Advances in Experimental Medicine and Biology 756

Mieczyslaw Pokorski *Editor*

Respiratory Regulation - The Molecular Approach

Respiratory Regulation - The Molecular Approach

Advances in Experimental Medicine and Biology

Editorial Board:
IRUN R. COHEN, The Weizmann Institute of Science, Rehovot, Israel
ABEL LAJTHA, N.S. Kline Institute for Psychiatric Research, Orangeburg, NY, USA
JOHN D. LAMBRIS, University of Pennsylvania, Philadelphia, PA, USA
RODOLFO PAOLETTI, University of Milan, Milan, Italy

For further volumes:
http://www.springer.com/series/5584

Mieczyslaw Pokorski
Editor

Respiratory Regulation - The Molecular Approach

Editor
Mieczyslaw Pokorski
Department of Respiratory Research
Polish Academy of Sciences
Medical Research Center
Warsaw, Poland

ISSN 0065-2598
ISBN 978-94-007-4548-3 ISBN 978-94-007-4549-0 (eBook)
DOI 10.1007/978-94-007-4549-0
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2012943591

© Springer Science+Business Media Dordrecht 2013
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed. Exempted from this legal reservation are brief excerpts in connection with reviews or scholarly analysis or material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work. Duplication of this publication or parts thereof is permitted only under the provisions of the Copyright Law of the Publisher's location, in its current version, and permission for use must always be obtained from Springer. Permissions for use may be obtained through RightsLink at the Copyright Clearance Center. Violations are liable to prosecution under the respective Copyright Law.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
While the advice and information in this book are believed to be true and accurate at the date of publication, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The book contains the chapters related to the molecular issues of respiratory regulation. The research presented herein was communicated and discussed at the International Conference 'Advances in Pneumology' which was held in Bonn, Germany, on June 17–18, 2011. The chapters are a selection of peer-reviewed manuscripts, mostly original research work, to demonstrate the topics discussed at the conference. Experimental pathophysiology research often seems not very interesting or even useful from the clinical standpoint. This basic research, however, meticulously builds up on previous studies and lays the foundation for clinical advances. The conference is thought as a merge between basic and clinical research concerning respiratory medicine, neural and chemical respiratory regulation, and the mutual relationship between respiration and other neurobiological functions. The essential topics of interest include a wide range of issues, most notably molecular aspects related to gene polymorphism underlying various diseases, cardiovascular-respiratory disorders, infections and inflammatory conditions exemplified by asthma and chronic obstructive pulmonary disease (COPD), respiratory allergy and cough, and immunology. In the chapters presented in this volume the cutting-edge knowledge is communicated and discussed by prominent experts in the areas of science outlined above. I want to thank the invited speakers and other presenters at the conference, as well as the authors and reviewers of the chapters; their contributions certainly will enhance the value of this volume. The clinical advances cannot be reached without the continual efforts to understand the underlying mechanisms of diseases.

The 2011 conference was the fruit of many collaborative efforts. The Local Organizing Committee was headed by Dr. Rüdiger Siekmeier of the Federal Institute for Drugs and Medical Devices (BfArM) in Bonn, Germany. I am indebted to him for his efforts and to all those who extended a helping hand and advice in the organization, in particular Prof. Dr. med. Kurt Rasche of HELIOS Klinikum Wuppertal Lungenzentrum, Klinik für Pneumologie, Allergologie, Schlaf- und Beatmungsmedizin and Anke Hastenrath of Wuppertal, and Dr. Tadeusz M. Zielonka of Warsaw Medical University and the Polish Respiratory Society in Warsaw, Poland.

I also want to thank the non-profit research and academic institution which kindly cooperated and supported the organization of the conference and the publication of this book; in particular the Medical Research Center of the Polish Academy of Sciences in Warsaw, the Polish Respiratory Society, and the Rhein-Ruhr-Stiftung in Essen. Finally, I am also grateful to Max Haring, Ph.D. and Tanja van Gaans of Springer for their expert management of the production process of this book.

Warsaw, Poland Mieczyslaw Pokorski

Contents

1 **Neutrophil Extracellular Trap in Human Diseases** 1
Magdalena Arazna, Michal P. Pruchniak, Katarzyna Zycinska, and Urszula Demkow

2 **Tiotropium Increases PPARγ and Decreases CREB in Cells Isolated from Induced Sputum of COPD Patients** 9
A. Holownia, R.M. Mroz, T. Skopinski, A. Kolodziejczyk, E. Chyczewska, and J.J. Braszko

3 **Expression of Ki-67, Bcl-2, Survivin and p53 Proteins in Patients with Pulmonary Carcinoma** 15
E. Halasova, M. Adamkov, T. Matakova, D. Vybohova, M. Antosova, M. Janickova, A. Singliar, D. Dobrota, and V. Jakusova

4 **Indacaterol Add-On Therapy Improves Lung Function, Exercise Capacity and Life Quality of COPD Patients** 23
R.M. Mroz, L. Minarowski, and E. Chyczewska

5 **Rapid DNA Extraction Protocol for Detection of Alpha-1 Antitrypsin Deficiency from Dried Blood Spots by Real-Time PCR** 29
R. Struniawski, A. Szpechcinski, B. Poplawska, M. Skronski, and J. Chorostowska-Wynimko

6 **CRAC Ion Channels and Airway Defense Reflexes in Experimental Allergic Inflammation** 39
M. Sutovska, M. Adamkov, M. Kocmalova, L. Mesarosova, M. Oravec, and S. Franova

7 **Experimental Model of Allergic Asthma** 49
S. Franova, M. Joskova, V. Sadlonova, D. Pavelcikova, L. Mesarosova, E. Novakova, and M. Sutovska

8 **Effects of Selective Inhibition of PDE4 and PDE7 on Airway Reactivity and Cough in Healthy and Ovalbumin-Sensitized Guinea Pigs** 57
Juraj Mokry, Marta Joskova, Daniela Mokra, Ingrid Christensen, and Gabriela Nosalova

9 **Naloxone Blocks Suppression of Cough by Codeine in Anesthetized Rabbits** 65
M. Simera, M. Veternik, and I. Poliacek

10 Influence of Sublingual Immunotherapy on the Expression of Mac-1 Integrin in Neutrophils from Asthmatic Children 73
Olga Ciepiela, Anna Zawadzka-Krajewska, Iwona Kotula, Beata Pyrzak, and Urszula Demkow

11 L-Arginine Supplementation and Experimental Airway Hyperreactivity 81
M. Antosova and A. Strapkova

12 Polyphenols and Their Components in Experimental Allergic Asthma 91
M. Joskova, V. Sadlonova, G. Nosalova, E. Novakova, and S. Franova

13 Green Tea and Its Major Polyphenol EGCG Increase the Activity of Oral Peroxidases 99
Baruch Narotzki, Yishai Levy, Dror Aizenbud, and Abraham Z. Reznick

14 Mangiferin and Its Traversal into the Brain 105
Dominika Zajac, Agnieszka Stasinska, Rene Delgado, and Mieczyslaw Pokorski

15 Plasma Selectins in Patients with Obstructive Sleep Apnea 113
S. Cofta, E. Wysocka, S. Dziegielewska-Gesiak, S. Michalak, T. Piorunek, H. Batura-Gabryel, and L. Torlinski

16 Blood Antioxidant Status, Dysglycemia and Obstructive Sleep Apnea 121
Ewa Wysocka, Szczepan Cofta, Tomasz Piorunek, Sylwia Dziegielewska-Gesiak, Wieslaw Bryl, and Lech Torlinski

17 Anti-natrium/Iodide Symporter Antibodies and Other Anti-thyroid Antibodies in Children with Turner's Syndrome 131
Anna M. Kucharska, Barbara Czarnocka, and Urszula Demkow

