. The last is a complex product of mitochondrial respiratory processes that affect energy production and utilization, as well as the maintenance and turnover of cellular molecules. The redox equilibrium is maintained by permanent ATP production, expenditure and consumption of available food substrates. The ADS is a GSH- and/or an NADPH-consuming system and thereby one of the determinants of the cell redox state[5].

In the homeostatic steady-state, heat production and its inevitable dissipation are balanced by different physiological systems that maintain an organism's thermal equilibrium. In endotherms, exposure to cold induces a defence reaction which is followed by oxidative stress and the depletion of redox equivalents. If cold persists over a long period, thermial adaptation and the establishment of a new homeostatic steady state occur. Adaptation also involves a general change in the ADS, however, this is a comparatively slow and tissue-specific process which is suited to the tissue's precise role in thermogenesis[6]. Hypothermia ensues if cold exposure persists and overwhelms the thermal capacity of an organism. Hypothermia is a state of severe stress that can lead to injury and death. Accidental hypothermia is accompanied by the depletion of ATP, metabolic suppression "retreat into hypometabolic states", elevated ROS production, oxidative stress and apoptosis and/or necrosis. Metabolic acidosis as well as ATP depletion induce an ionic imbalance, changes in membrane potential, Ca^{2+} influx into the cell and destabilization of lysosomes that release proteases which, in turn, promote the release of redox-active metals, notably iron which promotes ROS generation. However, in ectotherms, cold exposure invokes the slowing down of metabolism and a general metabolic rearrangement, as well as fine-tuning of physiological regulatory units to the hypothermic steady-state. Hibernators, being heterotherms, express seasonal responses to the cold. During endothermia (in spring, summer and early autumn), they efficiently protect themselves from the cold by increasing their heat production in a manner similar to endotherms, whereas during euthermia (in late autumn and winter), they hibernate[7, 8]. Both hypothermia and hibernation in ectotherms are very precisely coordinated and regulated processes that do not have any harmful effects upon arousal and re-warming. Heat production, ATP consumption and a brief but powerful respiratory burst are balanced towards full effectiveness without eliciting any injurious effects on either ectotherms or hibernators by virtue of the utilization of common regulatory elements and genes. These mechanisms have a potential therapeutic benefit in human diseases such as ischemia-reperfusion, traumatic CNS injury and neurodegenerative diseases[9].

Hibernators

Hibernation involves an active lowering of the body temperature and decrease of the metabolic rate according to seasonal and external temperature conditions. In winter, during hibernation, mitochondrial cytochrome activity is suppressed, respiration is lowered, circulation slows down and the organism becomes inactive. Due to a lowered body temperature the metabolic rate is very slow. The main mechanism of metabolic suppression involves reversible protein phosphorylation[10] that leads to the inhibition of enzymes of carbohydrate catabolism (e.g. glycogen phosphorylase, hexokinase, pyruvate dehydrogenase)

and a resulting preservation of carbohydrates. Protein phosphorylation also suppresses Na^+/K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase activities which significantly lower ATP expenditure as a result of decreased ion pumping[11]. At the biochemical level, preparation for hibernation involves changes in the composition of fatty acids in membranes, especially the ratio of n-6 to n-3 polyunsaturated fatty acids (PUFA)[12]. These changes protect membranes from temperature-induced lipid phase transition at lower temperatures and prevent cold denaturation of critical membrane proteins. Furthermore, during hibernation the metabolism shifts towards enhanced lipid catabolism and the use of ketone bodies (acetoacetate, B-hydroxybutyrate) which are produced from fatty acids by the liver as fuel[13]. DNA array screening has revealed during hibernation the upregulation of genes encoding for proteins involved in fatty acid transport and catabolism, carbohydrate catabolism, antioxidant defences, chaperone proteins, muscle restructuring proteins, transmembrane transporters, system regulators (growth, cell cycle, apoptosis, atrophy), and components of nonshivering thermogenesis[14]. In brown adipose tissue (BAT), the expression of uncoupling protein 1 (UCP1) which short-circuits the proton motive force without driving ATP metabolism and dissipating energy, becomes elevated.

There are also mechanisms that suppress ATP-expensive processes, such as gene transcription and mRNA translation, as well as different post-translational modifications of proteins and enzymes (phosphorylation, acetylation, SUMOylation), mechanisms involved in mRNA storage and differential expression of microRNA species[15]. This suggests that in hibernation, along with the suppression of ATP expenditure, processes that support the increased protection of molecules from damage and preserve their functionality are also important. Since arousal is accompanied by a brief and significant increase in respiration, the ADS assumes an important role in protection. Thus, oxidative stress due to excessive ROS generation during awakening is eliminated and redox stability is established. The physiological levels of activity of the ADS in the ground squirrel undergo seasonal changes that parallel the activities of other regulatory factors, such as monoamine oxidase (MAO)[16, 17]. However, in winter (during hibernation) the activity of the ADS is elevated compared to other seasons. Storey[9] has acknowledged Buzadžić et al.[18] as the first to report the elevation of antioxidant defences in the BAT of hibernators and to describe the link between high rates of oxygen consumption during arousal and increased ROS production. The elevation of the activity of the ADS includes increased activities of SOD, CAT and GPx, a rise in the glutathione levels in tissues with a high metabolic rate, as well as increased ascorbate and melatonin levels and SOD and CAT activities in plasma[19,20,21]. The upregulation of SOD, GPx and GST in the kidney, and of peroxiredoxin and metallothionein in the liver, have been observed[22]. The peroxiredoxins detoxify hydroperoxides and use thioredoxin as an electron donor[23]. It has been suggested that the expression of these genes is under the control of redox-sensitive transcription factors such as NFκB and Nrf2[24,25]. Thioredoxin regulates the DNA-binding activity of NF-κB by the reduction of a disulphide bond involving $cysteine_{62}$[26]. It has been proposed that ROS regulates the activities of upstream kinases that converge onto the NF-κB signalling pathway.

Cellular enrichment with polyunsaturated fatty acids and hyperoxia can induce the activity of activating protein-1 (AP-1) and NFκB; the hyperoxia-induced activation is mediated by the nitric oxide (NO)-sensitive pathway[27]. Although oxidizing conditions are necessary for the activation of NF-κB in the cytosol to allow for the translocation and dissociation from

inhibitory IκB, NF-κB must be maintained in a reduced state in the nucleus for activation to occur[28]. Modulation of the antioxidant/pro-oxidant equilibrium by alteration of the GSH/GSSG redox potential evokes a switch between HIF-1a and NF-κB regulation, an effect which is uncoupled from the normal pattern which follows changes in the prevailing pO_2 (Haddad 2004).

Ectotherms

The hypothermic state in ectotherms is often an integral part of their life cycles and not just induced by cold environmental conditions. These animals attenuate hypoxic stress by selecting a cooler environment[29]. Upon exposure to low temperatures, the metabolism in ectoothermic animals slows down and the animals become lazy and inactive. Reversible protein phosphorylation is the main mechanism for the metabolic reorganization and shift in metabolic priorities for ATP expenditure. These include the differential regulation of many processes that require considerable expenditure of ATP, such as ion pumping, glycolysis and lipolysis[30,31]. The metabolism of glucose shifts to the pentose phosphate pathway (PPP) as a result of increased glucose-6-phosphate dehydrogenase (G6PDH) activity, enabling the increased production of NADPH which is utilized for the synthesis and maintenance of both reduced glutathione (GSH) and thioredoxin[32]. The generation of oxidative stress after tissue reoxygenation is prevented by the elevation in the activity of the ADS and by the upregulation of both ferritin and transferrin receptor genes, thereby allowing for a more efficient storage of iron[33].

Several reptile species survive temperatures below 0^{o}C by freezing avoidance (supercooling) or freezing tolerance[34]. While the use of colligative cryoprotectants is undeveloped in reptiles, the metabolic and enzymatic adaptations that provide improved tolerance to anoxia, together with their ADS, are the central components of freezing survival. Water is retained by glycogen, thus preventing ice formation. However, glycogen breakdown is accelerated, as well as the production of fructose-1,6-bisphosphate[35]. Intracellular phosphorylated forms of fructose have significant hydroxyl radical scavenging properties, the ability to sequester ferrous ions and a high antioxidant potential[36,37,38]. DNA array screening has disclosed some of the possible mechanisms that are employed by reptiles to counteract the injurious effects of the ischemia resulting from plasma freezing. In hatchling painted turtles *Chrysemys picta marginata,* these include the freeze-responsive genes in the liver and heart that encode for the proteins involved in iron binding, enzymes of the ADS and serine protease inhibitors. UCPs are also expressed in the tissues of lizards after exposure to subzero temperatures[39]. In wood frogs, freeze-responsive genes include membrane transporters, proteins involved in adenosine receptor signalling, hypoxia tolerance, defences against the accumulation of advanced glycation end products (AGE) and antioxidant defences (GPx, GST, peroxiredoxin and glucose-6-phosphate dehydrogenase). The last enzyme generates the NADPH necessary to maintain reduced pools of glutathione and thioredoxin)[40]. Taken together, the response of the ADS appears to be directed at preventing the interaction of H_2O_2 and iron (Fenton chemistry). The other route of defence is the prevention of interaction between superoxide and NO, not just the prevention of peroxynitrite formation but maintenance of NO functionality. Studies of changes in gene expression revealed very similar patterns of expression in freezing tolerance and anoxia[35].

Insect freezing tolerance: deep hypothermia

According to their freezing tolerance, insects utilize different strategies for coping with the cold[41,42]. Research on insects has revealed that the ADS is very flexible, that it is involved in these strategies and that it is incorporated in different overlapping metabolic pathways[43,3]. All these processes are parts of a specific genetic programme beyond a simple reduction in metabolic rate.

When a freeze-intolerant species such as the Mediterranean borer (*Sesamia cretica*) was exposed to low temperatures, it exhibited a cold stress response that involved separate ADS components. However, a further decrease in external temperature exceeded its stress resistance capacities and resulted in death[44]. On the other hand, the freeze-tolerant *Ostrinia nubilalis* species responded to the cold by an elevation in the activity of the ADS. Since the metabolic level in the cold in these insects is very low, the elevation of the ADS suggests a mechanism that prepares the body for free radical attack during the thawing and re-warming processes. In *Ostrinia nubilalis* during entry into diapause, metabolic rearrangements toward the synthesis and accumulation of anhydroprotectors and anti-freeze compounds such as glycerol, ethylene glycol and trehalose occur, along with mechanisms responsible for the preservation of redox homeostasis and physiological concentrations of redox molecules (ascorbate, GSH, NADPH, H_2O_2 and NO), along with the promotion of the pentose phosphate pathway (PPP)[45]. These are critically important in cold-hardy insects for generating the reducing equivalent NADPH required in the synthesis of polyol cryoprotectants. For the synthesis of glycerol from glycogen, 86% of the total carbon flow must be routed through the PPP to generate the required reducing power. Furthermore, the metabolic pathways of ascorbate synthesis are linked to PPP, providing the connection between this metabolic pathway and the antioxidative system. In view of their large amounts and antioxidant properties, the anhydroprotectors and anti-freeze compounds are believed to be additional cellular free radical scavengers. However, it is questionable whether their antioxidant action is an additional physiological role, or if it is simply an opportunistic consequence of their chemical properties and relatively high concentrations under certain physiological conditions and developmental stage of insects[46,47,48].

Exposure of diapausing *Ostrinia* larvae to low temperature (8^{o}C to -12^{o}C) revealed different responses, suggesting a dual role for the ADS. In November, the ADS is active in protecting and promoting higher PPP activity. In February, the role of the ADS appears to focus on the preservation of reduced ascorbate, thus underlining the importance of reduced ascorbate in the physiology of this insect species[49]. However, the essential role of the ADS in free radical homeostasis is preserved since the level of ADS activity in the mitochondria of diapausing *Ostrinia* larvae parallel the changes in energy production and O_2 consumption and protect against oxidative stress[50]. On the other hand, the higher level of H_2O_2 at the temperature at which freezing tolerance is triggered indicates that H_2O_2 is involved in the metabolic adjustments found in cold-hardy insects[51]. H_2O_2 induces the intracellular accumulation of trehalose[52], an important anhydroprotectant found in many freeze-tolerant organisms. In addition, H_2O_2 can also be generated in cells via the polymerization reactions of quinonic melanogenic intermediates during melanin synthesis[53]. Increased levels of two characteristic melanin-related EPR signals (eumelanin and pheomelanin) coincide with the elevated H_2O_2 concentration. The observed increase in eumelanin suggests that it is responsible for the scavenging of radicals generated in response to exposure to cold stress. It has been proposed

that melanin production serves as a defence mechanism. By scavenging intracellular hydrogen peroxide, melanin protects cells against oxidative stress[43]. These results support the notion that the antioxidative defence in the larvae of *Ostrinia nubilalis* is closely connected to the metabolic changes that characterize diapause, i.e. the mechanisms of cold hardiness involved in diapause and processes responsible for maintaining a stable redox state.

Among the numerous genes that are involved in the survival of the Arctic springtail *Onychiurus arcticus* in extreme polar conditions is enabled in part by the genes that encode for antioxidant enzymes[54,55]. The survival strategy in *Onychiurus arcticus* is to reduce the amount of water in its body to almost zero ("protective dehydration"). Of the genes that participate in the process are those that encode for proteins that direct trehalose and glycogen pathways, the aquaporins, 6-transmembrane domain proteins involved in H_2O and H_2O_2 transport and components of the ADS, GSH and CAT. The elevation of expression of certain ADS genes in winter points once again to the physiological preparations for this period of activity. After the identification of genes in the Arctic springtail *Megaphorura arctica* that were upregulated during insect cryoprotective dehydration, it was suggested that the following cellular processes were induced: the production and mobilisation of trehalose, the protection of cellular systems via small heat shock proteins and tissue remodelling. The genes identified during recovery encode for the proteins involved in energy production, the initiation of protein translation and cell division, and potential tissue repair processes. Heat map analysis has identified a duplication of the trehalose-6-phosphate synthase (TPS) gene in *M. arctica* and 53 clones that were co-regulated with TPS, including a number of membrane-associated and cell signalling proteins. Q-PCR on selected candidate genes has also contributed to our understanding. Glutathione-S-transferase was identified as the major antioxidant enzyme that protects cells during stress, along with a number of protein kinase signalling molecules involved in recovery. Cold hardiness in the Arctic Collembola *Megaphorura arctica* (Tullberg) induces the reallocation of fatty acids between the two lipid pools as a response to a temperature reduction of 6°C. The hypometabolic state typifies cold adaptation and requires less energy than *de novo* synthesis of fatty acids. Thus the hypometabolic state has been proposed to be a part of the adaptive homeostatic response[56].
Exposure to cold/hypothermia by unknown mechanisms extends the lifespan in nematodes and is referred to as CHIL or cold-/hypothermic-induced longevity. The mutational inactivation of each SOD isoform significantly lowers the lifespan of worms by CHIL. However, CHIL paradoxically increases the lifespan while reducing resistance to oxidative stress, thereby disassociating oxidative stress resistance and lifespan[57].