18 Influence of Sera from Interstitial Lung Disease Patients on Angiogenic Activity of Mononuclear Cells 139
T.M. Zielonka, K. Zycinska, E. Radzikowska, M. Filewska, B. Bialas, M.H. Obrowski, E. Skopinska-Rozewska, and U. Demkow

19 Antiendothelial Cells Antibodies in Patients with Systemic Sclerosis in Relation to Pulmonary Hypertension and Lung Fibrosis 147
K. Lewandowska, M. Ciurzynski, E. Gorska, P. Bienias, K. Irzyk, M. Siwicka, K. Zycinska, P. Pruszczyk, and U. Demkow

20 Leptin Receptor in Childhood Acute Leukemias 155
E. Gorska, K. Popko, and M. Wasik

21 Expression of Cytotoxic T Lymphocyte Antigen-4 in T Cells from Children with Hashimoto's Thyroiditis 163
Anna M. Kucharska, Elzbieta Gorska, Maria Wasik, and Urszula Demkow

22 Exercise in Cold Air and Hydrogen Peroxide Release in Exhaled Breath Condensate 169
E. Marek, J. Volke, K. Mückenhoff, P. Platen, and W. Marek

23 Non-invasive Assessment of Exhaled Breath Pattern in Patients with Multiple Chemical Sensibility Disorder 179
Andrea Mazzatenta, Mieczyslaw Pokorski, Sergio Cozzutto, Pierluigi Barbieri, Vittore Veratti, and Camillo Di Giulio

24 Anti-inflammatory Treatment in Dysfunction of Pulmonary Surfactant in Meconium-Induced Acute Lung Injury ... 189
D. Mokra, A. Drgova, J. Kopincova, R. Pullmann, and A. Calkovska

25 Microcirculation in the Lungs: Special Features of Construction and Dynamics ... 197
K.P. Ivanov, I.L. Potekhina, Yu.S. Alyukhin, and N.N. Melnikova

26 Immunotargeting of the Pulmonary Endothelium *via* Angiotensin-Converting-Enzyme in Isolated Ventilated and Perfused Human Lung ... 203
Kai Nowak, Hans C. Kölbel, Roman P. Metzger, Christine Hanusch, Marc Frohnmeyer, Peter Hohenberger, and Siergiej M. Danilov

27 Angiogenic Activity of Sera from Interstitial Lung Disease Patients in Relation to Angiotensin-Converting Enzyme Activity ... 213
T.M. Zielonka, K. Zycinska, J. Chorostowska-Wynimko, M. Filewska, B. Bialas, M.H. Obrowski, E. Radzikowska, E. Skopinska-Rozewska, and U. Demkow

28 Development and Aging Are Oxygen-Dependent and Correlate with VEGF and NOS along Life Span ... 223
S. Zara, M. Pokorski, A. Cataldi, A. Porzionato, R. De Caro, J. Antosiewicz, and C. Di Giulio

29 Sarcoidosis and Tuberculosis: A Connection to the Human Leukocyte Antigen System ... 229
A. Dubaniewicz, A. Zimmermann, M. Smigielska, M. Dubaniewicz-Wybieralska, G. Moszkowska, J. Wysocka, K. Adamczyk-Bak, J.M. Slominski, and P. Deeg

30 Toll-Like Receptor-9 Polymorphisms in Sarcoidosis and Chronic Obstructive Pulmonary Disease ... 239
Stefan Pabst, Oxana Bradler, Adrian Gillissen, Georg Nickenig, Dirk Skowasch, and Christian Grohe

31 Association of Adiponectin Gene G276T Polymorphism with Atherogenic Indicators in Obese Children ... 247
Beata Pyrzak, Malgorzata Ruminska, Aneta Czerwonogrodzka-Senczyna, Anna Majcher, Alicja Wisniewska, Michal Brzewski, and Urszula Demkow

32 Relation of Fat-Mass and Obesity-Associated Gene Polymorphism to Fat Mass Content and Body Mass Index in Obese Children ... 255
Beata Pyrzak, Alicja Wisniewska, Anna Majcher, Andrzej Tysarowski, and Urszula Demkow

33 Rapid Test for Influenza in Diagnostics ... 263
Teresa Jackowska, Monika Grzelczyk-Wielgorska, and Katarzyna Pawlik

34 Infections with A(H1N1)2009 Influenza Virus in Poland During the Last Pandemic: Experience of the National Influenza Center ... 271
M. Romanowska, I. Stefanska, S. Donevski, and L.B. Brydak

35 Immune Response to Influenza Vaccine in Hemodialysis Patients with Chronic Renal Failure ... 285
Agnieszka Mastalerz-Migas, Andrzej Steciwko, and Lidia B. Brydak

36 Co-Infections with Influenza and Other Respiratory Viruses ... 291
I. Stefanska, M. Romanowska, S. Donevski, D. Gawryluk, and L.B. Brydak

37 Flow Cytometry in Detection of Abnormalities of Natural Killer Cell 303
K. Popko, I. Malinowska, E. Gorska, A. Stelmaszczyk-Emmel, and U. Demkow

38 sVEGF R1 and Tie-2 Levels During Chemotherapy of Lung Cancer Patients 313
R.M. Mroz, M. Korniluk, B. Panek, M. Ossolinska, and E. Chyczewska

39 Reliable Detection of Rare Mutations in EGFR Gene Codon L858 by PNA-LNA PCR Clamp in Non-small Cell Lung Cancer 321
Michal Skronski, Joanna Chorostowska-Wynimko, Ewa Szczepulska, Adam Szpechcinski, Piotr Rudzinski, Tadeusz Orlowski, and Renata Langfort

40 Neurological Paraneoplastic Syndromes in Lung Cancer Patients 333
P. Stefens-Stawna, T. Piorunek, H. Gabryel-Batura, W. Kozubski, and S. Michalak

41 Cardiovascular Side Effects of Aminophylline in Meconium-Induced Acute Lung Injury 341
D. Mokra, I. Tonhajzerova, J. Mokry, M. Petraskova, M. Hutko, and A. Calkovska

42 Proteomic Analysis of the Carotid Body: A Preliminary Study 349
C. Di Giulio, S. Angelucci, C. Di Ilio, E. Eleuterio, F. Di Giuseppe, M. Sulpizio, V. Verratti, M. Pecyna, and M. Pokorski

43 Effects of Body Positions on Respiratory Muscle Activation During Maximal Inspiratory Maneuvers 355
M.O. Segizbaeva, M.A. Pogodin, and N.P. Aleksandrova

Index 365

Chapter 1
Neutrophil Extracellular Trap in Human Diseases

Magdalena Arazna, Michal P. Pruchniak, Katarzyna Zycinska, and Urszula Demkow

Abstract NETosis is a unique death pathway that differs from apoptosis and necrosis and depends on the generation of reactive oxygen species (ROS) by NADPH oxidase. During this process, neutrophil extracellular traps (NETs) are created. NETs are extracellular structures composed of chromatin and variety of proteins from cells granules that bind and kill microorganisms. Recently, novel functions of NET have been proposed. It seems that neutrophil traps play an essential role during autoimmunity. They can induce and exacerbate diseases based on immune system malfunction.