Acknowledgements

This work was funded by the Ministry for Education and Science of the Republic of Serbia, grant No. 173014 entitled: "Molecular mechanisms of redox signalling in homeostasis, adaptation and pathology".

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Biomonitoring for Human and Environmental Health: Current Status and Perspectives in Radioecology

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Radiation protection has historically focused on humans with the assumption that human protection confers protection of non-human biota. There is current demand for the development of a system ensuring an adequate radiation protection of the non-human biota and their associated biotopes. The system of radiological protection of the environment that is currently under development is one contribution to the general need to adequately protect the environment against stress. It emphasises conceptual and methodological approaches (including biomonitoring) that are readily accessible today: reference organisms supported by individual – based traditional ecotoxicological data. To complete radioecological knowledge of transfers of radionuclides to biota it is necessary to reveal the distribution of the radionuclides in living organisms, i.e. their bioaccumulation at tissue, cellular and molecular levels. It is also important to develop an integrated approach of radiation protection which will enable the linkage across levels of biological organization.

Introduction

The basis of the environmental protection arises from the developing awareness that man is an inherent part of the biosphere which vital functions for the survival of humankind need to be respected. Unlike man's protection that is established on a linear logic – from a source to man, passing through the environment – environmental protection requires a system approach that incorporates feedback cycling, and taking into account the complex interactions between the biotope and the living populations[1]. The keyword of environmental protection is therefore the 'ecosystem', that is the biosphere's functional unit as a base for vital functions that condition man's survival in this biosphere[2]. In the global ecosystem – the biosphere – it is understood that man has become a participant whose impact is significant, but his survival remains linked to respecting the fundamentals laws that govern the homeostatic functioning of that ecosystems. Both man and environment will be protected given the acknowledgement that humankind is an inherent part of the biota forming the biosphere. Protection of the environment should be clearly demonstrated, independent of the presence of humans[3]. It is essential that the system of the environmental protection remains

compatible with that of the protection of man. It should also be inserted within the general context of environmental toxicology and even more so as certain radionuclides show, besides the radiological harm, a high chemical toxicity.

Bioindicators and biomonitors

Organisms, populations, biocenoses and the whole ecosystems are influenced by numerous biotic and abiotic stress factors which affect biological systems at different levels of organisation, from individual enzyme systems through cells, organs, single organisms and population to entire ecosystems. They react not only to single substances or parameters; they show species-specific and situation-specific sensitivity to the number of factors and parameters acting simultaneously at their location. Information on the sensitivity and specificity of such reactions provides a basis for planning the use and evaluating the results of effect-related biological measuring techniques[4,5]. Markert et al. (1997, 1999) define bioindicator as an organism (or part of an living organism or a community of organisms) that contains information on the quality of the environment (or a part of the environment) and biomonitor as an organism (or part of an living organism or a community of organisms) that contains information on the quantitative aspects of the quality of the environment[6,7]. In the case of active bioindication (biomonitoring) bioindicators (biomonitors) bred in laboratories are exposed in a standardised form in the field for a defined period of time. At the end of this exposure time the reactions provoked are recorded or the xenobiotics taken up by the organism are analysed. In the case of passive biomonitoring, organisms already occurring naturally in the ecosystem are examined for their reactions.

Various mechanisms contribute to the bioaccumulation of elements/compounds, depending on species-related interactions between the indicators/monitors and their biotic and abiotic environment, such as biomagnification (absorption of the substances from nutrients via the epithelia of the intestines) and bioconcentration (the direct uptake of the substances concerned from the surrounding media)[4]. A number of methods have been introduced as instruments of bioindication to investigate specific reactions to pollutant exposure at higher organisational levels of the biological system; most of these are biomarkers and biosensors. Biomarkers are measurable biological parameters at the suborganismic (genetic, enzymatic, physiological, morphological) level in which structural or functional changes indicate environmental influences in general and the action of pollutants in particular in qualitative and sometimes also in quantitative terms. A biosensor is a measuring device that produces a signal in proportion to the concentration of a defined group of substances through a suitable combination of selective biological systems, e.g. enzyme, antibody, membrane, organelle, cell or tissue, and a physical transmission device. Bioindication is an integrated consideration of various bioindicative test systems which attempts to produce a picture of a pollution situation and its development in the interests of prophylactic care of health and the environment.

To be biologically relevant, a bioindicator should exhibit changes in response to a stressor, have low natural variability, have measurable changes, exhibit persistent changes that are most likely attributable to the stressor, encompass variations in scale and complexity, and embody biologically relevant changes[8]. The changes should be measurable before the damage is irreversible, either to the indicator species itself or to species that depend on it. To be suitable for application as biomonitor for air particulate matter, specific requirements have to be met by a biological tissue or monitor. According to Sloof (1993), general criteria of

primary importance are: a) the response of the organism to the quantity of the elements to be monitored should be known; b) the organisms should be common in the area of interest c) availability is required at any time or season; d) the monitor should be tolerant to pollutants at relevant levels[9]. Additional requirements specific for monitoring atmospheric trace elements are: e) the element uptake should be independent of local conditions other than the levels of the elements to be monitored; f) the element uptake should not be influenced by regulating biological mechanisms or antagonistic or synergistic effects; g) the biomonitor should average the element concentrations over a suitable time period as a result of integrated exposure over a time period; h) the organism should not take up appreciable amounts of elements from sources other than atmospheric; i) the organisms should have low background concentrations; j) the sampling and sample preparation should be easy and quick; k) the accumulation should reach to concentration levels which are accessible by routine analytical techniques.

Biomonitoring methodologies

A number of methods are now developed for biomonitoring at different levels of biological organization, which include ecological survey procedures for identifying changes in the abundance and diversity of species comprising communities and chemical and biomonitoring procedures for determining the concentrations and bioavailability of anthropogenic contaminants. These methods also include biochemical, physiological and behavioral biomarkers that signal exposure to and in some cases adverse effects from pollution[10].

Biomarkers have found to be ideal candidates for radiological exposures in humans because radiation interacts with DNA (a biological target), resulting in lesions or strand breaks (a functional or anatomical change), which lead to mutagenesis and/or carcinogenesis (an adverse response in the organism)[11]. Biomarkers are also actively being researched as promising tools for determining ecological risks to non-human biota from radioactive contaminants[12,13]. Advances in the recovery efficiency and identification of molecular damage have led to the use of biomarkers as suitable indicators or an early warning of ecological risks to exposed biota, with the tacit assumption that such damage is indicative of damage to the individual's health[14]. The use of biomarkers in ecological risk analyses requires a linkage between molecular-level effects and quantifiable observed in individuals and populations.

Biomonitoring using biomarkers of genotoxicity. These methodologies divide into two classes: (1) biochemical and molecular approaches, which include analysis of deoxyribonucleic acid (DNA) adducts and strand breaks and (2) cytogenetic approaches, which include analyses of sister chromatid exchanges (SCEs), of micronuclei (MN), and of chromosomal aberrations[10].

The analysis of DNA adducts is based on the fact that some chemicals react with DNA to form covalent bonds, and these DNA adducts may be differentiated from normal fragments of DNA by size[10]. Physical and chemical genotoxins can influence the integrity of the genetic material, and the appearance of DNA strand breaks is a sensitive indicator of genetic damage. DNA strand breaks can have major effects on chromatin and nuclear structure, since a break can allow an entire region of chromatin between nuclear attachment sites to lose superhelicity with relaxation of DNA structure, decrease in density of DNA packing/stacking, and resultant local nuclear swelling[15]. To improve cancer prevention in workers chronically exposed to low level ionising radiation in nuclear power plants,

occupational medicine applies essentially biomonitoring of exposure and effects. It has been shown that biomarkers for genotoxic effects (DNA breaks and alkali-labile sites and micronucleus and non-disjunction frequencies) could be fully validated for biomonitoring workers chronically exposed to ionising radiation[16]. Angelis et al. (1999) demonstrated that the sensitivity of the single-cell gel electrophoresis (comet) assay allows rapid prediction of genotoxic potential of compounds and has been shown to be useful for biomonitoring of environmental pollution[17-19]. The comet assay assessment of DNA damage in *Allium cepa* growing in soil of high background radiation area has shown the significant positive correlation of the DNA damage in nuclei of the root cells with ^{226}Ra specific activity in the soil[20]. The zebrafish *Dania rerio* is a suitable biological model to study effects at the molecular and individual level following exposure to ionising radiation. The most important advantage comparing with other vertebrates is the homology between human and zebrafish genomes[21]. Simon et al. (2011) have investigated the embryo/larval toxic and genotoxic effects in zebrafish after chronic exposure to gamma radiation and have confirmed that this vertebrate is the optimal biomonitor of aquatic environments, capable of furnishing measurable responses to genotoxicity[22].

The SCEs are the cytological manifestation of the interchanges between DNA replication products at apparently homologous loci. Numerous studies have demonstrated a dose-response relationship for a wide variety of chemicals and contaminants[10].

Micronuclei are normally formed by broken parts of the chromosomes from daughter nuclei at mitosis and exist separately from the main nuclei of the cell. MNs are induced by physical and chemical agents and can be scored during the interphase stage of the eukaryotic cell cycle. Compared with other cytogenetic methods, MN assay is considered to be relatively simple and fast and could be applied to a wide range of different species without any requirement for a detailed knowledge of the chromosome complements (karyotype)[10]. Kuglik et al. (1994) have shown a very good correlation between the micronucleus frequency and the increasing dose of ^{60}Co gamma rays from 10 to 300 mGy[23]. Minouflet et al. (2005) have demonstrated the efficiency of micronucleus test for assessing the genotoxic effects in *Vicia faba* induced by low doses of ^{137}Cs[24]. The investigation of impact of low doses of tritium on the marine mussel (*Mytilus edulis*) revealed a dose-dependent increase in the response for both the MN test and the comet assay[25]. The micronucleus assay have demonstrated to be useful method for examing the dose-responses in fibroblasts from ungulate species exposed to high doses of ionising radiation[14]. Cytogenic assays provide feasible biological effects-based alternatives that are more biologically relevant than traditional contaminant concentration-based radioecological risk assessment.

Analysis of chromosomal aberrations is considered to be the most important genetic end-point because they are associated with the initiation and progression of malignancies, congenital abnormalities, and reproductive wastage[10]. The incidence of cytogenic anomalies in seedling root and needle meristem of Scotch pine (*Pynus sylvestris*) growing at sites in the Chernobyl nuclear power plant 30 km zone have found to increase with the radiation exposure[26]. The higher level of chromosome mutagenesis have been found in *Polycheta* and *Malacostraca* collected in the areas of geothermal springs in Ikaria Island, Greece[27]. Cytogenic analysis have shown several-fold higher chromosomal aberrations in bone marrow cells of tundra vole (*M. Oeconomus*) living under the increased natural radiation background for a long time in comparison with those for reference animals[28].

Biomonitoring using biochemical biomarkers. Biochemical changes associated with pollutant exposure might include: (1) the inhibition or induction of enzymes, (2) modulation of cellular

defenses involved in chemical chelation/storage of contaminants, (3) induction of specific enzymes involved in the metabolism of pollutants and the appearance of associated metabolites, and (4) structural changes in proteins or lipids and the appearance of protein adducts[10]. The majority of radiation effects at low dose exposures were found to be induced not by radiation directly, but indirectly through changes in the regulatory and immune system, antioxidant status of organism and genome destabilization[29]. The lipid peroxidation proceeds in all types of cellular metabolism and plays an important role in the regulation of cellular metabolism in intact animals and under the impact of various damaging factors, including ionising radiation[30]. The results of long-term investigations revealed significant alterations in lipid peroxidation regulation in tissues of tundra vole (*M. Oeconomus*) trapped in the radioactively contaminated areas compared with the reference site[28].

Biomonitoring using histological biomarkers. Measurement of histological change offers several advantages over other approaches for detecting environmental stress: target organs, cells and sometimes organelles can be identified in vivo, sample collection and storage are relatively simple in the field, both short- and long-term effects may be identified, histochemical methods may indicate routes of exposure[31]. The histological changes in earthworm *Eisenia fetida* have been observed at the uranium concentrations in soil of about 300 mg kg^{-1}, indicating severe degradation of chloragogenous tissues[32]. Renal injury observed in most mammalian toxicity studies is usually characterised histologically by cellular injury and tubular necrosis[33].

Biomonitoring using physiological biomarkers. Physiological responses offer a major advantage for biomonitoring because the effects of pollutants are usually rapid. Thus physiological responses offer a real-time measurement of exposure or toxic effect. Pollutants can cause a variety of respiratory, cardiovascular, osmoregulatory, neurological, and/or endocrine disturbances. It has been demonstrated that depending on dose, route of administration and chemical form of administered uranium, exposure to uranium compounds can produce effects in a variety of mammalian organ systems, including kidney, liver, lungs, cardiovascular and central nervous system[34].

Animal behaviour. A number of animal behaviors have potential as biomonitors of exposure, including avoidance of the pollution gradient, changes in feeding activity, predator avoidance, and reproductive and swimming behaviors[10].

Biomonitoring in radioecology – the concept of a reference organism

The common approach to the radiological protection of humans and the environment[35] includes safeguarding the environment by preventing or reducing the frequency of effects likely to cause early mortality, reduced reproductive success, or DNA damage effects in individual fauna and flora, to a level where they would have a negligible impact on conservation of species, maintenance of biodiversity, or the health and status of natural habitats or communities. To derive the reasonably complete information for a few types of organisms that are typical of the main environment types a set of reference animal and plant approach is established[36]. Each reference organism would serve as a primary point of reference for assessing risks to organisms with similar life cycles and exposure characteristics. For each reference animal and plant organism a set of data on basic life cycle biology, pathways of exposure to radiation, dose models and radiation effects on individuals should be obtained. Such data sets would also serve as default values for use in various assessment scenarios. When applying different assessment models, the size and shape of

target organism must be taken into consideration and geometries within dose models must be set. The criteria suggested by Pentreath and Woodhead (2001) in selection of relevant reference animals and plants included: (1) the extent to which they are typical of a particular ecosystem, (2) extent to which they are likely to be exposed to radiation, (3) stage(s) in their life cycle likely to be of the most relevance for evaluating total dose rate or dose rate, and of producing different types of dose-effect responses, (4) extent to which their exposure to radiation can be modelled using simple geometries, (5) possibility to identify radiation effects in an individual organism, (6) amount of radiobiological information already available, (7) their amenability to future research and (8) extent to which both decision makers and the public are likely to know what these organisms actually are[37].

The ICRP produced derived consideration reference level (DCRL) for each reference animal and plant[38] which is defined as a band of dose rate within which there is likely to be some chance of deleterious effects of ionising radiation occurring to individuals of that type of RAP that, when considered together with other relevant information, can be used as a point of reference to optimise the level of effort expended on environmental protection, dependent upon the overall management objectives and the relevant exposure situation. Being designed on the concept similar to that for human radiation protection, the reference organism approach ensures integration the protection of both humans and non-human biota into the single system (Fig. 1).