Keywords NETosis • Neutrophil extracellular trap • Autoimmune disease • Immunity • Neoplasms

1.1 Introduction

Neutrophils are the most abundant group of cells specialized in defense against pathogens such as bacteria, fungi, and protozoa. They are one of the first responders among inflammatory cells during infections. They migrate towards blood stream to the site of inflammation in response to chemical agents in the process called chemotaxis. After migration to infection site, they act in synergy with several other cells amplifying inflammatory reactions. They synthesize and release specific cytokines which activate endothelial cells, macrophages, and mast cells. However, the main task of neutrophils is to attack microorganisms with the use of their unique repertoire of activities: phagocytosis, degranulation and generation of reactive oxygen species (ROS). It is very important that after elimination of inflammatory factor, neutrophils should be safely removed as it is necessary to keep cellular homeostasis under physiologic conditions. Apoptosis is a physiologic suicide mechanism contributing to cells removal from inflamed tissue. This effect inhibits releasing hazardous intracellular contents. Recently, a new microbicidal mechanism has been described. NETosis is a unique cell death distinct

M. Arazna (✉) • M.P. Pruchniak • U. Demkow
Department of Laboratory Diagnostic and Clinical Immunology of Developmental Age, Warsaw Medical University, 24 Marszalkowska St, 00-576 Warsaw, Poland
e-mail: magdaarazna@gmail.com

K. Zycinska
Department of Family Medicine, Internal and Metabolic Diseases, Warsaw Medical University, Systemic Vasculitis Outpatient Clinic, Czerniakowski Hospital, Warsaw, Poland

M. Pokorski (ed.), *Respiratory Regulation - The Molecular Approach*,
Advances in Experimental Medicine and Biology 756, DOI 10.1007/978-94-007-4549-0_1,
© Springer Science+Business Media Dordrecht 2013

from necrosis and apoptosis. This phenomenon was observed in human, bovine, and fish neutrophils (Brinkmann et al. 2004; Köckritz-Blickwede and Nizet 2009; Villanueva et al. 2011; Yousefi et al. 2009). In this mechanism, neutrophils die after releasing 'neutrophil extracellular traps' (NETs), a web-like structure. Mast cells, eosinophils, and plant cells also are able to form extracellular traps (Köckritz-Blickwede and Nizet 2009; Papayannopoulos at al. 2010). Probably, mast cells play more important role than neutrophils in the web formation, during allergic disease (Köckritz-Blickwede and Nizet 2009).

1.2 Characterization of Neutrophil Extracellular Traps

NETs are composed of granular and nuclear constituents of neutrophils (Wartha et al. 2007). Most of proteins and enzymes which act inside the web originate from primary, secondary and tertiary granules. The most common enzymes are elastase, cathepsin G, myeloperoxidase, proteinase-3, bactericidal permeability increasing protein, lactofferin, gelatinase (Wartha et al. 2007; Soehnlein 2009; Li et al. 2010). They cause death of bacteria or at least inhibit their growth. The nuclear components are chromatin fibers and histones (h1, h2a, h2b, h3, h4) (Wartha et al. 2007). The first one creates a backbone on which proteinaceous effectors can reside. No membrane fragments, membrane proteins, and cytoplasmic markers are present in this structure (Wartha et al. 2007). In 2004, Brinkmann et al. (2004) using electron microscopy observed and measured all structures for the first time. They noted that DNA stretches have a diameter of 15–17 nm and a globular protein creates specific domains of around 25 nm. Due to its size, they might aggregate to larger elements of diameters of up to 50 nm. DNase causes disintegration of these unique structures (Wartha et al. 2007). The major components of mast cell extracellular traps are DNA, histones, and mast cell-specific granule proteins such as tryptase. It is important that DNase alone is not sufficient to dismantle NET. In addition, myeloperoxidase can cause degradation of tryptase (Köckritz-Blickwede and Nizet 2009). The origin of DNA in neutrophils and mast cell is nuclear. After releasing the chromatin neutrophils die. Eosinophils form this structure from mitochondrial DNA, although they survive after catapulting their genetic material. Moreover, they react faster, as they need only a few seconds to form a web (Köckritz-Blickwede and Nizet 2009).

The release of NETs can be stimulated by bacteria, fungi, protozoa, and some mediators (e.g., interleukin-8, lipopolysaccharide (LPS), phorbol esters (PMA), and hydrogen peroxide). Also, complement factor (Li et al. 2010) in synergy with GM-CSF or interferon γ/α (INF- γ/α) may play a crucial role in this process. Direct exposure to a variety of different microbial pathogens, both Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Mycobacterium tuberculosis, Escherichia coli*, or *Leishmania amazonesis* is sufficient to trigger NET formation (Brinkmann et al. 2004; Köckritz-Blickwede and Nizet 2009; Ramos-Kichik et al. 2009; Urban et al. 2006). Also, fungi like *Candida albicans* stimulate neutrophils in the same manner. Depending on the target, neutrophils extracellular traps act in two ways. They can simply immobilize microorganisms or kill them after capturing. These processes can be blocked pharmacologically by cytochalasin D or by DNase enzyme. Only mature and non-defective granulocytes may express the functional machinery required for the transduction of indispensable signals for NET generation. Depending on the stimulator, the time frame to form NETs varies from 10 min to 4 h. After bacterial stimulation or activator such as phorbol esters, NETs are released within 2–4 h, but neutrophils activated by platelet cells stimulated with LPS, react faster (Köckritz-Blickwede and Nizet 2009).

The role of ROS in innate immunity was recognized for the first time in phagocytes undergoing a 'respiratory burst' upon activation. This process is connected with the superoxide-generating enzyme NADPH oxidase (Nox2). This enzyme is essential for microbial killing and till now, lack of its function was only related to enhanced susceptibility to microbial infections (Rada and Leto 2008).

New evidence strongly indicates that creation of NETs is associated with ROS. It has been noted that hereditary dysfunction of NADPH oxidase prevents neutrophils from creating extracellular traps. Also, a pharmacological influence on ROS enzymes blocks NETosis. As above mentioned, NET formation requires reduced NADPH oxidase activity. Marcos et al. (2010) postulated that chemokine receptors (CXCR) can be related during NETosis. Their studies have shown that promotion of CXCR1, but not CXCR2, signaling pathways provokes the neutrophil trap formation. Whereas CXCR1 stimulate ROS production, CXCR2 have no effect on NADPH oxidase activity and generation of ROS, so it strongly indicates a tight relationship between ROS and NETosis. However, how do ROS promote NET formation remains still unclear (Villanueva et al. 2011; Marcos et al. 2010).

NETs are an important component of immune defense, especially in severe infections. This mechanism is implicated in the pathogenesis of sepsis and autoimmunity (Papayannopoulos at al. 2010). Research describes NETs in the course of appendicitis, pre-eclampsia, systemic lupus erythematosus (SLE), chronic granulomatous disease, small vessels vasculitis, or intestinal spirochetosis (Wartha et al. 2007; Köckritz-Blickwede and Nizet 2009). There is increasing evidence that NETs play an important role in noninfectious diseases. For example recent studies show a commitment of these structures during insemination (Wartha et al. 2007). Sperm cells are caught and clogged into web in female reproductive system, which prevents conception. Recently, more bacterial virulence factors and their defense systems have been identified. Pathogens can resist NET-mediated killing by adding positive charge to their cell surface (Wartha et al. 2007). They can produce a special outer capsule, which reduces bacterial trapping and generate DNases or variety of other enzymes which are able to digest NET structures (Wartha et al. 2007; Köckritz-Blickwede and Nizet 2009). For example, *Streptococcus pneumoniae* causes NET degradation by DNase EndA. Moreover, these bacteria produce capsules rich in D-analynated lipoteichoic acids (LTA) which modify the surface charge; thus this structure prevents entrapment by extracellular traps (Köckritz-Blickwede and Nizet 2009).

1.3 NETs in Neoplasm of Developmental Age

Neoplasms of developmental age account for 0.5–2.0% all cancers causes in human population. Clinical manifestation, progression rate and genetic predisposition are different in children compared with adults. Leukemia, due to maturation arrest and uncontrolled proliferation of cells in bone marrow, is the most common pediatric neoplastic disease. Acute lymphocytic leukemia (ALL) approaches 80% of leukemia cases in children (Bleyer 1990). ALL leads to the accumulation of malignant lymphoblasts in bone marrow and peripheral blood. Acute myeloid leukemia (AML) and acute non-lymphoblastic leukemia (ANLL) occur rarely.