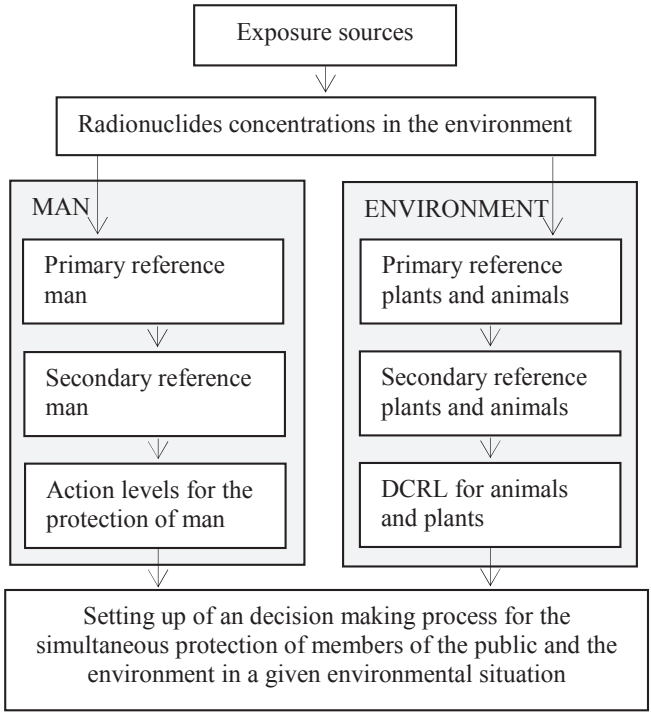

Fig. 1. The integrated approach to the protection of man and the environment[35]

An ability to predict radionuclide concentration in biota is a requirement of any method assessing the exposure of biota to ionising radiation[39]. It is regular practice within the currently available approaches, to quantifying transfer to non-human biota by using the concentration ratio (CR), i.e. the ratio between activity level of radionuclide of interest in biota and that in environmental medium. A review of the available data for many potential radionuclide - biota combinations, which may be required for quantitative estimation of the radionuclide transfer between ecological compartments, highlights numerous data gaps for terrestrial species. Since the deposition of strontium and plutonium isotopes was much lower in sites remote from Chernobyl and since the analysis requires much effort, the data on activity levels of these radionuclides in plants and animals of forest ecosystems are rather scarce when compared to radiocaesium data.

The integrated approach to the environmental effects of contaminants emitting ionising radiation, with an emphasis on biota and ecosystems, has been developed in the frame of European Community 6th Framework funded project 'The Environmental Risk from Ionising Contaminants: Assessment and Management (ERICA)'[41]. Central to the ERICA integrated approach is the quantification of environmental risk whereby data on environmental transfer and dosimetry are combined to provide a measure of exposure which is compared to exposure levels at which detrimental effects are known to occur[42]. In view of the large data sets underpinning the assessment approach and the potential to introduce errors when performing numerous calculations, a supporting computer-based tool (the ERICA Tool) has been developed. The reference organisms for freshwater, marine and terrestrial ecocystems used in the ERICA Tool are listed in Table 1.

Table 1. Reference organisms used in the ERICA Tool[42]

Freshwater	**Marine**	**Terrestrial**
Amphibian	(Wading) bird	Amphibian
Benthic fish	Benthic fish	Bird
Bird	Bivalve mollusc	Bird egg
Bivalve mollusc	Crustacean	Detritivorous invertebrate
Crustacean	Macroalgae	Flying insect
Gastropod	Mammal	Gastropod
Insect larvae	Pelagic fish	Grass and herb
Mammal	Phytoplankton	Lichen and bryophyte
Pelagic fish	Polychaete worm	Mammal
Phytoplankton	Reptile	Reptile
Vascular plant	Sea anemone/true coral	Shrub
Zooplankton	Vascular plant	Soil invertebrate
	Zooplankton	Tree

Although a relatively large amount of research has been conducted on radionuclide transfer to biota, there is still a scarcity of data for some biota species and for some radionuclides. Radioecological studies following the Chernobyl accident have provided data dominated by

radiocaesium and radiostrontium and there is still a lack of data on other radionuclides from elements such as radium, thorium and uranium.

Few data are available to quantify the transfer of both natural and anthropogenic radionuclides to detritivorous invertebrates to facilitate estimation of the internal dose to such biota in models used to assess radiation exposure. To enhance the available data, activity concentrations of ^{137}Cs, ^{40}K, ^{90}Sr, $^{239+240}$Pu, ^{241}Am, ^{235}U and ^{238}U were measured in ants (*Formicidae*) and corresponding undisturbed soil collected from the Zlatibor Mountain in Serbia and ant/soil CRs calculated[43]. The mean of the ant ^{137}Cs CR values of 0.04 was an order of magnitude lower than the mean CR value of 0.13 used in the ERICA database. It was, however, well within the large range of 0.0005-1.6. For $^{239+240}$Pu, the mean CR of the ant samples was 0.14, which is an order of magnitude higher than the mean CR value of 0.039 in the ERICA database; however, these data fall at the higher end of the large range in CR values (0.0004-0.3). For ^{241}Am, the CR values were close to the mean CR value of 0.1 in the ERICA database. For ^{228}U, the mean of our four CR values presented in Table 3 is 0.02. This compares well to the single CR value available of 0.01 for a worm[44] used in the ERICA Tool default database for soil invertebrates. The CR values for $^{239+240}$Pu and ^{241}Am were higher than those for ^{137}Cs. This is the reverse of what would be expected as caesium is more environmentally mobile than the actinide elements. The differences could be explained by physiological characteristics, plutonium and americium have been shown to accumulate in the exoskeleton and internal organs and the digestive system may still contain food thus overestimating the activity concentrations in the ants[45]. Possible external soil contamination may also be expected to lead to higher CR values for environmentally immobile radionuclides such as actinides. All the CR values are included in a future revision of the ERICA Tool database and will particularly improve the information available for natural radionuclides.

The use of CRs to estimate whole-body activity concentrations and, consequently, adsorbed dose rates for internal exposure, is obviously a simplistic approach that amalgamates many biogeochemical processes. However, to enable consideration of the wide range of radionuclides and wildlife species that may need to be considered in assessments, it currently represents a pragmatic approach that meets the needs of 'prospective' and 'current' exposure assessments[46]. The estimation of whole-body activity concentrations and, consequently, whole-body dose rates also enables comparison with the available effects data, which originate from studies of external gamma exposure[47].

Chemical and biochemical aspects of radiocaesium accumulation in mosses

To complete radioecological knowledge of transfers of radionuclides to biota it is necessary to reveal the distribution of the radionuclides in living organisms, i.e. their bioaccumulation at tissue, cellular and molecular levels. Due to their peculiar anatomical, morphological and physiological characteristics (lack of protective cuticle and thickened cell walls, numerous cell wall constituents with negatively charged groups, mineral nutrition mainly from wet and dry deposition), mosses are extensively used as bioindicators of environmental contamination by a variety of radionuclides and heavy metals[48-50]. They have been widely used as reference organisms in the assessments of radiation exposure of non-human biota[42,51]. Mosses are lower plants without true roots, shoots or vascular system[4]. They grow in groups forming turfs, cushions or other forms, colonising stones, bark, skeletons, fresh water. The life history

of mosses involves an alternation between sporophytic and gametophytic generations that differ in form and function. The actual plant is represented by the gametophytic generation. In terrestrial species, all nutrients are mainly absorbed directly from the atmosphere across the cell wall. The accumulation rates of metals can be very high and the concentrations found in mosses can reach potentially toxic levels without apparent damage to the plant itself[52]. The capability to retain metals is both metal and species specific.

The accumulation capacity of mosses is severalfold higher than those observed in vascular plants growing on the same habitat. The morphology of mosses does not vary with seasons, allowing for steady accumulation of elements throughout all seasons[53]. Mosses usually show considerable longevity, which assist their use as long-term integrators of atmospheric deposition.

Even though mosses were extensively used and studied as bioindicators, the mechanisms of trace element uptake, retention and tolerance are still not sufficiently known. These organisms exhibit a range of reactions at a cellular level that might be involved in the detoxification and, thus, tolerance to a stress caused by the environmental pollution[54].

Ion exchange. Experimental investigation of ^{137}Cs binding strength in mosses, as well as the specifity of binding has been conducted using aqueous solutions of salts and acids. The efficiency of different cations to exchange with $^{137}Cs^+$ in mosses has been investigated: (1) chlorides of the monovalent cations (ammonium, sodium, potassium and caesium), (2) inorganic ammonium salts (chloride, thiocyanate, sulphate, persulphate, phosphate and molybdate) and (3) organic salts of the monovalent cations (ammonium, sodium and potassium acetate, tartarate, citrate and oxalate). Ammonium cation proved to be the most suitable cation to substitute $^{137}Cs^+$ in moss, followed by sodium, potassium and caesium[55]. In the series of different ammonium salts, ammonium phosphate solution was the most effective for $^{137}Cs^+$ displacement, followed by ammonium chloride, thiocyanate and sulphate. As for the efficiency of organic salts, ammonium oxalate reduced the presence of $^{137}Cs^+$ in the analysed organisms to the greatest extent, followed by other oxalates and ammonium acetate. The ability to exchange $^{137}Cs^+$ for H^+ in mosses has been further tested, using different inorganic acids (hydrochloric, sulphuric, phosphoric and boric). All inorganic acids, except boric, were able to significantly decrease the amount of $^{137}Cs^+$ in mosses[55]. Using phosphoric acid 30% more $^{137}Cs^+$ has been extracted than in distilled water. The chemical behaviour of caesium is similar to that of potassium and structures that bind Cs^+ in a cell are those with the greatest affinity for K^+. Physiological studies on higher plants demonstrated that Cs^+ and K^+ competed for influx, suggesting that the influx of these cations is mediated by the same mechanism(s)[56,57]. The studies on accumulation of ^{137}Cs in the algae *Nitella flexilis* and *Chara coralline* have shown that caesium is accumulated in the cell wall and then enters a cell through potassium channels in the plasma membrane[58,59]. Potassium channels are also permeable to other monovalent cations, like Na^+, Li^+, Rb^+, NH_4^+, Tl^+, as their size (0.19-0.30 nm) is similar to that of K^+ (0.27 nm). Potassium channels, however, conduct these cations at different rates[60]. Besides the radius of the hydrated ion, the three dimensional structure of the binding sites is of the greatest importance. The selectivity in ion conduction is based on different affinities of the binding sites for cations and different rates of their translocation. To investigate the influence of moss species to ion exchange efficiency, experiment has been conducted with three different moss species: *Hypnum cupressiforme*, *Leskella nervosa* and *Hylocomium splendens*. Although a general trend of the ion exchange efficiency was kept, examined moss species exhibited different behaviour, in quantitative terms, upon subjection to salt and acid solutions[55]. Different species of the same type, within

moss classification, collected at the same time and location, had significantly different specific radioactivities and desorption rates of ^{137}Cs. It is, therefore, obvious that biodiversity is a variable that must be taken into consideration when discussing the type and strength of $^{137}Cs^{+}$ binding.

Interaction of $^{137}Cs^{+}$ with organic molecules. Further investigation has been focused on closer determination of the relation between organic molecules and $^{137}Cs^{+}$ in mosses. Samples were treated with 3 % trichloro-acetic acid (TCA) in order to cause partial decomposition of cell walls and membranes, allowing leakage of cellular components without complete damage of the skeletal structure[61]. The TCA extracts contained approximately 60 – 70 % of the initial ^{137}Cs activity. Solubilized substances were gradually precipitated by increasing concentrations of ethanol. Precipitates obtained after addition of ethanol up to the final concentration of 50 %, and further 75 %, were almost inactive in respect to ^{137}Cs, but dry resins remaining after evaporation of the solutions contained most of the extracted ^{137}Cs (40 – 50 % of the initial ^{137}Cs activity). Precipitates obtained upon ethanol addition contained polypeptides and acid polysaccharides[62]. Dry resins remaining after solvent evaporation contained peptides, small saccharides and other organic and/or inorganic compounds. Mosses remaining after TCA treatment were neutralized and subjected to extraction in pure water. Again, ethanol was added to solubilizates in order to precipitate extracted molecules, and residual solutions were evaporated to dryness. The second extraction step solubilized approximately 20 – 30 % of the initial ^{137}Cs activity and, again, dry resins contained most of the extracted ^{137}Cs. Neutral molecules, predominantly polysaccharides, were constituents of the second extracts and they were precipitated with ethanol[62]. Less than 10 % of the initial ^{137}Cs activity and approximately 70 % of the initial mass was measured in the remainings of mosses. The results obtained have shown that almost all ^{137}Cs (93-94% of the initial activity) can be extracted using neutral and acidic aqueous solutions without complete destruction of the moss tissue. Fractional precipitation of aqueous extracts with ethanol indicated that essential biomolecules, polypeptides and polysaccharides, do not bind significant amounts of ^{137}Cs. The great tolerance of mosses towards radionuclides and other toxic substances could be partly explained by this finding.

Entrapment of the solubilized $^{137}Cs^{+}$ in a crystalline lattice. Previous investigations[55] have shown that solutions of ammonium oxalate and phosphoric acid (at a concentration of 5 % each) exhibit specific characteristics in respect to caesium isolation from mosses. Further research has been conducted to determine experimental conditions to achieve maximal extraction of caesium from mosses without significant destruction of the plant and to obtain specimens that could be further analysed. Caesium was extracted from the moss at 22-25 °C with three different types of aqueous solution: ammonium oxalate, phosphoric acid and a mixture of two. Different concentrations of the solutes, solution volumes and extraction periods were tested. After extraction, an incubation of the solution at 4 ^{0}C for 24 hours, resulted in formation of transparent, colourless crystals. These crystals contained 75 – 80 % of the solubilized $^{137}Cs^{+}$. The relative amount (percentage) was constant regardless of the actual quantity of the caesium in the solution prior to precipitation, suggesting the equilibrium between the crystalized and the soluble Cs^{+} in the ratio of approximately 4:1. Crystals were subjected to crystallographic and elemental chemical analysis. Elemental analysis reported the presence of 20.98 % of carbon, 4.68 % of hydrogen, 6.04 % of nitrogen, less than 0.01 % of phosphorus and the rest, 68.30 %, was assumed to be oxygen. The closest chemical formula for the found composition is $C_4H_{11}NO_{10}$, which corresponds to the mixed crystal $NH_4HC_2O_4 \cdot H_2C_2O_4 \cdot 2H_2O$[63]. Radioactivity levels of $^{137}Cs^{+}$ in the crystal lattice

demonstrated that most of the extracted caesium precipitated. This method of desorbing caesium from mosses without destruction of the plant itself, besides of its simplicity, offers the additional advantage of allowing a choice between direct analysis of the crystal, for example by energy dispersive X-ray fluorescence spectrometry[64], or analysis after solubilization, by an ion-exchange system, or by atomic absorption spectrometry[65].

Intracellular compartmentalization of $^{137}Cs^{+}$. For better understanding of the high tolerance of mosses to radiocaesium and other toxic substances the distribution and cellular compartmentation of ^{137}Cs has been investigated[61]. The ability of a cell wall and plasma membrane to bind and, in a way, "immobilize" ^{137}Cs, thus avoiding translocation into intracellular space, has been investigated using three different moss samples. Cellular fractionation was performed by differential centrifugation of tissue homogenates obtained from[66]. The presence of ^{137}Cs was monitored by measuring the radioactivity level in each fraction. The greatest amount of ^{137}Cs was found in fractions containing plasma membranes, ribosomes, endoplasmatic reticulum and microsomes, which precipitated after ultracentrifugation at 100 000 g and it ranged from 30 to 45 % of the initial radioactivity. Cell walls were measured to have approximately 25 to 30 % of the initial ^{137}Cs. Plastids, chromoplasts and mitochondria, isolated as one fraction after centrifugation at 10 000 g, had 17 – 25 % of the initial ^{137}Cs and the remaining cytosol had 10 – 13 %. A small quantity of ^{137}Cs in the soluble fraction of the cell lysate, thus, indicates that relatively small amount of the radiocaesium is present as a free cation in a cell. The cell wall also has found to be an efficient barrier against the penetration of heavy metals into the protoplasm of the moss cell[67,68]. Burton (1979) found that *Fontinalis antipyretica* maintained 80-90% of its accumulated Zn in the cell walls[69]. The cellular fractionation demonstrated that the relative distribution of ^{137}Cs between subcellular fractions is genus independent. This finding pointed out the similar chemical structure of ^{137}Cs-binding molecules in different mosses. Their distribution between various cellular compartments is not found to be species specific.

The problem of linkage across levels of biological organisation

The effects of ionising radiation can be observed at all levels of biological organisation, from molecular to ecosystem level. Radiation effects on organisms can be traced to molecular and cellular responses, as radiation impact does not necessarily lead to observable effects on population or ecosystem. Although all levels are interrelated, the measurable attributes of the levels differ[27,70].

Several important underlying assumptions and uncertainties need to be carefully considered when performing ecological extrapolations from individual effects to population effects[71]: (a) different stages in the life cycle of an organism can be differentially sensitive; (b) the radiation sensitivity and influence of specific life history traits on population growth can differ; (c) differences in life cycles between species can affect extrapolation to the population level; (d) population density dependent effects and (e) relating effects in individuals to effects on the structure and function of the ecosystem.

Extrapolation form individual/population up to community and ecosystem involves additional processes, such as trophic and competitive relationships, that are not accounted for at lower levels[72]. Furthermore, such processes have also been demonstrated to drive indirect effects[73]. Numerous experimental results reveal that extrapolation of data acquired on individuals to the population often proves to be problematic. The gamma radiation of a simplified model ecosystem containing an algae population of *Euglena* (producer), a

protozoan population of *Tetrahymena* (consumer) and a bacteria population of *Escherichia coli* (decomposer) has shown that nonsensitive species can be affected by the radiation in an indirect manner, by means of the perturbation of trophic equilibria[74,75].

Although conceptual linkages between molecular effects to population and ecosystem level effects are generally lacking, there has been limited progress in this area[76]. For example, environmental biodosimetry can quantify radiation-induced chromosome translocations[13]. If translocations occur in germ cells, reproductive success may be impaired which, in turn, may alter population demography, thereby demonstrating ecological relevance of a molecular biomarker[76].

In most cases linkages across varying levels of biological organisation are complex and may be obscured by the interdependance of populations or communities, as manifested by indirect effects and compensatory interactions[11]. The importance of life history traits to an organism's response to its operative environment has been well established[14]. Basic physiological and life history trait differences determine the direction and amount of individual response to changes in contaminants within an organism's environments.

The manner in which radiation effects in individuals translates to the ecosystem scale depends on numerous processes which involve interactions between populations of different species. Other contaminants whose combined effects on the internal processes are practically unknown almost always accompany radioactive pollution to which they may be subject.

Conclusion

A number of biomonitoring procedures for detecting the impacts of pollutants, ranging from molecular- to community-level approaches have been developed. The reliability of the future radiation protection system will only be ensured if relevant research will be undertaken to overcome current deficiencies: to complete radioecological knowledge of the distribution of the radionuclides bioaccumulated in living organisms, to study the effects of this bioaccumulation in a context of multiple contamination at low chronic doses, to identify the characteristics of the effects on the scale of the ecosystem.

Acknowledgements

This work was supported by the Ministry of Education and Science of the Republic of Serbia (Project No. III 43009).

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The effects of nitric oxide donors and the other bioactive substances on the energy and oxidative-antioxidative metabolism of blood cells and cancer cell lines.

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The important significance of red blood cells (RBCs, erythrocytes and reticulocytes) in the basic physiology, pathophysiology of anaemia and drug biometabolism in the context of energy and oxidative-antioxidative metabolism was discussed in this overview. Experimentally induced anaemia by daily bleeding and phenylhydrazine-hydrochloride (PHZ) treatment give a significant amount of reticulocytes in peripheral blood (over than 80% in PHZ treatment). The reticulocytes are adequate model system and a valuable source of information for investigations of mitochondrial processes, considering energy and redox metabolism, as well as apoptosis. We discuss the properties of nitric oxide (NO), as significant mediator of physiological process and source of reactive nitrogen species. Our results showed that exogenous NO donors significantly inhibited energy production and induced oxidative stress in rat reticulocytes, as consequence of mitochondrial dysfunction. We also showed that comparative studies of NO donors effects on erythrocytes and reticulocytes, may give important data about biometabolism of investigated NO-released drugs. In addition, the oxidative stress as cause of preeclampsia, pregnancy disfunction, and cancer were discussed. We showed important role of erythrocytes as oxidative stress biomarkers in preeclampsia. Our results represented antiproliferative and proapoptotic activities of new bioactive substances, potentially anticancer drugs, on colon and breast cancer cell lines, and these effects were discussed on the basis of prooxidative/antioxidative metabolism.

Introduction - Red blood cells maturation, energy and oxidative-antioxidative metabolism

In mammals, the process of erythropoiesis included maturation of haematopoietic stem cells through increased haemoglobin synthesis and loosing of genetic materials and all organelles into mature erythrocytes. The last but one stadium of this differentiation, the reticulocytes do not posses a full range of metabolic pathways compared to proliferating cells, because some

pathways are missing due to the loss of the nucleus, the endoplasmic reticulum and the Golgi apparatus. However, reticulocytes are still equipped with a set of metabolic pathways, due to the presence of mitochondria and ribosomes[1]. Glycolysis is the only energy producing process in mammalian erythrocytes, while in reticulocytes energy is provided by glycolysis - 10 %, as well as, by oxidative phosphorylation (OxP) in mitochondria - 90 %[1]. The primary consumers of oxygen and primary ATP generators are mitochondria and are a permanent source of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells. In consideration of their primary role in ATP production, mitochondrial dysfunction is an irreversible step in the pathway to cell death[2,3].

The main role of red blood cells (RBCs, including erythrocytes and reticulocytes) is transport of hemoglobin which supplies oxygen to all tissues in the body. RBCs also serves as system which transports and metabolizes many drugs and active substances applied in the body. The combination of several factors, such as active metaloprotein (hemoglobin), which functions as an oxidase and peroxidase, high pressure of oxygen in the circulation, membrane proteins and unsaturated fatty acids, which can be oxygenated, creates the environment for potentially harmful reactions for RBCs[4]. Such conditions may induce physiological aging and cell death, but in terms of oxidative stress, it leads to premature dysfunction and death of RBCs. Oxygen is a primary oxidant in metabolic reactions which are required in order to obtain energy by oxidising various organic molecules. Oxidative stress is a consequence of these reactions and can be defined as an impaired balance between development of reactive oxygen and nitrogen species on the one side and antioxidant defence mechanisms on the other side[5]. The main source of ROS in cells are mitochondria, peroxisomes, microsomes, cell membranes, membrane bound enzymes (cyclooxigenase and lipooxigenase)[3]. Antioxidant defence system (AOS) has been developed in all aerobic organisms to prevent, limit or repair damage caused by activities of ROS[5]. Primary antioxidant protection consists of low molecular, nonenzymatic and enzymatic components. Nonenzymatic components are divided in: substances soluble in water (selenium, vitamin C, glutathione (GSH), methionine, albumin, bilirubin, biliverdin, uric acid, transferin, laktoferin, ceruloplazmin, histidine and feritin) and substances soluble in fats (vitamin A, vitamin E and coenzyme Q). The enzymatic components include: superoxide dismutase (SOD), catalase (CAT), glutathione-peroxidase (GSH-Px), glutathione-reductase (GR) and glutathione-S-transferase (GST) [5]. The redox state of a biological system is kept within a narrow range under normal conditions. Cells and tissues have the mechanisms to restore redox state after temporary exposition to high concentrations of ROS.

The most important mechanisms of redox homeostasis is based on ROS-associated induction of redox-sensitive signal cascade that leads to a higher expression of antioxidative enzymes or elevated intensity of cistine transport system, which keeps the high level of gluthatione in cells. Since mitochondria are major site of free radical generation, they are highly enriched with antioxidants including gluthatione and enzymes, such as Mn-containing superoxide dismutase (Mn SOD) and GSH-Px, which are presented on the both sides of their membranes in order to minimise oxidative stress in the organelle[5].

Red blood cells – experimentally induced reticulocytosis. Phenylhydrazine-hydrochloride effects.

In mammals, erythropoiesis is extravascular. The process of erythropoiesis includes maturation of hematopoietic stem cells into mature erythrocytes through increased hemoglobin synthesis and loss of genetic materials and all organelles. Under normal conditions, reticulocytes are the youngest erythrocytes released from the bone marrow into circulating blood. They mature for 1-3 days within the bone marrow and circulate for 1-2 days before becoming mature erythrocytes[6]. In animal systems, erythropoietic study using blood cells from the peripheral circulation is possible only through the induction of physiological stress, providing the release of a large number of immature cells into peripheral blood. In experimental conditions, the hemolytic anaemia induced by bleeding or by phenylhydrazine-hydrochloride (PHZ) treatment. Excessive bleeding is the most common cause of anaemia. When blood is lost, the body quickly pulls water from tissues outside the bloodstream in an attempt to keep the blood vessels filled. As a result, the blood is diluted, and the haematocrit (the percentage of red blood cells in the total blood volume) is reduced. Eventually, increased production of red blood cells may correct the anaemia. Over time, bleeding can reduce the amount of iron in the body, so that the bone marrow is not able to increase production of new red blood cells to replace the lost ones[6].

The reticulocytes are adequate model system and a valuable source of information for many metabolic pathways occurring, for investigations of mitochondrial processes, considering energy and redox metabolism, as well as apoptosis[7,8,9]. In our performed studies, we try to clearly distinguish and defined the metabolic properties of reticulocytes induced by daily bleeding and by PHZ treatment. In order to investigate daily changes of hematological parameters in bleeding induced anemia, we treated Wistar albino male rats by daily bleeding (1.5-2 mL of blood from tail vein for 8 days). Blood samples were taken before (0 day) and 1-8th day of bleeding. The values of haematocrit, haemoglobin and erythrocytes count significantly decreased after the second, sixth and second day of bleeding, respectively. The number of leukocytes and platelets, as well Heinz body levels significantly increased after the third and second day of treatment. Percentage of reticulocytes significantly increased from the second day and had the maximum level (32.55±0.96%) on the eighth day[10].

Phenylhydrazine and its derivatives are well-known agents that induce deleterious changes of RBC properties and lead to haemolysis and/or phagocytosis. It is generally assumed that all these changes are mediated by formation of superoxide anion radicals ($O_2^{\cdot -}$), hydrogen peroxide, phenyl radicals and other reactive species derived from PHZ[11,12]. In RBCs, PHZ reacts with oxyhaemoglobin to form of methaemoglobin, oxidises to haemichrome, and causes Heinz body formation, inducing membrane alterations due to lipid peroxidation and changes in membrane proteins[4]. Studies *in vitro* showed that PHZ induced significant changes in energy metabolism of RBCs in humans[13] and rabbits[14], as well as stimulation of oxidative pentose pathway in RBCs of rats[15]. Studies of other authors showed that PHZ--induced reticulocytes maturated into erythrocytes normally *in vitro*[16], as well *in vivo*[15,17]. The amount of bleeding-induced reticulocyte generation is a 30-40% increase[10,14], whereas PHZ-induced reticulocytosis in rats is over to 80%[15,18-25].

In order to exact definition of reticulocyte model system, the question which we try to resolve in performed studies is whether PHZ-induced reticulocytosis produce the energy and redox functional cells. We compared *in vivo* effects of PHZ treatment with bleeding on

respiratory processes, energy production and redox status in reticulocytes of rats[24,25]. Obtained data showed that bleeding-induced experimental anaemia in rats was followed by relatively low amount of reticulocytes and low level of haematological parameters in peripheral blood (strong anaemia). The RBCs obtained after bleeding had stable energy production and redox status. On the other hand, anaemia caused by PHZ yielded a greater degree of reticulocytes with lower haematological anaemic problems. There is inhibition of energy production in PHZ-induced reticulocytes, as consequence of appeared oxidative stress in these cells[25]. However, the energy processes in PHZ-induced reticulocytes were stable enough, resulting in maturation of these cells into normal erythrocytes[15]. When two anemic groups of animals are compared, there is accumulation of ROS and less effective glutathione antoxidative system in plasma of PHZ-treated rats, as well as, hemorrhagic shock-induced lipid peroxidation in plasma of bleeding rats. All these data indicate higher oxidative stress in plasma and RBC of PHZ-treated group compared to bleeding-treated group[24]. Our recent studies showed that PHZ-induced reticulocytes are an experimental system with up-regulated oxidative stress, but when compared with untreated erythrocytes, reticulocytes are a system adapted to one[22,23]. Taking all together, these studies contributes to better definition of metabolic processes in PHZ-induced reticulocytes, as simple model system for investigation of anemia, apoptosis and mitochondria-based processes[24,25].

Nitric oxide (NO) and effects of exogenous NO donors on energy and oxidative-antioxidative metabolism of RBCs.