The diagnosis of leukemia relies on immunocytochemistry, immunophenotyping, cytogenetics, and gene rearrangement detection. Nowadays, there are many innovative, specific molecular and cellular techniques to confirm the diagnosis. The improved sensitivity of applied tests has made possible to establish an early presence pathological cells in bone marrow and peripheral blood.

Leukemia is the most common cause of disease-related death in childhood. The patients with acute leukemia are at high risk for infections complications (Hirotsu and Akatsuka 1991; Maranda et al. 2010). Serious, life threatening infections remain a major cause of morbidity and mortality in this group of patients. In 1970–1990 large scale clinical trials regarding therapy in ALL reported that 90% of patients die within 4 years. In the 1990s, more than 70% of all children with ALL, who were younger than 10 years at the time of diagnosis, reached a complete and durable remission due to modern therapy. Nowadays, in the group of children with acute lymphocytic leukemia, the mortality rate from infection during chemotherapy is around 1–3% (Hirotsu and Akatsuka 1991). Neutropenia, the use of corticosteroids, broad spectrum antibiotics, central venous lines, presence of mucositis, and surgery procedures are among common underlying causes of infections in leukemic

patients (Hirotsu and Akatsuka 1991; Klaassen et al. 2000). Furthermore, other factors such as impaired functional capabilities of neutrophils may contribute to severe infections before and after chemotherapy. Several studies have shown the presence of defective neutrophil function (i.e., ROS formation) in patients with acute leukemia (Hubel et al. 1999). However most of them were performed in adult population and only a few in children, who usually undergo more intensive chemotherapy compared with adults.

Multidrug chemotherapy combinations are standard regiments in the treatment of leukemia in children significantly improving survival of patients. Chemotherapeutic agents act by the multiple mechanism, including incorporation into DNA and inhibition of DNA replication, cell membrane damage or free radical generation (Bleyer 1990). Invasive infections are the common complications of chemotherapy in leukemic children. To-date, no study has been undertaken to explore if NET formation is defective in patients with acute leukemias and how this mechanism is altered by chemotherapy.

NET formation is initiated by the production of ROS. ROS constitute an essential signal leading to the induction of unique cell death program connected with NETs (Köckritz-Blickwede and Nizet 2009; Yousefi et al. 2009). This is a physiologic suicide mechanism for elimination of neutrophils from inflamed tissue and it has been named NETosis. Oxidative burst may be hampered by chemotherapeutic agents, bacterial products and inflammatory mediators released by leukemic blasts and tumor infiltrating immune cells. Interference with ROS generation by chemotherapeutic agents may block the formation of the web. Moreover, NET formation requires fully mature neutrophils, thus immature or defective neutrophils released from leukemic bone marrow may not express the functional machinery required for the transduction of external signals (Martinelli et al. 2004). The negatively charged DNA backbone of NETs also provides a framework for the activation of the contact system, including serum-derived serine proteases, factor XI, factor XII, and plasma kallikrein, together with the non-enzymatic high-molecular-weight-cofactor and kininogen (Oehmcke et al. 2009). NETs also produced after platelet-mediated neutrophil activation and the accumulation of NETs and their component proteases may promote vascular endothelial injury and ischemia (Clark et al. 2007; Oehmcke et al. 2009). These events may contribute to cardiovascular complication including thrombosis in leukemic patients.

The immune system is a complex and decentralized machinery present in multicellular organisms and is composed of many types of proteins, cells, organs, and tissues. Its main goal is to provide a generic and immediate defense against pathogens like bacteria, fungi, and protozoa. In addition, it can identify and destroy tumor or virus carrying cells. Due to its complex nature every disorder can provoke different reaction: autoimmune diseases, immunodeficiency, cancer, or inflammatory diseases (Beck and Gail 2007).

1.4 NETs in Autoimmunity

Autoimmunity is defined as a failure in self-recognition of the body. This kind of failure of immune regulation is responsible for autoimmune diseases. During immune system malfunction, a variety of antibodies attack host structures. Not only do these antibodies influence in a direct manner various body parts, but they also can recruit cytotoxic T cells which multiply devastating effect. Recently, it has been postulated that NETs can be related to autoimmune diseases either through the initiation or propagation of the disease (Papayannopoulos and Zychlinsky 2009). In this paper we will focus on two immune-related syndromes: ANCA-associated vasculitis and systemic lupus erythematosus.

Anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) is a life threatening autoimmune disease characterized by necrotizing vasculitis of small and medium sized vessels. Pathogenesis of AAV is complex, with a number of overlapping effector limbs. ANCAs are of major importance for disease and cause vasculitis interacting with neutrophils upon specific triggers

(Ozaki 2007; Jennette and Falk 1998; van Rossum et al. 2005; Wilde et al. 2010). ANCAs with specificity for either proteinase-3 (PR3-ANCA) in Wegener's granulomatosis or myeloperoxidase (MPO-ANCA) in Churg-Strauss syndrome are a hallmark of AAV and had a pivotal role in disease development (Gómez-Puerta et al. 2009; Wilde et al. 2010; van Rossum et al. 2005). ANCAs themselves are thought to be pathogenic (Ozaki 2007). Furthermore, ANCAs promote deregulation of neutrophils and monocytes facilitating endothelial damage. The endothelium is also activated and neutrophil adherence is enhanced. The initial damage leads to a cascade of reactions resulting in leucocyte tissue infiltration (Gómez-Puerta et al. 2009). T-cell driven granuloma formation and further damage. ANCAs bind to neutrophils and endothelial cells having differential but synergistic effects on both cell types (Wilde et al. 2010). ANCAs bind to membrane-bound PR3/MPO on neutrophils. This interaction with ANCAs results in activation and finally in release of cytotoxic superoxide and serine proteases (such as PR3). Membrane-bound MPO-PR3 is expressed constitutively by neutrophils and can be enhanced by proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon-γ (INF-γ). Priming of neutrophils also enhances adhesion to endothelial cells along with a further increase of membrane MPO/PR3 expression (van Rossum et al. 2005; Rarok et al. 2003). Thus, deregulation occurs in close contact with the vascular endothelium, resulting in vasculitic damage. The interaction of ANCAs with endothelial cells enhances expression of adhesion molecules like E/P- selectin and vascular cell adhesion molecule (Ara et al. 2001). In the late 1980s, it was discovered that PR3 was the main antigen for cytoplasmic-ANCA, whereas MPO was shown to be the antigenic target of perinuclear-ANCA in patients with vasculitis. Recent findings bring up a new hypothesis on the induction of ANCAs by immune responses against Gram-positive or Gram-negative bacteria. Pathogens like *Staphylococcus aureus* bear genetic sequences that are complementary to the human PR3 gene pointing to an exogenous origin of cPR3 transcripts. Chronic nasal carriage of *S. aureus* has been demonstrated to increase the risk for disease relapse. T cells are also usually found within granulomas in lesions present in AAV (Stegeman et al. 1994; Tadema et al. 2010). Elevated levels of markers of T-cell activity, such as soluble interleukin-2 (IL-2) receptor, neopterin and soluble CD30 as measured in the circulation have been shown to be associated with disease activity (Wilde et al. 2010; Schmidt et al. 1992).

As above mentioned, the anti-neutrophil cytoplasmic autoantibodies formed during small-vessel vasculitis have major effects upon neutrophils; thus they can be potent NETosis inductors. Two types of ANCA antibodies are abundant in AVV. c-ANCA are specific for the serine proteinase-3. pANCA called MPO-ANCA type has specificity for myeloperoxidase. The interaction of p-ANCA and c-ANCA with neutrophils surface results in the production of ROS (Papayannopoulos and Zychlinsky 2009; Kallenberg 2010). Due to the fact that production of ROS plays an essential role in the neutrophil traps-creation pathway, it is believed that ANCA are strongly associated with continuous NETs formation. Moreover, microbial factors, in particular *Staphylococcus aureus* and Gram-negative bacteria, simultaneously with anti-neutrophil cytoplasmic autoantibodies seem to be involved in AVV induction (Kallenberg 2010). On the other hand, it has been shown that NET can occur in an autoinflammatory structure in the absence of microbial infection. Unfortunately, the basic mechanism that includes exacerbation of AVV and neutrophil extracellular traps is still not known (Kessenbrock et al. 2009).

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune connective tissue disease characterized by various manifestations often demonstrating a waxing-waning course. It is a Type III hypersensitivity reaction caused by antibody-immune complex formation (Ballestar et al. 2006; Rahman and Isenberg 2008). SLE does run in families, but no single casual gene has been identified. Genes which contain risk variants for SLE are IRF5, PTPN22 (Orozco et al. 2011; Rahman and Isenberg 2008). The second mechanism may be due to environmental factors such as viruses and bacteria (Sebastiani and Galeazzi 2009). Many patients with SLE exhibit symptoms that involve skin and joints, other symptoms of SLE vary widely between patients. SLE most often harms the heart, lungs, liver, kidneys and nervous system. The course of the disease is unpredictable, with periods of

illness (flares) alternating with remissions (Rahman and Isenberg 2008). The disease occurs nine times more often in women than in men, especially in women in child-bearing years ages 15–35 and is also more common in those of non-European descent (Krishnan and Hubert 2006; Tandon et al. 2004). Serologic markers play an important role in the assessment of disease activity in SLE, including assessment of anti-double-stranded DNA and complement levels. Serological markers are critical for the understanding of the pathogenesis of the disease. Serologic markers have an imperfect correlation with disease activity and cannot substitute alone for a direct assessment of clinical benefit (Rahman and Isenberg 2008). Antinuclear antibody (ANA) testing and anti-extractable nuclear antigen (ant-ENA) form the mainstay of serologic testing for SLE (Agarwal et al. 2009). Clinically, the most widely used method is indirect immunofluorescence. The pattern of fluorescence suggests the type of antibody present in the patient's serum. ANA screening yields positive results in many connective tissue disorders and may occur in normal individuals. Subtypes of antinuclear antibodies include anti-Smith (Sm) and anti-double stranded DNA (dsDNA) antibodies (which are linked to SLE) and anti-histone antibodies (which are linked to drug-induced lupus). Anti-dsDNA antibodies are highly specific for SLE. They are present in 70% of cases, whereas they appear in only 0.5% of people without SLE. Other ANAs that may occur in SLE sufferers are anti-U1 RNP (also in systemic sclerosis), SS-A (or anti-Ro), and SS-B (or anti-La also in Sjögren's syndrome). The lupus erythematosus (LE) cell test was previously commonly employed for the diagnosis, but it is no longer in use because the LE cells are only found in 50–77% of SLE cases, and they are also found in other syndromes (Rahman and Isenberg 2008; Buyon and Clancy 2003).

Recent evidence indicates that neutrophils traps may play an important role in the induction of autoimmune responses and organ damage and in the pathogenesis of systemic lupus erythematosus (Villanueva et al. 2011). As above mentioned, the backbone of the neutrophil extracellular trap consists of double stranded DNA which is a prime target for auto-antibodies present in SLE. The possible mechanism includes the formation of NET-antibody complexes. The accumulation of these structures can provoke vascular endothelium damage and can lead to thrombosis or heart-failure (Oehmcke et al. 2009). Moreover, blockade of NETs degradation has been observed in some SLE cases. Specific antibodies bind to NET structures, where they can be recognized by DNAse enzymes. Preventing DNase1 access to NETs stops *in situ* clearance. A prolonged life time of neutrophil traps is devastating for the SLE patient because dangerous complexes are being created in enormous amounts. Villanueva et al. (2011) postulate that a distinct neutrophil fraction, called low density granulocytes (LDGs), is strongly involved in NETs formation. LDGs are found in peripheral blood of adult SLE patients. These specific cells are not characterized by any specific surface markers. They can differ from normal neutrophils only by their density, although LDGs nuclear morphology suggests that this population of cells is slightly immature. The mechanisms by which LDGs are more potent to make NETs are still unclear, but a crucial role for elastase, reactive oxygen species, and even the cyto-skeleton has been proposed (Villanueva et al. 2011; Denny et al. 2010). On the other hand, it is certain that immature neutrophils cannot create NESs due to their lack of specific receptors. Even though low density granulocytes are considered to have immature phenotype, they still have ability to create extracellular traps. This phenomenon can be contributed to a high level of expression of mRNA that encodes the neutrophil serine proteases; the process which is strongly associated with NETosis (Villanueva et al. 2011).

Since we have learned about neutrophil extracellular traps just about several years ago, we still have gaps in the basic knowledge. During recent years, we have discovered that these DNA-traps are not only related to one type of cells, but at least to three types of granulocytes. We know what basic agents induce NETosis, thus we can procure its creation *in vitro*. These unique structures seem destined to surprise us many more times.

Conflicts of interest: The authors declare no conflicts of interest in relation to this article.

References

Agarwal, S., Harper, J., & Kiely, P. D. (2009). Concentration of antibodies to extractable nuclear antigens and disease activity in systemic lupus erythematosus. *Lupus, 18*, 407–412.

Ara, J., Mirapeix, E., Arrizabalaga, P., Rodriguez, R., Ascaso, C., Abellana, R., Font, J., & Darnell, A. (2001). Circulating soluble adhesion molecules in ANCA-associated vasculitis. *Nephrology, Dialysis, Transplantation, 16*, 276–285.

Ballestar, E., Esteller, M., & Richardson, B. C. (2006). The epigenetic face of systemic lupus erythematosus. *Journal of Immunology, 176*, 7143–7147.

Beck, G., & Gail, S. H. (2007). Immunity and the invertebrates. *Scientific American, 275*, 60–66.

Bleyer, W. A. (1990). Acute lymphoblastic leukemia in children. Advances and prospectus. *Cancer, 65*, 689–695.

Brinkmann, V., Reichard, U., Goosmann, Ch, Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., & Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science, 303*, 1532–1535.

Buyon, J. P., & Clancy, R. M. (2003). Maternal autoantibodies and congenital heart block: Mediators, markers, and therapeutic approach. *Seminars in Arthritis and Rheumatism, 33*, 140–154.

Clark, S. R., Ma, A. C., Tavener, S. A., McDonald, B., Goodarzi, Z., Kelly, M. M., Patel, K. D., Chakrabarti, S., McAvoy, E., Sinclair, G. D., Keys, E. M., Allen-Vercoe, E., DeVinney, R., Doig, Ch. J., Green, F. H. Y., & Kubes, P. (2007). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nature Medicine, 13*, 463–469.

Denny, M. F., Yalavarthi, S., Zhao, W., Thacker, S. G., Anderson, M., Sandy, A. R., McCune, W. J., & Kaplan, M. J. (2010). A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *Journal of Immunology, 184*, 3284–3297.

Gomez-Puerta, J. A., Hernandez-Rodríguez, J., Lopez-Soto, A., & Bosch, X. (2009). Respiratory disease antibody-associated vasculitides and antineutrophil cytoplasmic. *Chest, 136*, 1101–1111.