Nitric oxide (NO), a short-lived free radical, has the diversity of physiological function and general ubiquity. NO is small hydrophobic molecule with chemical properties that make it uniquely suitable as both an intra- and intercellular messenger[26,27]. NO is synthesized during the enzymatic conversion of l-arginine to l-citrulline by nitric oxide synthase (NOS). There are three types of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In mononuclear phagocytes (monocytes and macrophages) iNOS is responsible for the production of a large amount of NO[26] which may react with superoxide anion radical to generate RNS. Primary reactions of NO are almost exclusively limited to other species possessing unpaired electrons, such as the iron in hem proteins, as well as nonhem iron (proteins with iron-sulfur centers), thiols, molecular oxygen and superoxide[26,28]. Recent studies showed that influence of NO on processes in mitochondria, particularly on respiratory chain, had many consequences in function of cell, even cell death. Namely, nanomolar concentrations of NO immediately, specifically and reversibly inhibit cytochrome oxidase in competition with oxygen, while higher concentrations of NO and its derivatives (peroxynitrite, nitrogen dioxide or S-nitrosothiols – R-SNO) can cause irreversible inhibition of the respiratory chain[2]. On the other hand, NO induced stimulation of glycolysis[29] and direct influence of NO on glycolytic enzymes were documented only for glyceraldehyde 3-phosphate dehydrogenase (GAPD) [30].

Physiologically, $NO^{\cdot}$ is a key component of the respiratory cycle and is the third gas transported by erythrocytes[31]. In addition, red blood cells, including reticulocytes and mature erythrocytes are the main site of NO metabolism, either endogenously synthesised, or exogenously supplied by NO donors. NO reacts with oxy- and deoxy-Hb in RBCs, generating methemoglobin (MetHb) and nitrozylhemoglobin, respectively[27,31]. NO is thereby inactivated

due to these reactions. The third kind of reaction between NO and Hb involves the SH-groups of the globin chains and the subsequent generation of S-nitrosohemoglobin, the form that retains NO-regulator function[27,31]. In addition, the metabolic pathways for endogenous synthesis and degradation of NO are closely connected with the metabolic pathways of redox and anti-oxidative metabolism particularly in erythrocytes and reticulocytes[3]. High concentrations of NO in reactions with $O_2^{\cdot-}/O_2$ generate reactive nitrogen species, which affect almost all molecules in cells[28]. On the other hand, a carefully-controlled endogenous balance of NO production and its clearance allows NO (in low doses) to be an anti-oxidative and a preventive agent[28]. According to literature data, glutathione is included in NO metabolism[32]. Generally, glutathione-dependent enzymes of AOS have SH-groups in active centres. NO-species in the primary reaction with SH-groups inactivated activities of these enzymes[33]. Data of Niketić et al.[34] showed that NO did not affect CuZn SOD, while it inhibited Mn SOD activity with liberation of NO^+ and NO^- ions.

Diverse and important physiological roles of NO implicate that exogenous donation of NO may be useful in the treatment of some disease states[35,36]. NO donors are the compound that releases active mediator NO. Today, there are a lot of drugs that react as NO donors. On the basis of the different pathways of NO formation from NO donors, they divided in some different classes: organic nitrates, sydnonimines, NONOates, sodium nitroprusside[35]. In biological systems, the redox form of NO (NO^+, $NO^{\cdot}$, NO^-) which released makes a difference to the NO donor's reactivity towards other biomolecules[35].

Nitroglycerine (NTG) has been used in the treatment of headache and angina pectoris long before its biochemical significance was understood. Today it is known that the action of NTG is mediated by the liberation of NO, after enzymatic biotransformation in cultured vascular smooth muscle cells as well as endothelial cells from different species[37]. Previously, GST[38], the cytochrome P-450 system[39] and xanthine oxido-reductase[40] were candidates for the NTG bio-transformation enzyme. Recently, Chen et al.[41] and Sydow et al. [42] identified mitochondrial aldehyde dehydrogenase (mtLDH) as a NTG reductase that specifically catalyzed the formation of 1,2-glyceryl dinitrate and nitrite. In addition, according to Feelisch and Kelm[37], direct interaction of NTG with low-molecular-weight thiols may produce vasodilator S-nitrosothiols. Furthermore, bio-transformation of NTG by erythrocytes is, et least in part, due to the interaction with hemoglobin[43,44].

Molsidomine (MO) has been used in the treatment of coronary heart disease for the last few decades. The main mechanism underlying its action as a vasodilator is its biotransformation (by esterases) into the metabolite 3-morpholinosydnonimine (SIN-1), which spontaneously liberates nitric oxide. The activation of soluble guanylate cyclase by NO results in an increase in the intracellular level of cyclic guanosine monophosphate and subsequent smooth muscle relaxation[45,46,47]. Investigations into the molecular mechanism of molsidomine/SIN-1 action have indicated that molecular oxygen plays a key role in the initiation of sydnonimine decomposition[47]. SIN-1 has also been shown to liberate superoxide anions concomitantly with NO, which rapidly react to form peroxynitrite[45,47]. However, $O_2^{\cdot-}$ present at the site of NO generation from SIN-1 interferes with the pharmacological effects of this drug and superoxide dismutase (SOD) may prevent the breakdown of NO by $O_2^{\cdot-}$[48].

Transition metal NO complex, sodium nitroprusside (SNP) spontaneously releases NO, mainly as nitrosonium ion – NO^+[49], which generates R-SNO in reaction of nitrosation with

low molecular weight thiols and which may store, or release NO[50]. SNP also gives rise to important quantities of cyanide ions, which react with hem-containing proteins[51].

The aims of our performed studies were to investigate the role of nitric oxide donors (nitroglycerine, isosorbide dinitrate-ISDN, molsidomine, SIN-1 and sodium nitroprusside) on rat RBC energy and oxidative-antioxidative metabolism. Our obtained results showed that all investigated NO donors induced stimulation of glycolysis and shortening of the ATP-turnover time in rat erythrocytes through mediation of NO as effector molecule[29]. In reticulocytes, investigated NO donors induced (*i*) inhibition of total and coupled, as well as stimulation of uncoupled mitochondrial respiration, (*ii*) stimulation of glycolysis, (*iii*) decrease of total energy production (*iv*) increased catabolism of adenine nucleotides, (*v*) decreased ATP production and concentration, and (*vi*) prolonged ATP-turnover time. All of these changes were mediated by NO as effector molecule; NTG and SNP were the most potent NO donors in induction of mentioned effects[18-21].

In the comparative investigation of NO donors effects on redox status in rat erythrocytes and reticulocytes, we can identified some products and metabolic pathways of NO donors biotransformation in these cells. NTG bio-transformation is primarily connected with Hb in erythrocytes. NTG-induced oxidation of Hb resulted in MetHb formation and $O_2^{\cdot -}$ generation, which caused lipid peroxidation[22]. On the other hand, two pathways of NTG bio-transformation exist in reticulocytes: one causing RNS production and the other connected with Hb (as in erythrocytes). The results of this study indicate the significant role of mitochondria in the enzymatic bio-transformation of NTG. NTG caused oxidative stress in rat erythrocytes and reticulocytes leading to significant oxidative damage in these cells, which is one of the essential reasons to suggest only acute clinical treatment with NTG[22].

Our unpublished data showed that GSH plays a critical role in biotransformation of NTG in erythrocytes. Reduced glutathione in the reaction with NO (released from NTG) generated GSNO, which has important biological functions as relatively stable storage and transport form of NO. In this way, the erythrocytes are important in the biotransformation of NTG and preservation of NO bioactivity.

Investigation of MO and SIN-1 effects showed that there are two metabolic pathways for MO biotransformation: one leading to NO and NO^- generation in erythrocytes and reticulocytes and another via SIN-1 metabolism that is present in reticulocytes. The main difference between MO and SIN-1 action was that SIN-1 induced oxidative damage in RBCs. These biochemical effects might account for the clinical effectiveness of MO in coronary heart disease[23].

According to our data, SNP spontaneously liberated nitric oxide as NO^- ion in rat erythrocytes and reticulocytes. In addition, applied experimental doses of SNP induced strong nitrosative and oxidative stress in these cells[52].

The one segment of our investigations was followed effects of NO donors on antioxidative defence system in rat erythrocytes and reticulocytes. On the basis of obtained data, we can conclude that all investigated NO donors induced (i) oxidative stress in rat RBC, (ii) RBC mobilized non-enzymatic components of AOS in order to induce cells protection against oxidative damage effects of NO donors. In reticulocytes, NO donors inhibited MnSOD, which was followed by stimulation of CuZnSOD[52-55].

Effects of cisplatin (cisPt) and selenium on redox status of rat RBC

Cisplatin (*cis*-diamminedichloroplatinum II, cisPt) is one of the most potent antitumor agents. Cisplatin induces production of ROS in renal epithelial cells mainly by decreasing the activity of antioxidant enzymes and by depleting intercellular concentrations of reduced glutathione[56]. Thiols such as the sulfur of GSH bind to the platinum molecule, replacing one of the chloride ions and preventing binding to other cellular nucleophiles[57]. These ROS can also, cause extensive tissue damage through reactions with all biological macromolecule, e.g., lipids, proteins and nucleic acids, leading to the formation of oxidized substances such as the membrane lipid peroxidation product malondialdehyde (MDA) [5] and failure of the antioxidative defense mechanism against free radical-mediated organ damage.

The selenium (Se) is an essential dietary trace element which plays an important role in a number of biological processes. As an integral part of the glutathione peroxidases and thioredoxin reductase, Se interacts with nutrients that affect cellular redox status (i.e., pro-oxidant/antioxidant balance). Selenoenzymes are also known to play roles in carcinogen metabolism, in the control of cell division, oxygen metabolism, detoxification processes, apoptosis induction and the functioning of the immune system[58]. Red blood cells (RBC) are the main place of inorganic Se utilization and reduction by GSH[59,60]. There is a great deal of evidence indicating that Se supplementation at high levels reduces the incidence of cancer in animals. Many experimental studies in animals have demonstrated the ability of Se to prevent carcinogenesis, and epidemiological studies have suggested that a decreased Se status in humans is associated with an increased risk of cancer[61]. Different therapeutic adjuvants have been tested in an attempt to reduce the nephrotoxicity of cisplatin. Burk et al. [62] showed that heavy metals, among them platinum, disclosed an interaction with Se and formed a complex of metal-Se with reduced toxicity.

In our performed investigations, we evaluated possible protective effects of Se on hematological and redox status parameters, as well on the activity of antioxidative enzymes in RBC of rats acutely and chronically treated with cisPt. Based on these results we can conclude that (*i*) acute cisPt treatment did not affect RBC maturation and redox status, except in glutathione metabolism, (*ii*) acute Se treatment stimulated erythropoiesis, increased lipid peroxidation and altered glutathione metabolism, favouring oxidizing state, and (*iii*) co-treatment with Se and cisPt and their synergistic effects may partially participate in protection against cisPt induced toxicity, and Se in high acute doses acts as prooxidant and as antioxidant[63]. Our unpublished data showed that chronic cisPt treatment induced anemia, generation of reactive species and increase of glutathione status. Based on the presented results it can be argued that it is necessary to take into account dose-dependence between antioxidants and chemotherapeutic agents as an important precondition for effective treatment of patients with cancer.

Oxidative stress as cause of many diseases. Preeclampsia.

Reactive oxygen and nitrogen species are play a significant and multiple roles in normal cellular signalling mechanisms and physiological control of cell function. However, an excessive and/or sustained increase of reactive species production has been implicated in the

pathogenesis of many diseases, such as cancer, atherosclerosis, neurodegenerative diseases, ischemia/reperfusion injury, preeclampsia[3,26,28].

Preeclampsia is a specific syndrome of human pregnancy and a leading cause of maternal and fetal morbidity and mortality. It is diagnosed by new development of hypertension (≥140/90 mmHg) and proteinuria (≥0.3 g/24 h) in the second half of gestation. Other manifestations of preeclampsia include generalized vasoconstriction, increased vasoactivity, reduced perfusion to organs and platelet activation. After delivery, these signs remit[64].

Etiopathogenesis of preeclampsia remains to be elucidated. There is substantial evidence that the diverse manifestations of preeclampsia derive from pathologic changes within the maternal vascular endothelium. Evidence points to the placenta as a key source of factors that lead to the maternal endothelial cell dysfunction in preeclampsia[65]. Several hypotheses invoke oxidative stress as a cellular process contributing to endothelial dysfunction in preeclampsia and a plausible convergence point for interaction of the fetoplacental unit and maternal predisposing factors involved in the disorder[65,66]. Nitric oxide potently relaxes arterial and venous smooth muscles and less strongly inhibits platelet aggregation and adhesion[26]. Considering that oxidative stress can cause endothelial dysfunction in preeclampsia, the administration of antioxidants could decrease oxidative stress and its clinical manifestation. Based on the literature data, the administration of antioxidant supplements, such as vitamin C and vitamin E does not reduce the risk of preeclampsia[67].

Estradiol is the most potent sex steroid hormone of placental origin and is essential for the maintenance of pregnancy and the delivery time. Estrogens, like vitamin E, as hydrogen donors from their phenol-hydroxyl ring, have antioxidant effects. Free radical scavenging by estrogens is one of their non-genomic (estrogens-receptor independent) mechanisms of cytoprotection[68]. Evidence *in vitro* showed that estradiol was the only steroid with the antioxidant role in pregnancy[69].

We have shown that markers of oxidative stress (H_2O_2, peroxynitrite, lipid peroxides and GST) are higher in erythrocytes and are linked to MAP in preeclampsia. Also, we have proposed that oxidative stress in erythrocytes may mark endothelial cell dysfunction in preeclampsia[70]. Our study[71] provides the first *in vivo* evidence for antioxidative effect of estradiol in the erythrocytes and its positive effect on blood pressure in women with preeclampsia. The results demonstrated positive correlation between the reduction of oxidative stress marker concentrations in erythrocytes and the reduction of blood pressure during estradiol therapy in preeclampsia. We suggest that a rapid increase of NO bioavailability after intramuscular administration of estradiol leads to reduction of blood pressure in preeclampsia. This might be mediated by the direct oxyradical scavenging activity of estradiol in erythrocytes. Considering key role of placenta in initiation of oxidative stress in maternal circulation, we also investigated relationship between RiAU (resistance index of the fetal umbilical artery) and concentration of GSH and GSSG (indicator of oxidative stress) in maternal circulation. On the basis of our results, RiAU increases and is negatively correlated with GSH in preeclampsia. This suggests that increased concentration of GSH in maternal plasma is protective mechanism against oxidative injury in human umbilical arteries[72].

Oxidative stress and cancer. Bioactive substances.

Neoplastic diseases are a life threatening and mutilating diseases and therefore represent a significant health problem in whole world. Cancer affects people at all ages with the risk for most types increasing with age. Cancer caused about 13% of all human deaths. In the last a few decades, there was arising evidence that oxidative stress plays a significant role in pathophysiology of cancer (see review in 3). ROS are potential carcinogens, since they facilitate mutagenesis, tumor promotion and progression, and growing-promotioning effects of ROS are related to redox-responsive signalling cascades. Droge[3] also suggested that pro-oxidative shift in the systemic thiol/disulfide redox state and elevated mitochondrial ROS production are in the basis of cancer pathology. The elevation of mechanisms of prooxidative/antioxidative metabolism that included in apoptosis and migration process of tumor cells are the main goals of our current and future scientific investigations.