Hirotsu, T., & Akatsuka, J. (1991). Infectious complications in childhood leukemia. *Acta Paediatrica Japanese, 33*, 564–572.

Hubel, K., Hegener, K., Schnell, R., Mansmann, G., Oberhause, F., Staib, P., Diehl, V., & Engert, A. (1999). Suppressed neutrophil function as a risk factor for severe infection after cytotoxic chemotherapy in patient with acute nonlymphocytic leukemia. *Annals of Hematology, 78*, 73–77.

Jennette, J. C., & Falk, R. J. (1998). Pathogenesis of the vascular and glomerular damage in ANCA-positive vasculitis. *Nephrology, Dialysis, Transplantation, 13*, 16–20.

Kallenberg, C. G. (2010). Pathophysiology of ANCA-associated small vessel vasculitis. *Current Rheumatology Reports, 12*, 399–405.

Kessenbrock, K., Krumbholz, M., Schonermarck, U., Back, W., Gross, W. L., Werb, Z., Grone, H. J., Brinkmann, V., & Jenne, D. E. (2009). Netting neutrophils in autoimmune small-vessel vasculitis. *Nature Medicine, 15*, 623–625.

Klaassen, R. J., Goodman, T. R., Pham, B., & Doyle, J. J. (2000). 'Low-risk' prediction rule for paediatric oncology patients presenting with fever and neutropenia. *Journal of Clinical Oncology, 18*, 1012–1019.

Kockritz-Blickwede, M., & Nizet, V. (2009). Innate immunity turned inside-out: Antimicrobial defense by phagocyte extracellular traps. *Journal of Molecular Medicine, 87*, 775–783.

Krishnan, E., & Hubert, H. B. (2006). Ethnicity and mortality from systemic lupus erythematosus in the US. *Annals of the Rheumatic Diseases, 65*, 1500–1505.

Li, P., Li, M., Lindberg, M. R., Kennett, M. J., Xiong, N., & Wang, Y. (2010). PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *The Journal of Experimental Medicine, 207*(9), 1853–1862.

Maranda, L., Seweryn, M., Giebel, S., Holowiecki, J., Piatkowska-Jakubas, B., Wegrzyn, J., Skotnicki, A., Kielbinski, M., Kuliczkowski, K., Paluszewska, M., Jedrzejczak, W. W., Dutka, M., Hellmann, A., Flont, M., Zdziarska, B., Palynyczko, G., Konopka, L., Szpila, T., Gawronski, K., Sulek, K., Sokolowski, J., Kloczko, J., Warzocha, K., & Robak, T. (2010). Infectious complications in patients with acute myeloid leukemia treated according to the protocol with daunorubicin and cytarabine with or without addition of caladribine. A multicenter study by the Polish Adult Leukemia Group (PALG). *International Journal of Infectious Diseases, 14*, e132–e140.

Marcos, V., Zhou, Z., Yildirim, A. O., Bohla, A., Hector, A., Vitkov, L., Wiedenbauer, E. M., Krautgartner, W. D., Stoiber, W., Belohradsky, B. H., Rieber, N., Kormann, M., Koller, B., Roscher, A., Roos, D., Griese, M., Eickelberg, O., Doring, G., Mall, M. A., & Hartl, D. (2010). CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. *Nature Medicine, 16*, 1018–1023.

Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P. A., Baumann, C., Fey, M. T., Dummer, R., Simon, H. U., & Yousefi, S. (2004). Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. *Journal of Biological Chemistry, 279*, 44123–44132.

Oehmcke, S., Morgelin, M., & Herwald, H. (2009). Activation of the human contact system on neutrophil extracellular traps. *Journal of Innate Immunity, 1*, 225–230.

Orozco, G., Eyre, S., Hinks, A., Bowes, J., Morgan, A. W., Wilson, A. G., Wordsworth, P., Steer, S., Hocking, L., Thomson, W., Worthington, J., & Barton, A. (2011). Study of the common genetic background for rheumatoid arthritis and systemic lupus erythematosus. *Annals of the Rheumatic Diseases, 70*, 463–468.

Ozaki, S. (2007). ANCA-associated vasculitis: Diagnostic and therapeutic strategy. *Allergology International, 56*, 87–96.

Papayannopoulos, V., & Zychlinsky, A. (2009). NETs: A new strategy for using old weapons. *Trends in Immunology, 30*, 513–521.

Papayannopoulos, V., Metzler, K. D., Hakkim, A., & Zychlinsky, A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *The Journal of Cell Biology, 191*(3), 677–691.

Rada, B., & Leto, T. L. (2008). Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contributions to Microbiology, 15*, 164–187.

Rahman, A., & Isenberg, D. A. (2008). Systemic lupus erythematosus. *The New England Journal of Medicine, 358*, 929–939.

Ramos-Kichik, V., Mondragon-Flores, R., Mondragon-Castelan, M., Gonzalez-Pozos, S., Muniz-Hernandez, S., Rojas-Espinosa, O., Chacon-Salinas, R., Estrada-Parra, S., & Estrada-García, I. (2009). Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis*. *Tuberculosis, 89*, 29–37.

Rarok, A. A., Limburg, P. C., & Kallenberg, C. G. (2003). Neutrophil-activating potential of antineutrophil cytoplasm autoantibodies. *Journal of Leukocyte Biology, 74*, 3–15.

Schmidt, W. H., Heesen, C., Csernok, E., Rautmann, A., & Gross, W. L. (1992). Elevated serum levels of soluble interleukin-2 receptor in patients with Wegener's granulomatosis. Association with disease activity. *Arthritis and Rheumatism, 35*, 1088–1096.

Sebastiani, G. D., & Galeazzi, M. (2009). Infection-genetics relationship in systemic lupus erythematosus. *Lupus, 18*, 1169–1175.

Soehnlein, O. (2009). An elegant defense: How neutrophils shape the immune response. *Trends in Immunology, 30*(11), 511–512.

Stegeman, C. A., Tervaert, J. W., Sluiter, W. J., Manson, W. L., de Jong, P. E., & Kallenberg, C. G. (1994). Association of chronic nasal carriage of *Staphylococcus Aureus* and higher relapse rates in Wegener granulomatosis. *Annals of Internal Medicine, 120*, 12–17.

Tadema, H., Abdulahad, W. H., Lepse, N., Stegeman, C. A., Kallenberg, C. G., & Heeringa, P. (2010). Bacterial DNA motifs trigger ANCA production in ANCA-associated vasculitis in remission. *Rheumatology, 50*, 689–696.

Tandon, A., Ibanez, D., Gladman, D. D., & Urowitz, M. B. (2004). The effect of pregnancy on lupus nephritis. *Arthritis and Rheumatism, 50*, 3941–3946.

Urban, C. F., Reichard, U., Brinkmann, V., & Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cellular Microbiology, 8*(4), 668–676.

van Rossum, A. P., Limburg, P. C., & Kallenberg, C. G. (2005). Activation, apoptosis, and clearance of neutrophils in Wegener's granulomatosis. *Annals of the New York Academy of Sciences, 1051*, 1–11.

Villanueva, E., Yalavarthi, S., Berthier, C. C., Hodgin, J. B., Khandpur, R., Lin, A. M., Rubin, C. J., Zhao, W., Olsen, S. H., Klinker, M., Shealy, D., Denny, M. F., Plumas, J., Chaperot, L., Kretzler, M., Bruce, A. T., & Kaplan, M. J. (2011). Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *Journal of Immunology, 187*, 538–552.

Wartha, F., Beiter, K., Normark, S., & Henriques-Normark, B. (2007). Neutrophil extracellular traps: Casting the NET over pathogenesis. *Current Opinion in Microbiology, 10*, 52–56.