Bioactive Substances (BAS), chemically synthesized or isolated from biological specimens, may show significant biological effects *in vivo* or *in vitro* in biological model systems (definition in accordance with our investigations). BAS could also become potential medicines with improved characteristics in comparison with existing relevant classes of drugs. Understanding of BAS mechanisms of action on physiological, genetic and molecular levels is of crucial importance in defining biological effects and future adequate application in patients' therapy. Importance of molecular markers for individual therapy, in the first place in patients with cancer, is obligatory course in area of biomedical research in the world.

Cisplatin is one of the most potent antitumor agents. Its activity has been demonstrated against a variety of tumors, particularly for head and neck, testicular, ovarian, bladder and small cell lung cancers[73]. The clinical success of cisPt for the treatment of cancer is clear, but severe side effects (nephrotoxic and hepatotoxic) and intrinsic or acquired resistance limits its application in high doses[74]. Cisplatin therapeutic effects are based on the interaction with DNA in the cell, preventing proliferation[75], as well as, by inducing apoptosis in tumours cells[76]. On the other hand, cisPt is highly mutagenic, inducing chromosome aberrations in peripheral blood lymphocytes in patients and in rat bone marrow cells[77-79]. Since the discovery of cisPt, the development of new analogue platinum or palladium complexes in order to improve of cisPt side effects and with good antitumor activity has been an empirical tasks[80-83]. The new selenium complexes, as potentially antioxidative substances and their application in anticancer therapy are also investigated[84].

Our investigation were followed a series of twelve new synthesized 3-substituted-5,5-diphenylhydantoins. Their antiproliferative effects against HCT-116 human colon carcinoma cells were evaluated to determine structure-activity relationships. The introduction of a substituent at position N3 produced a trend of changes in the antiproliferative potencies of compounds analogous to that in their anticonvulsant activities. The exception was the derivative bearing a benzyl group, which demonstrated a significant antiproliferative effect even in lower concentrations[85].

Our unpublished data showed that new platinum(IV) complexes had good antiproliferative effects on colon cancer (HCT-116) and breast cancer (MDA-MB-231) cell lines. The combined *in vitro* treatment with these platinum complexes and methanol mushroom extracts of *Phellinus linteus* had better synergistic antiproliferative activities and inducing apoptosis of investigated cancer cell lines.

Through medical history, nature has long been shown an excellent and reliable source of new drugs, including anticancer agents. It is well established that plants have always been useful sources of antitumor or cancer prevention compounds[86]. Approximately more than 60% of currently used anticancer chemotherapeutic drugs are derived in one way or another from natural sources, including plants. Large groups of different phenolic compounds from plants are important and essential anticancer agents[87,88]. Lichens are unique life form of symbiosis between fungi (mycobionts) and algae and/or cyanobacteria (photobionts). Strong antioxidant power and antitumour potential of lichen flora are investigated[89-91]. We evaluated antioxidative, antimicrobial and antiproliferative potentials of methanol extracts of lichen species *Parmelia sulcata, Flavoparmelia caperata, Evernia prunastri, Hypogymnia physodes* and *Cladonia foliacea*. Antioxidant capacities of lichen extracts were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging. *Hypogymnia physodes* with the highest phenolic content showed the strongest DPPH radical scavenging effect. Antiproliferative activity of lichen extracts were explored on colon cancer adenocarcinoma cell line HCT-116 by MTT viability assay and acridine orange/ethidium bromide staining. Methanol extracts of *Hypogymnia physodes* and *Cladonia foliacea* showed better cytotoxic activity than other extracts. All lichen species showed the ability to induce apoptosis of HCT-116 cells[92].

In performed investigation, we tested antioxidative and antiproliferative activities of some plant species and their methanol extracts. The antiproliferative activity of methanolic extracts from different Teucrium species was determined using MTT cell viability assay, where IC50 value was used as a parameter for cytotoxicity. The type of cell death was explored by fluorescence microscopy using acridin orange/ethidium bromide method. MTT assay showed that all extracts significantly reduced cell viability in a dose-dependent manner, with very low IC50 values. The highest content of phenolic compounds and the best cytotoxic activity on HCT-116 cells after 24 h of exposure was in T. chamaedrys extract, with IC50 values of 5.48 x 10-9 μg/ml. After 72 h methanolic extract of T. arduini appeared to have the best cytotoxic activity on HCT-116, with IC50 values of 0.37 μg/ml. Treatments caused typical apoptotic morphological changes in HCT-116 cells and showed high percentage of apoptotic cells. The results of presented research indicate that some Teucrium extracts are very rich source of phenols, which may directly contribute to high antiproliferative and proapoptotic activity[93]. The methanolic extract of *Allium flavum* has good antiproliferative activity, with IC_{50} values of 28.29 for 24 h and 35.09 for 72 h on HCT-116 cell line. Based on these results, *A. flavum* is a potential source of phenols as natural antioxidant, antibacterial and anticancer substance of high value. Phenolic content of extracts depend on the solvents used for extraction[94]. Our unpublished data showed that *Ligustrum vulgare* L. is a considerable source of natural bioactive substances with antiproliferative activity on HCT-116 cells and has a very good synergistic effect with new Pd (N, N'-bis (3-aminopropyl) oxamide) complex.

Acknowledgements

The author would like to thank Prof. dr Milosav M. Kostić, Prof. dr Andraš Š. Štajn, Prof. dr Radoslav V. Žikić, Prof. dr Mihajlo B. Spasić and Dr Zorica Saičić for the mentorship in my scientific work and to thank cooautors (especially to my younger colleagues) of the published papers which were used in this presentation.

This work was supported by the Ministry of Science of the Republic Serbia in a past decade, and currently by Ministry of Education and Science of the Republic of Serbia (Project No. III41010).

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Mitochondrial redox metabolism in oxyphilic thyroid cancer

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Oxyphilic tumors of the thyroid gland are characterized by the presence of Hürthle cells - mitochondrion-rich, eosinophilic epithelial cells. Hürthle cells are characterized by profound aberrations in the nuclear and mitochondrial genome and by alterations in oncogenes, tumor suppressor genes and other key genes involved in energy metabolism, proliferation and apoptosis. The mitochondrial energetic impairment and defective ATP synthesis in oxyphilic cancer is associated with the alterations of the mtDNA sequence. Two mutations within the mitochondrial genome were detected in the oxyphilic thyroid cancer cell line, and it is suggested that these two mutations are sufficient to explain the defective ATP synthesis. Oxyphilic thyroid cancer cells are characterized by deregulated bioenegetic and redox state. Our data of comparative analysis performed in thyroid cancer cell lines showed that oxyphilic cells produce much higher amounts of ROS compared with a line derived from a non-oxyphilic thyroid cancer. A novel biochemical rationale, based on fundamental differences in cancer versus normal cell oxidative metabolism, for combining oxidative stressors with radiotherapy and chemotherapy, may lead to designing of more effective treatment strategies for numerous malignancies, including oxyphilic thyroid cancer. Redox and metabolic parameters have a significant impact on the sensitivity of thyroid malignancies to a broad range of treatments including ionizing radiation, traditional cytotoxic chemotherapy, targeted therapy, and immunotherapy. Further and more detailed understanding of molecular and genetic defects underlying the malignant metabolic phenotype of oxyphilic thyroid cancer is required as a key to sustainable and efficient cancer therapy.

Introduction

Oxyphilic neoplasms in the thyroid gland represent a distinct subtype within the group of follicular tumors, according to the World Health Organization Committee for the Histological Typing of Thyroid Tumors [(1)]. Hürthle cells (Askanazy cells, oxyphilic cells) are thyroglobulin producing, mitochondria-rich, thyroid epithelial cells. They are found in a variety of thyroid conditions, including Hashimoto thyroiditis, Graves's disease, nodular

goiter, and thyroid neoplasms. Oxyphilic features have also been described in parathyroid, pancreatic, hepatic, renal, pituitary and salivary gland tissue [2].

The term Hürthle cell tumor, which is widely used in the pathology literature, designates oncocytic neoplasms in the thyroid gland. Oncocyte (from the Greek word onkoustai, to swell) is the generally accepted term for those cells exhibiting the characteristic phenotype, featuring a finely granular eosinophilic cytoplasm in histology sections and an increase in the number of mitochondria ultrastructurally. In accordance with the high mitochondrial density in oxyphilic tumors, transcript levels of subunits of complexes of the oxidative phosphorylation system are increased [3]. Hürthle cell tumors are defined as being composed of at least 75% Hürthle cells and are classified as benign (Hürthle cell adenomas [HCA]) or malignant (Hürthle cell carcinomas [HCC]) [2-5]. These two entities are distinguished based on the identification of capsular or vascular invasion, or on the presence of metastatic disease [3-5].

Cytogenetic characteristics of oxyphilic thyroid cancer

Great attention has been paid in recent years to combining morphological and genetic characteristics in oxyphilic tumors and to the further elucidation of pathogenesis of these neoplasms. DNA content profiles after flow cytometry are commonly abnormal. Thyroid oncocytic neoplasms, including histologically benign tumors, are often aneuploid or polyploid, but the demonstration of aberrant DNA content does not help in differentiating adenomas from carcinomas [6,7].

No specific pattern of chromosomal aberration has been described for thyroid oncocytic tumors, so that, unlike the case of renal neoplasms, a classification according to large genetic rearrangements is not feasible [8]. Wada et al. [9] have reported a plethora of chromosomal aberrations in a relatively small set of samples (13 carcinomas and 15 adenomas), involving gains and losses of genetic material from both arms of chromosomes 1, 2, 5, 7, 12, 17, 19, 20 and 22, detected by comparative genomic hybridization (CGH) [9].

LOH on chromosomes 2q21 and 19p13.2 has been analyzed in a cohort of 70 sporadic oncocytic tumors by our group [10]. A statistically significant LOH in the oncocytic sample set with respect to the control set (follicular non-oncocytic tumors) was obtained for the 2q21 region. The number of patients samples showing LOH for the 19p13.2 region did not differ in a statistically significant way from the number of controls. However, LOH was more frequent in oncocytic than in non-oncocytic samples, suggesting that the lack of significance might be due to the relatively small number of cases analyzed. The LOH in the two regions supports the idea that a tumor suppressor gene may be lost in the tumorigenesis process leading to the oncocytic transformation [10]. This hypothesis has been supported also by the finding that, in one affected individual of the large family where the TCO locus has been identified, FISH analysis in the tumor specimen revealed an LOH of the chromosome 19p13.2 region, supporting the linkage data [11].

Mitochondrial genome mutations in oxyphilic thyroid cancer

Mitochondrial abnormalities have been described in many thyroid tissues [12]. It is known that rapidly growing cancer cells have an increased glycolytic rate [13]. Tumor cells have been associated with changes in mitochondrial size, number, distribution, morphology, membrane lipid composition, membrane potential, loss of electron transport components, deficiencies in energy-related functions, and impaired protein synthesis [14-18].

The majority of proteins in mitochondria are encoded by the nuclear genome, and intergenomic communication is necessary for mitochondrial synthesis and function. The oxidative phosphorylation (OXPHOS) activity occurs within the mitochondrial environment; these organelles are to a large extent the "power plant" of the cell [17]. Under normal physiological conditions, a small fraction of the oxygen consumed by mitochondria is converted to superoxide anions, H_2O_2 and other reactive oxygen species (ROS). Mitochondria are the major intracellular source and primary target of ROS, which are generated under normal conditions as by-products of aerobic metabolism in animal and human cells. It has been established that defects in the respiratory chain lead to enhanced production of ROS and free radicals in mitochondria. In addition, H_2O_2 has been proposed to be involved in signal transduction pathways. Recently H_2O_2 has been proposed to be involved in the communication between mitochondria and the nucleus. The mtDNA copy number has been suggested to be increased by a feedback mechanism that compensates for defects in mitochondria harboring mutated mtDNA and a defective respiratory system [18].

The mitochondrial energetic impairment in oxyphilic cancer is associated with the alterations of the mtDNA sequence [19]. The aberrant mtDNA sequence was first discovered by Tallini et al, namely the common deletion in a panel of oxyphilic thyroid cancer samples [20]. Two mutations within the mitochondrial genome were detected in the oxyphilic thyroid cancer cell line XTC.UC1 [21]. First was a single base pair homoplasmic insertion in a C-homopolymer in the ND1 OXPHOS complex I gene, causing a premature stop codon that explained the absence of the protein. The second was a non-conservative heteroplasmic missense mutation in the CYTB complex III gene. The authors suggested that these two mutations are sufficient to explain the defective ATP synthesis already reported in the same cell line by our group [22]. In a recent review, the ratio between the frequency of reported and expected mutations per mitochondrial gene indicated that ND1 is a hotspot for somatic changes, since its mutations occur three times more frequently than expected on the basis of the gene length. Similar, although less striking, tendency to accumulate somatic changes were observed for ND4, ND5 and ATP6 genes [23].

In a context of oxyphilic phenotype expression and mtDNA mutated genotype correlation with oxyphilic changes, such as increased mitochondrial biogenesis and malignant transformation, recent data imply that in the up-regulation of biogenesis are involved the genes responsible for DNA repair and senescence, such as TP53 and Rb, together with the PGC1α/β induction upon mtDNA mutations in ND5 and COI [23]. Further investigation may

provide additional data on mtDNA mutations involvement in oxyphilic malignant transformation.

Mitochondrial bioenergetic hallmarks of cancer

The importance of mitochondria in cell physiology and cancer metabolism was emphasized by pioneering observation of Nobel Prize laureate Otto Warburg [24]. His hypothesis that in the presence of oxygen, the rapidly growing tumor cells consume glucose at a higher rate than normal cells, profoundly influenced the cancer metabolism investigations [25]. The first incontestable examples of causality between mitochondrial dysfunction and tumorigenesis were only discovered less than a decade ago when mutations in succinate dehydrogenase (SDH) or fumarate hydratase (FH), both enzymes of the TCA cycle, were found to be the initiating events of familial paraganglioma or leiomyoma and of papillary renal cell cancer, respectively [25]. Therefore, the mitochondrial impairment observed in many tumours could be the consequence of complex metabolic shifts that, while conferring survival and replicative advantages to cancer cells.

Cancer cells must adapt their metabolism to produce all molecules and energy required to promote tumour growth and to possibly modify their environment to survive. These metabolic peculiarities of cancer cells are recognized to be the outcome of mutations in oncogenes and tumour suppressor genes which regulate cellular metabolism [26]. Cancer cell mitochondrial metabolism is an emerging target for the translational cancer research and numerous small molecule inhibitors of this metabolism are in pre-clinical or clinical development [27]. Programmed cell death, including apoptosis, autophagy and necrosis, have arisen as attractive targets for cancer therapy. The role of mitochondria in programmed cell is well established in all subsequent steps. The stress signals induce changes in mitochondrial membrane permeability, resulting in the release of cytochrome c from the mitochondrial intermembranous space and activation of a proteolytic cascade of cysteine proteases called caspases [28].

Oxidative stress accompanied by calcium overload and ATP depletion induces the mitochondrial permeability transition (mPT) with formation of pathological, non-specific mPT pores (mPTP) in the mitochondrial inner membrane. Opening of the mPTP with a high conductance results in matrix swelling ultimately inducing rupture of the mitochondrial outer membrane and releasing pro-apoptotic proteins into the cytoplasm. The ATP level is the determining factor in deciding whether cells die through apoptosis or necrosis [29]. Cancer cells exhibit less sensitivity to pore opening which can be in part explained by increased expression of mPTP compounds/modulators and metabolic remodeling. Since the main goal of chemotherapy is to provoke apoptosis, mPT induction may represent an attractive approach for the development of new cancer therapeutics to induce mitochondria-mediated cell death and prevent cell differentiation in carcinogenesis [30]. Cancer cells are armed with a

variety of adaptive responses and carry mutations, such as defective apoptotic machinery, that further confer survival advantage. The defects in the apoptosis-inducing pathways can eventually lead to the expansion of a population of neoplastic cells resistant to chemotherapy and irradiation, as is the case of oxyphilic thyroid cancer [31]. In our study of oxyphilic thyroid cancer cells, we have used the TMRM (tetramethylrhodamine methyl ester) as a probe that was accumulates in polarized mitochondria and was released upon depolarization. The addition of arachidonic acid (AA) induced the massive, cyclosporin A (CsA) – sensitive depolarization in thyroid cancer cells. These results may imply that the mtPTP AA – induced opening mediates the mitochondrial *in situ* depolarization. The fluorescence changes detected by microscopy corresponded with the mtPTP opening, further confirmed by CsA addition that blocked the mtPTP opening. mtPTP opening is voltage dependent process, thus FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone, a protonophore and uncoupler of mitochondrial oxidative phosphorylation in mitochondria) induced depolarization favoures the mtPTP opening. Our results indicate that the depolarization in XTC.UC1 and B-CPAP cell lines was not followed by mtPTP opening. This may indicate the prevaling influence of mtPTP opening – inhibitory factors and apoptosis resistance in thyroid cancer cells, despite the depolarization induced by FCCP [22].

Thus, improving therapeutic efficacy and selectivity and overcoming drug resistance are the major goals in developing anti cancer agents today [32]. Restoration of cell death pathways via the targeting of mitochondrial proteins is an attractive concept that emerged following the identification of the central orchestrators of this pathway. Indeed, this pathway is frequently impaired in cancer cells and contributes to the development of resistance to conventional chemotherapy. Several small molecules, targeted anti/pro-oxidants and antisense oligonucleotides have been designed to activate pro-apoptotic proteins as well as to block anti-apoptotic proteins and are currently under clinical evaluation. Results of clinical trials will determine whether the promise that these strategies hold will be realized for a significant improvement in the clinical management of cancers that are refractory to conventional interventions [32, 33].

Redox metabolism and mitochondrial dysfunction in oxyphilic thyroid cancer

During the electron transport chain (ETC), electrons are occasionally captured by oxygen to produce superoxide anion radicals. Within the mitochondria, these superoxide radicals are converted to hydrogen peroxide by the action of manganese superoxide dismutase. Complexes I, II, and III are all capable of generating ROS during oxidative phosphorylation (OXPHOS) [34]. It has been proposed that chronic mtDNA damage causes a vicious cycle of ROS production and serves to amplify oxidant injury during disease. Numerous studies

reviewed recently by De Moura et al [35], provided evidence that dysfunctional mitochondria, alterations in mitochondrial dynamics, increased ROS, mtDNA damage, and the loss of energy production are important contributors to the pathophysiology associated with several neurodegenerative diseases and cancer [35].

Transcriptional profiling revealed coordinated up-regulation of oxidative metabolism genes in thyroid oncocytic tumors in comparison with the normal thyroid tissue, which was followed by the under-expression of anti-apoptotic protein coding gene Bcl-2 [36]. The canonical role of Bcl-2 as an oncoprotein and anti-apoptotic factor is classically attributed to its protective effect on the integrity of the outer mitochondrial membrane and the prevention of the mtPTP opening. Interestingly, novel data demonstrated that Bcl-2 maintains the intracellular redox status at a level optimal for cell survival, and increases the flux of oxygen through the mitochondrial electron transport chain. Bcl-2 is often overexpressed in variety of cancers, unlike the oxyphilic thyroid cancer, and its overexpression could be a function of a mild pro-oxidant intracellular milieu coupled with bioenergetic proficiency that allow tumor cells to thrive even under non-favorable conditions [28]. Oxyphilic thyroid cancer cells are characterized by deregulated bioenegetic and redox state, which is in accordance with the altered Bcl-2 expression levels [22, 36].

The occurrence of mtDNA mutations in oxyphilic thyroid cancer, implying an incomplete or partial assembly of complex I, raises the question of whether this may contribute to ROS generation. Zimmermann et al. speculated that the observed lack of complex I staining in oncocytic thyroid may have a role in tumor formation through the increase in ROS production and, simultaneously, a downstream inhibition of pro-apoptotic pathways, although the nuclear-coded complex I subunits called into play are not apoptotic effectors, but rather caspases targets [37].

Lack of data on the role of ROS in oncocytic tumors is mainly due to the fact that studies on tumor biopsies are not feasible and that cell models for in vitro studies are very scarce. Investigation of OXPHOS function and ROS production showed that the XTC.UC1 cells exhibit a dramatic decline of ATP synthesis supported by NAD – dependent substrates, while in the mitochondria isolated from these cells the Complex I activity is strongly depressed (21, 38). Accordingly, the XTC.UC1 cells produce much higher amounts of ROS compared with a line derived from a non-oxyphilic thyroid cancer [22].

Our data of comparative analysis performed in thyroid cancer cell lines, suggested that enhanced oxidative stress and not deficient respiratory activity *per se* is the stimulus triggering over-expression of plasma membrane oxidative enzymes [39]. Dichlorophenol indophenol (DCIP) reduction by intracellular pyridine nucleotides was investigated in two different lines of cultured cells characterized by enhanced production of reactive oxygen species (ROS) with respect to suitable controls. Our aim was to analyze the possible correlation between the oxidative stress and the activity of plasma membrane oxidoreductase (PMOR), an enzyme system that functions as a cellular redox sensor [40, 41]. The first line

denominated XTC.UC1 was derived from a metastasis of an oxyphilic thyroid tumor characterized by mitochondrial hyperplasia and compared with a line (B-CPAP) derived from a papillary thyroid carcinoma with normal mitochondrial mass. The second line (170 MN) was a cybrid line derived from rho0 cells from an osteosarcoma line (143B) fused with platelets from a patient with a nucleotide 9957 mutation in mitochondrial DNA (encoding for cytochrome c oxidase subunit III) in comparison with the parent 143B line. The experimental cell lines had no major decreases of electron transfer activities with respect to the controls; both of them, however, exhibited an increased peroxide production. The XTC.UC1 cell line exhibited enhanced activity with respect to control of dicoumarol-sensitive DCIP reduction, identified with membrane bound DT-diaphorase, whereas dicoumarol insensitive DCIP reduction was not significantly changed. On the other hand the mtDNA mutated cybrids exhibited a strong increase of both dicoumarol sensitive and insensitive DCIP reduction [39].

Additional data on mtDNA alterations involvement in ROS production showed that the different degree of heteroplasmy of the 3571insC ND1 mutation does not influence ROS amounts. This was explained with a differential expression of ROS detoxifying enzymes such as manganese superoxide dismutase and catalase in presence/absence of complex I [42]. In similar malignant model of oncocytic tumors of the salivary glands, the oxygen peroxide scavenging enzyme peroxiredoxin I was shown to be overexpressed. Its overexpression in oncocytes is related to its ability to decompose mitochondrial-derived H_2O_2 and that it could provide to the cells a protective role in an environment that, by continuously producing potential DNA-damaging ROS, predisposes to genome instability and cellular transformation. This result led the authors to suggest that detoxifying mechanisms may be up-regulated in these neoplasms likely carrying mitochondrial dysfunctions and electrons leakage [43].

Conclusion

Inducing apoptosis has long been a central goal of chemotherapy and radiation treatment. The anti-cancer activity of a variety of therapies is partially based on the concept of selectively inducing an “oxidative catastrophe” in cancer cells to eliminate the malignant cells by apoptosis or mitotic linked cell death [44]. Developing novel combined-modality therapeutic approaches based on understanding of the involvement of redox biology in apoptosis of malignant cells is a promising approach for improving clinical responses [33, 44]. A novel biochemical rationale (based on fundamental differences in cancer versus normal cell oxidative metabolism) for combining oxidative stressors with radiotherapy and chemotherapy, may lead to designing of more effective treatment strategies for numerous malignancies, including oxyphilic thyroid cancer. Therefore, redox and metabolic parameters have a significant impact on the sensitivity of thyroid malignancies to a broad range of

treatments including ionizing radiation, traditional cytotoxic chemotherapy, targeted therapy, and immunotherapy. Further and more detailed understanding of molecular and genetic defects underlying the malignant metabolic phenotype is required as a key to sustainable and efficacious cancer therapy.

Acknowledgements

The author would like to thank Prof dr Giovanni Romeo and Prof dr Giorgio Lenaz, University of Bologna, Italy for the mentorship and to thank cooautors of the published papers which were used in this review. This work was supported by the Ministry of Education and Science of the Republic of Serbia (Project No. OI 173014).

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Matrix metalloproteinase-9, TNF alpha- and TNF receptor gene polymorphisms in juvenile idiopathic arthritis

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Tumor necrosis factor-α (TNF-α) is likely to have a primary role in the pathogenesis of juvenile idiopathic arthritis (JIA), including matrix metalloproteinase-9 (MMP-9) production. Etanercept is one of several TNF inhibitors approved for treatment of JIA. Genetic contribution of TNF-α- and TNF receptor (TNFRII) gene polymorphisms in patients with JIA on response to TNF blocking agents, as well as MMP-9 production, is not yet well established. We have investigated whether the TNF-α-308G/A and TNFRII 676T/G polymorphisms can influence MMP-9 level and clinical response to etanercept in JIA patients, after 1 year of treatment. Clinical assessment was performed according to ACR Pedi 50 improvement criteria. Patients with the -308GG genotype achieved an ACR Pedi 50 response significantly more frequently than those with the TNF-α-308AA genotype. There was no statistically significant change of ACR Pedi 50 response in patients with TNFRII676TT genotype in comparison to patients with 676TG/GG genotypes. It was found that etanercept significantly reduced MMP-9 level in children with polyarticular JIA and -308GG genotype, while in patients carriers of 676TT and 676TG/GG genotypes, only a trend was observed. Our results correlate with findings that the -308A allele is associated with a lower response to etanercept treatment.

Introduction

Juvenile idiopathic arthritis (JIA) is the most common chronic inflammatory disease in childhood and can lead to severe disability. The term JIA encompasses a group of clinically heterogeneous disorders with arthritis that begin prior to age 16 years, are of unknown cause and present with joint pain, stiffness and swelling that persists for longer than 6 weeks. According to the International League of Associations for Rheumatology (ILAR) classification, JIA is subclassified into seven distinct categories by the use of definitions and exclusion criteria. The ILAR classification is mainly based on the number of joints affected, the presence of extra-articular manifestations, the presence or absence of rheumatoid factors

and HLA-B27 and finally the family history. Polyarthritis affects five or more joints during the first 6 months of the disease and is one of the most aggressive disease subtype (1).

Although the aetiology of JIA is still unknown, the inflammation that occurs in response to the autoimmune reaction is quite well described. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that has a complex role in the pathogenesis of JIA. This cytokine has been found in elevated levels in the circulation and synovial fluid of patients with JIA (2). TNF is synthesized by several cells, notably monocytes and macrophages and may play a role in leukocyte activation and migration, the acute-phase response and apoptosis. TNF-α starts life as a membrane-bound protein but a soluble fragment is cleaved off by the TNF-α converting enzyme (TACE). This soluble fragment binds to its two receptors, the 55-kDa and the 75-kDa TNF-α receptor (TNFRI and TNFRII respectively), resulting in activation of downstream effector pathways. Receptor binding induces apoptosis, c-Jun N-terminal kinase-stress activated protein kinase (JNK-SAPK) or NFkB activation. Activated JNK induces production of activator protein-1 (AP-1). NFkB and AP-1 enter nuclei and induce the transcription of genes associated with inflammation, including those coding for TNF. The production of TNF and other inflammatory cytokines serves to recruit other inflammatory cells, which in turn release cytokines and subsequently amplify the immune response. Furthermore, TNF-α is a potent stimulator of mesenchymal cells, such as synovial fibroblasts, osteoclasts, and chondrocytes, that release tissue-destroying enzymes (3).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are capable of degrading all major components of the extracellular matrix. The whole group can be divided into subclasses, such as collagenases, gelatinases, strome-lysins, and membrane-type MMPs. MMP-9 belongs to the gelatinase subfamily (gelatinase B) of the MMPs, and is believed to be primarily responsible for the degradation of laminin and type IV collagen, the major component of basement membranes. The promoter region of MMP-9 posseses several functional enhancer element-binding sites, such as NF-κB and AP-1 sites, indicating that these molecules are crucial transactivators for the expression of MMP-9 induced by TNF-α (4). A number of studies have demonstrated that MMP-9 is an important mediator in inflammatory and connective tissue diseases and is thought to be one of the main mediators of joint damage in rheumatoid arthritis (RA) (3, 5, 6), but its role in JIA still remains unknown.

Intensive studies on TNF-α -driven inflammation processes have led to the development of TNF-α blockers for JIA treatment. At present, there are three TNF inhibitors approved by the US FDA for treatment of JIA (etanercept, infliximab, adalimumab). In Serbia, etanercept is approved for treatment of polyarticular JIA patients aged from 4 to 25 years. Etanercept is a fusion protein consisting of two identical chains of the recombinant extracellular TNF receptor II monomer fused with the Fc domain of human IgG1. Etanercept binds TNF-α and lymphotoxin and inhibits their activity. In a single placebo controlled trial with 69 children with polyarticular disease who were refractory to previous treatment, etanercept proved to be effective. Efficacy seemed to last over a period of up to 8 years (7). However, response to these therapies is heterogeneous with roughly two-thirds of patient response and one-third non-response. Given the destructive nature of JIA, the risk of adverse effects, and considerable costs for TNF-α blocker therapy, there is a strong need to identify predictors of response prior to start the treatment. The fact that these drugs target TNF-α has led to interest

in TNF-α itself and TNF receptors as candidate genes for pharmacogenetic association studies.

The TNF-α gene is located on chromosome 6. Several single-nucleotide polymorphisms (SNPs) have been identified in the TNF-α promoter (8). Among these, a common polymorphism in the promoter, a G to A substitution at position −308 (rs 1800629), has been studied intensively. It is not clear whether the TNF-α promoter −308G/A polymorphism has a functional significance, but there are some indications that the A allele could be associated with greater levels of TNF-α transcription (9-11).