Wilde, B., Thewissen, M., Damoiseaux, J., van Paassen, P., Witzke, O., & Tervaert, J. W. (2010). T cells in ANCA-associated vasculitis: What can we learn from lesional versus circulating T cells? *Arthritis Research & Therapy, 12*, 204.

Yousefi, S., Mihalache, C., Kozlowski, E., Schmidt, I., & Simon, H. U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death and Differentiation, 16*, 1438–1444.

Chapter 2
Tiotropium Increases PPARγ and Decreases CREB in Cells Isolated from Induced Sputum of COPD Patients

A. Holownia, R.M. Mroz, T. Skopinski, A. Kolodziejczyk, E. Chyczewska, and J.J. Braszko

Abstract Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation and chronic inflammation of airways and lung parenchyma. Our aim was to assess two important elements of intracellular signaling involved in regulation of inflammation in COPD in patients subjected to long-acting beta2-agonist or long-acting beta2-agonist plus long-acting antimuscarinic: peroxisome proliferator-activated receptor gamma (PPARγ) protein, which has antiinflammatory and immunomodulatory properties and cAMP response element binding protein (CREB) and activated (CREB-P) protein which has histone acetyltransferase activity and increases histone acetylation and transcriptional activation of chromatin. Twenty one stable COPD patients (18 males and 3 females, mean age 65 years) receiving 12 μg B.I.D formoterol were assayed before and after 3 month add-on therapy, consisting of 18 μg Q.D. tiotropium. In all patients, sputum induction, spirometry, lung volumes, and DLCO were performed before and after therapy. Sputum cells were isolated and processed to isolate cytosolic and nuclear fractions. PPARγ, CREB, or CREB-P proteins were quantified in subcellular fractions using Western blot. Tiotropium add-on therapy improved respiratory parameters: FEV1 and lung volumes. After therapy mean expression of PPARγ in cell nuclei was significantly increased by about 180%, while CREB and phosphorylated CREB levels in cytosol and nuclei were decreased by about 30%. Our data show that the mechanism whereby tiotropium reduces exacerbations may be associated not only with persistent increase in airway functions and reduced hyperinflation mediated by muscarinic receptors, but also with possible anti-inflammatory effects of the drug, involving increased PPARγ and decreased CREB signaling.

Keywords COPD • CREB • Histone acetylation • PPARγ • Tiotropium

A. Holownia (✉) • T. Skopinski • A. Kolodziejczyk • E. Chyczewska • J.J. Braszko
Department of Clinical Pharmacology, Medical University of Bialystok,
Waszyngtona 15a, 15-279 Bialystok, Poland
e-mail: holow_sinai@hotmail.com

R.M. Mroz
Department of Chest Diseases and Tuberculosis, Medical University of Bialystok,
Waszyngtona 15a, 15-279 Bialystok, Poland

M. Pokorski (ed.), *Respiratory Regulation - The Molecular Approach*,
Advances in Experimental Medicine and Biology 756, DOI 10.1007/978-94-007-4549-0_2,
© Springer Science+Business Media Dordrecht 2013

2.1 Introduction

Irreversible and progressive airflow limitation is a landmark of chronic obstructive pulmonary disease (COPD), the only major disease with an increasing death rate (Viegi et al. 2007). In COPD, airflow obstruction is caused by increased activity of parasympathetic system and chronic inflammation of the airways and lung parenchyma, very frequently associated with chronic tobacco smoking (Viegi et al. 2007). COPD is considered as a fatal disease, but it can be managed, controlled and slowed down, however a necessary step is smoking cessation. In pharmacotherapy of moderate to severe COPD long-acting bronchodilators are used (Global Strategy for the Diagnosis, Management and Prevention of COPD 2008). Currently approved drugs for the treatment of COPD are: long-acting beta2-agonists (LABA), i.e., formoterol, salmeterol, indacaterol, combined with long-acting antimuscarinic agents (LAMA) such as tiotropium bromide (Meyer et al. 2011). Formoterol, a selective LABA, increases adenylyl cyclase and cyclic adenosine monophosphate (cAMP) resulting in relaxation of bronchial smooth muscles (Kaur et al. 2008). Tiotropium acts as antagonist of M3 and M1 muscarinic receptors, modulating inositol 1,4,5-trisphosphate (IP3) and 1,2-diacyl-glycerol (DAG) signaling pathways (Casarosa et al. 2010). Drug binding produces prolonged improvement in clinical respiratory parameters and usually a single inhaled dose reverses compromised respiratory function (Kato et al. 2006). Our previous data indicate that tiotropium altered pharmacodynamic parameters of cholinergic M3 receptors and increased histone acetylation in chromatin of inflammatory cells migrating to the airways of COPD patients (Holownia et al. 2010). However, the possible anti-inflammatory mechanisms related to tiotropium are unknown. Our aim was to assess important elements of cytosolic and nuclear inflammatory signaling - expression and activation (Ser133 phosphorylation) of cAMP response element binding protein (CREB), and peroxisome proliferator-activated receptor gamma (PPARγ) in cells isolated from induced sputum of COPD patients before and after tiotropium therapy. CREB is an end-point and integration site of several signaling pathways, with histone acetyltransferase (HAT) activity (Lim et al. 2011), whether PPARγ acts as nuclear hormone receptor regulating glucose metabolism and the expression of inflammatory cytokines with possible histone deacetylase (HDAC) activity (Miard and Fajas 2005).

2.2 Methods

2.2.1 Subjects and Treatment

Twenty one (18 males and three females, mean age 65 years) COPD patients with stable disease, defined according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (Global Strategy for the Diagnosis, Management and Prevention of COPD 2008) were enrolled into the study. All patients with COPD had airflow limitation (FEV1 $<$ 80% predicted, FEV1/FVC $<$ 70%, GOLD stage 2–4) and received stable formoterol therapy for 4 weeks preceding inclusion. All subjects were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current medical treatment. Exclusion criteria included the following: other systemic diseases, other lung diseases apart from COPD and lung tumors, pulmonary infection and antibiotic treatment 4 weeks before inclusion, no inhaled or oral glucocorticosteroids in the 3 months preceding inclusion. All patients gave their informed consent after a full discussion of the nature of the study, which had been approved by a local Ethics Committee. No patient in the study had symptoms nor was treated for COPD exacerbation during at least 2 months preceding the day of inclusion.

The lung function and DLCO tests were performed with body box (Elite DL, Medgraphics, USA). The measurement was performed using standard protocols according to American Thoracic Society guidelines. Twenty one patients underwent 4 week stable therapy with 12 μg B.I.D. formoterol and subsequently were subjected to sputum induction. Next patients were treated for 12 additional weeks with add-on 18 μg Q.D. tiotropium and their sputum was collected.

2.2.2 Sputum Induction and Processing

Sputum was induced by the inhalation of a 4.5% hypertonic aerosol saline solution, generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland) (Loh et al. 2005). Throughout the procedure, subjects were encouraged to cough and to expectorate into a plastic container. Three flow volume curves were performed before and after each inhalation, and the best FEV1 was recorded. Induction of sputum was stopped if the FEV1 value fell by at least 20% from baseline or if troublesome symptoms occurred. Samples were processed within 15 min after the termination of the induction.

Induced sputum samples were solubilized in equal volumes of 0.1% dithiothreitol (Sigma Chemicals Co, Poznan, Poland) in Hanks solution and incubated for 15 min in an ice bath. Cell suspension was then rinsed twice with Hanks solution, filtered by a nylon membrane and centrifuged (1,000 rpm) on Histopaque 1077. Isolated cells were further processed to obtain cytosolic and nuclear fractions.