TNFRII (p75) is coded by gene (TNFRSF1B) located on chromosome 1. SNP within exon 6 (676T/G) of the TNFRII gene (rs1061622) causes a non-conservative amino acid substitution (methionine (M) to arginine (R)) at codon 196 (M196R) within the fourth cysteine-rich domain of the extracellular domain and might affect the binding site for TACE and consecutive solubilization of TNFR (sTNFR) (12). Considering the fact that the soluble TNFRs neutralize the biological activity of TNF in the liquid phase by competing with its membrane-bound counterparts, it is hypothesized that this SNP could affect clinical response to TNF inhibitors. As identification of new diagnostic markers may be helpful in formulating the future strategies of JIA treatment, the aim of this study was to evaluate whether TNF-α-308G/A and TNF-RII676T/G SNPs can influence MMP- 9 level and etanercept treatment response of JIA patients.

Patients and experimental procedures

Sixty six JIA patients, with active polyarticular disease course, defined by the presence of five or more joints with active arthritis, were enrolled. Joints were defined as active by the presence of swelling or, if no swelling was present, by the limitation of motion accompanied by pain, tenderness, or both. Patients had active disease despite standard disease modifying antirheumatic drugs (methotrexate 15–20 mg/m^2/week) and steroid therapy. In order for patients to be eligible for treatment with etanercept, failure to respond or intolerance to methotrexate was required. Etanercept therapy was given at the dosage of 0.4 mg/kg subcutaneously twice a week. All JIA patients were included in the etanercept national registry survey, willing to donate paired blood samples before starting etanercept therapy and 12 months afterward. Clinical response to etanercept was assessed after 12 months of therapy according to American College of Rheumatology Pediatric 50% (ACR Pedi 50) improvement criteria, defined as three or more criteria improved by at least 50% with respect to baseline and no more than one core set criteria worsened by 30% (13). The control group consisted of 65 healthy children, matched by age and sex with the patients. Genomic DNA was isolated from the whole blood samples using QIAamp DNA Blood Mini Kit (Quiagen GmbH, Hilden, Germany). The subjects were genotyped for −308G/A promoter polymorphism of the TNF-α gene by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) as described previously (14). After RFLP analysis using enzyme *Nco*I, the presence of the -308A allele resulted in an uncut, full-length product (117 bp), while the presence of the -308G allele was detected as fragments of 97 and 20 bp on the gel pattern obtained. PCR-RFLP analysis was also performed in order to genotyping for TNFRII676T/G SNP, with the same PCR conditions as for TNF-α-308G/A SNP. A fragment of 242 bp of the TNFRII gene was amplified using the forward primer 5'-TTCTGGAGTTGGCTGCGTGT-3' and the

reverse primer 5'-ACTCTCCTATCCTG'CCTGCT-3'. PCR product was digested with 1 U of *HIN*1II enzyme (Fermentas GmbH, St.Leon-Rot, Germany) at 37°C overnight. The 242-bp PCR product was uncleaved in the 676T allele and cleaved into two fragments of 133 and 109 bp in the 676G allele. Plasma total MMP-9 level was determined using the sandwich enzyme-linked immunosorbent assay (ELISA; Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. The allele and genotype frequencies were determined in the patients and controls and were compared with the values predicted by the Hardy–Weinberg equilibrium by means of the χ^2 test. We performed a χ^2 test to compare TNF and TNFRII polymorphisms with clinical response after 12 months (ACR Pedi 50). Quantitative data were compared using Student's *t* test. Statistical analyses were performed using the SPSS version 17.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

The genotype frequency distributions of the TNF-α-308 polymorphism in the patients were significantly different from those of the controls ($\chi^2 = 12.120$; $P = 0.002$). The frequency of the -308A allele was significantly higher in JIA patients compared to controls (39% vs. 26%; $\chi^2 = 5.207$; $P = 0.022$). There were no differences from the genotype frequency distributions of the TNFRII676T/G polymorphism ($\chi^2 = 0.673$; $P = 0.714$), as well as 676G allele frequency in JIA patients compared to healthy subjects ($\chi^2 = 0.003$; $P = 0.954$; Table 1).

Table 1. Genotype frequencies of the TNF-α-308G/A and TNFRII676T/G SNPs in children with polyarticular JIA and controls

Genotype	JIA (n=66)	Controls (n=65)	P value (*df*=2)
-308GG	19 (28.8%)	37 (56.9%)	
-308GA	42 (63.6%)	22 (33.8%)	0.002
-308AA	5 (7.6%)	6 (9.3%)	
676TT	47 (71.2%)	42 (64.6%)	
676TG	16 (24.2%)	19 (29.2%)	NS
676GG	3 (4.6%)	4 (6.2%)	
Allele	n (%)	n (%)	*df*=1
-308G	80 (61.0)	96 (74.0)	
-308A	52 (39.0)	34(26.0)	0.022
676T	110 (83.0)	103 (79.0)	
676G	22 (17.0)	27 (21.0)	NS

df degree of freedom, NS not significant

The clinical response to etanercept was analyzed by calculating ACR Pedi 50 response at 12 months. Patients with the genotype −308GG more frequently responded to etanercept treatment than patients who had the −308GA/AA genotype, but this observation did not reach significance ($P = 0.246$). In comparison to the patients with -308AA genotype, patients with the -308GG genotype achieved ACR Pedi 50 response significantly more frequent ($\chi^2 = 4.367$; $P = 0.037$). There was no statistically significant change of ACR Pedi 50 response in patients with TNF-RII676TT genotype in comparison to patients with 676TG/GG genotypes ($\chi^2 = 0.756$; $P = 0.385$) and 676GG genotype ($\chi^2 = 0.278$; $P = 0.598$; Table 2). Three out of 66 patients (4.5%) did not respond to therapy.

Table 2. ACR Pedi 50 response at month 12

-308GG	-308GA/AA	P value	676TT	676TG/GG	P value
18/19 (94.7%)	41/47 (87.2%)	NS	43/47 (91.5%)	16/19 (84.2%)	NS
-308GG	-308AA	P value	676TT	676GG	P value
18/19 (94.7%)	3/5 (60%)	0.037	43/47 (91.5%)	3/3(100%)	NS

Patients with the genotype −308GG showed a significant decrease of MMP-9 level after 1 year of treatment with etanercept compared to the value from before ($P = 0.036$; Fig. 1).

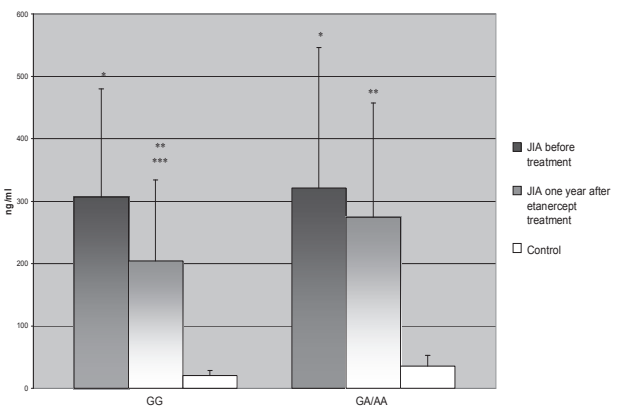

Fig. 1. MMP-9 level in children with polyarticular JIA and controls with TNF−308GG and GA/AA genotypes. *.**P<0.001 vs. control; ***P= 0.036 JIA 1 year after etanercept treatment vs. JIA before treatment

There was a decrease of MMP-9 levels after treatment, but not statistically significant in patients with the genotypes 676TT and 676TG/GG (Fig. 2).

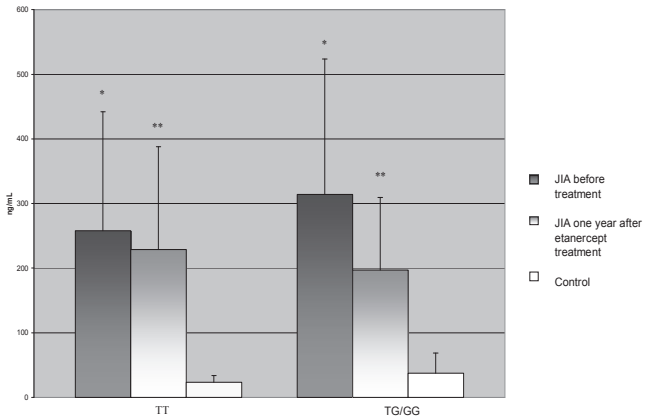

Fig. 2. MMP-9 level in children with polyarticular JIA and controls with TNFRII 676TT and TG/GG genotypes. *,**P<0.001 vs. control

Discussion and Conclusion

TNF blocking agents are among the most effective therapies for JIA, but not all patients have a good response. From a clinical point of view, there is an increasing need to predict responsiveness to identify the patients more proper for this therapy, to define timing of treatment and to avoid the complications due to the prolonged treatment in patients with a low-clinical response. Results of previous studies indicate TNF-α and TNFRII genes as candidate genes potentially involved in the modulation of clinical response to TNF blocking agents (15, 16). On this basis, we evaluated the potential association between selected SNPs in the TNF-α and TNFRII genes and clinical response to etanercept in JIA patients. The results of the present study showed that patients with the genotype −308GG more frequently reached a response to etanercept therapy than patients with the genotype −308AA, while compared to patients with the −308GA/AA genotypes, only a trend was observed. These results are consistent with findings of Schmeling and Horneff considering 101 JIA patients from the German registry (17). Also, Ozen et al. (18) showed that the TNF-α-308 polymorphism was associated with a poor prognosis in JIA in a Turkish group of patients, but not in a Czech group. On the other hand, Cimaz et al. (19) found no association between TNF-α-308G/A polymorphism and response to etanercept treatment. Our results might be due to the high level of TNF-α production associated with −308A allele carrier state (9, 20). Indeed, some studies considering RA patients have suggested that this SNP associated with high production of TNF is also associated with worse outcome of TNF suppression (21). On

the other hand, this polymorphism may be in linkage disequilibrium with other genes. Thus, this polymorphism may be one of the many genetic factors affecting response to etanercept. Moreover, there is also less agreement as to whether the 676T/G (M196R) polymorphism in the TNFRSF1b gene is related to the anti-TNFα response in rheumatism. Results of this study showed no statistically significant changes of ACR Pedi 50 response in patients with TNF-RII676TT genotype in comparison to patients with 676TG/GG genotypes and 676GG genotype. Also, there were no differences from the genotype frequency distributions of the TNFRII676T/G polymorphism as well as 676G allele frequency in JIA patients compared to healthy subjects, supporting the findings of Zeggini et al. (22), the only study on this SNP in JIA patients until now. According to our knowledge, there were no studies in JIA patients analyzing the influence of this SNP on response to etanercept and other TNF blocking agents. Thus, a comparison of our data is only possible with few studies in adults. In particular, two studies on a relatively small number of RA patients (66 and 105 Italian Caucasians respectively) found a lower degree of response to anti-TNF-α treatments (etanercept and infliximab) in patients carrying the G allele (23, 24), but this association has not been confirmed in a more recent paper on 234 Duch RA subjects treated with infliximab and adalimumab (25). A number of factors might explain these discrepancies, such as differences in ethnicity, differences in pharmacocinetics between monoclonal antibodies (infliximab and adalimumab) and the soluble receptor (etanercept), as well as the small sample size, and finally different response to therapy in adults and patients with JIA. As in previous studies only 19 and 55 adult RA patients respectively were treated with etanercept, further research studying TNFRII676 SNP in JIA patients is necessary to investigate it's potential role in response to this TNF inhibitor. Thus, it is unlikely that one independent polymorphism is responsible for anti-TNF response. Chatzikyriakidou et al. (26) suggested that a combined study of polymorphisms TNF-RII676T/G and TNF-α-857C/T could predict anti-TNF response in patients with RA.

As we previously described, considering clinical improvement, MMP-9 level showed significant decrease in responders to etanercept treatment, while in patients with low response and no response, the decrease of MMP-9 level was not statistically significant (14). With enlarged number of patients in comparison to our previous study, the same trend was observed. Etanercept significantly reduces the level of MMP-9 in patients with −308GG genotype. To our knowledge, this is the first study to investigate the influence of TNFRII676T/G SNP on MMP-9 level either before or after etanercept treatment in JIA patients. We found a decrease of MMP-9 levels after etanercept treatment in carriers of 676TT and 676TG/GG genotypes, but the decrease did not reach significance. Binding of activated TNF-α to TNFRI and TNFRII can result in the activation of signaling events, with consecutive activation of transcription factors, which then induce numerous genes, such as MMP. TNF-α-mediated induction of MMP-9 gene expression is well described and could be responsible for the overexpression and activity of this molecule as found in the synovial cells (27, 28). Lim et al. (29) showed that TNF-α signaling, specifically through TNFRII, is required for MMP-9 expression in mouse macrophages. Lee et al. (30) suggest that MMP-9 expression by LPS was reduced significantly in mouse macrophages treated with neutralizing anti-TNF-α or anti-TNFR1/2 antibody. Also, it is found that treatment of cholangiocarcinoma cell line, CCKS1 with TNF-α substantially activated NFκB, MAPK and Akt signalings which in turn activated MMP-9 secretion and in vitro invasiveness of CCKS1,

while pretreatment of cells with anti-TNFRII neutralizing antibody inhibited the TNF-α-dependent signaling and MMP-9 secretion and subsequently blocked invasion in vitro [31].
Our findings could provide some support to previous studies which describe an association of TNF with MMP-9 production, as all patients either before or after treatment had higher levels of MMP-9 in all genotypes of TNFRII676T/G SNP in comparison to control. On the other hand, neither allelic nor genotypic frequencies exhibited significant differences when JIA patients were compared to controls. Also, no defferencies in clinical response were seen among JIA patients carriers of different genotypes. Therefore, it might be possible that this SNP does not affect TNFRII solubilization and clinical response as well as MMP-9 production in JIA patients. According to a relatively small sample size of this study, it would be interesting to investigate this hypothesis in the near future.
In conclusion, the present study demonstrated the decrease of MMP-9 level in children with polyarticular JIA and TNF-α-308GG genotype after etanercept treatment. Patients with the genotype −308GG more frequently reached a response to etanercept than patients with the genotype −308AA. On the other hand, TNFRII676T/G polymorphism does not appear to be associated with clinical response to etanercept. However, further studies are needed to confirm the specific role of these polymorphisms and MMP-9 as potential genetic or biochemical predictors of the responsiveness to anti-TNF drugs in JIA.

Acknowledgements

The author would like to thank Prof. dr Dusica Pavlovic for the mentorship in PhD thesis and to thank coauthors of the published paper which was used in this presentation.
This work was supported by the Ministry of Education and Science of the Republic of Serbia (project no. III41018).

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