To isolate subcellular fractions, sputum cells were centrifuged, resuspended in cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 50 mM dithiothreitol, 100 mM phenanthroline, 1 mg/ml pepstatin, 100 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 100 mM 3,4-dichloroisocoumarin, 10 mM NaF, 100 mM sodium orthovanadate, 25 mM b-glycero-phosphate and centrifuged at 14,000 × *g* for 5 min at 4°C (Mroz et al. 2007). Cells were then lysed in a solution of the same buffer containing 0.2% (v/v) Nonidet P- 40 for 10 min on ice and centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was collected as a cytosolic extract. The remaining pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 100 mM 3,4-dichloroisocoumarin), incubated for 15 min at 4°C, and centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant including soluble nuclear protein was collected as a nuclear extract.

Cytosolic and nuclear fractions were evaluated for the expression of CREB and CREB-P proteins, while PPARγ was assessed only in nuclear fractions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots. Sample proteins were separated in reducing conditions, transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with specific rabbit monoclonal antibodies against human CREB or PPARγ proteins (Abcam, Cambridge, USA). After washing, bound antibody was detected using appropriate secondary anti-rabbit antibody (Abcam, Cambridge, USA) linked to horseradish peroxidase. The bound complexes were detected using enhanced chemiluminescence (ECL, Amersham, GE Healthcare, Little Chalfont, UK) and quantified using Quantity One software (BioRad, Warsaw, Poland). The constitutively expressed protein – β-actin, served as a loading control and the data were quantified in respect to β-actin expression. For the negative control study, membranes were treated similarly but without the addition of primary antibody. Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan, Poland).

Statistical analysis was performed using statistical package – Statistica (Statsoft, Cracow, Poland) using nonparametric Wilcoxon test for paired data. The data were expressed as means ± SD. $P < 0.05$ was as considered statistically significant.

2.3 Results

Table 2.1 and Fig. 2.1 show respiratory parameters: forced expiratory volume in one second (FEV1), inspiratory capacity (IC), residual volume (RV) and residual volume divided by total lung capacity (RV/TLC) and biochemical data: cytosolic and nuclear CREB and phosphorylated CREB as well as nuclear PPARγ levels in cells isolated from induced sputum of COPD patients before and after tiotropium ad-on therapy. Representative Western blot pictures of CREB, CREB-P and PPARγ protein are shown (Fig. 2.1). Therapy improved lung function parameters. After therapy, mean FEV1 increased by 14% ($P < 0.05$), IC increased by 14% ($P < 0.05$), residual volume was reduced by 13% ($P < 0.05$), and RV/TLC decreased by 11% ($P < 0.05$) from baseline.

Table 2.1 Respiratory parameters: forced expiratory volume in 1 s (FEV1), inspiratory capacity (IC), residual volume (RV) and residual volume divided by total lung capacity (RV/TLC) and cytosolic and nuclear CREB and phosphorylated CREB as well as nuclear PPARγ levels (relative units) in cells isolated from induced sputum of COPD patients before and after tiotropium add-on therapy

	Formoterol(F)		Formoterol + Tiotropium (FT)	
FEV1 (L)	1.52 ± 0.18		1.73 ± 0.19*	
% predicted	52%		56%	
IC (L)	1.87 ± 0.13		2.14 ± 0.22*	
% predicted	62%		71%	
RV (L)	3.39 ± 0.21		2.92 ± 0.23*	
% predicted	152%		132%	
RV/TLC	53 ± 6%		47 ± 7%*	
	Cytosol	Nuclei	Cytosol	Nuclei
CREB	100.0 ± 21.3	100.0 ± 25.4	75.4 ± 18.4*	64.3 ± 26.2*
CREB-P	100.0 ± 37.2	100.0 ± 31.3	71.2 ± 19.3*	112.4 ± 7.5
PPARγ	100.0 ± 33.9	–	281.0 ± 45.7**	–

*P < 0.05; **P < 0.01 – comparing to corresponding data from F-monotherapy

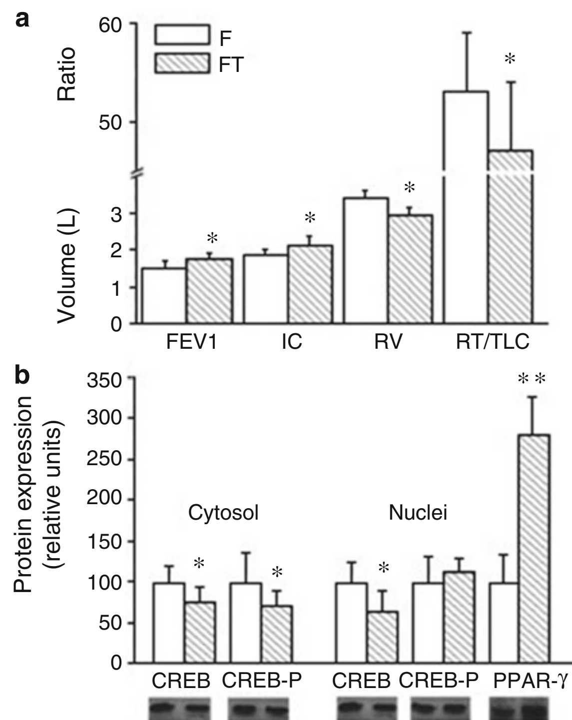

Fig. 2.1 **(a) Respiratory parameters:** forced expiratory volume in one second (*FEV1*), inspiratory capacity (*IC*), residual volume (*RV*) and residual volume divided by total lung capacity (*RV/TLC*); **(b) Cytosolic and nuclear CREB, phosphorylated CREB, and nuclear PPARγ** (relative units) in cells isolated from induced sputum of COPD patients before and after tiotropium add-on therapy. Representative Western blot pictures of cytosolic and nuclear CREB, CREB-P, and PPARγ protein are also shown. *F* formoterol, *FT* formoterol + tiotropium; *P < 0.05 and **P < 0.01 compared with the corresponding data from F-monotherapy

Tiotropium decreased expressions of CREB and phosphorylated CREB in cytosol by about 25% ($P<0.05$) and 29% ($P<0.05$) for cytosolic CREB and CREB-P, respectively. In cell nuclei CREB was also decreased after tiotropium therapy by about 36% ($P<0.05$), but CREB-P levels were not altered. Comparing to values from patients on formoterol monotherapy, tiotropium significantly increased PPARγ in cell nuclei (increase by about 180%, $P<0.01$).

2.4 Discussion

We have previously shown that in cells isolated from induced sputum of COPD patients treated with tiotropium, acetylated H3 histone levels are significantly higher (Holownia et al. 2010). This observation may be important to inflammatory signaling, because acetylated histones represent a type of epigenetic tag which is responsible for gene transcription within chromatin. Histone acetylation mediated by HAT neutralizes the positive charge on the histone molecules (Khan and Khan 2010). As a consequence, chromatin is transformed into a more relaxed structure, associated with greater levels of gene transcription which is relevant to inflammatory signaling. In asthma, bronchial tissue and alveolar macrophages have increased HAT and decreased HDAC1 expression (Grabiec et al. 2008). In COPD formoterol and glucocorticosteroids were found to increase HAT-active CREB, especially in the cytosol of sputum cells (Mroz et al. 2007). In our patients cytosolic CREB (both inactive and phosphorylated) was slightly reduced after antimuscarinic therapy. In cell nuclei, unphosphorylated CREB was also lowered but phosphorylated, active CREB was not decreased after LAMA treatment. Given the important role of CREB in adrenergic signaling (Kaur et al. 2008), it seems that described changes may reflect adaptation of adrenergic receptors to chronic stimulation of β_2 receptors by formoterol. It is interesting to note that the PPARγ agonist rosiglitazone can decrease adrenoceptor desensitization and increase salbutamol effects on airway smooth muscle (Fogli et al. 2011). On the other hand, it was shown, that downregulation of PPARγ increased airway inflammation (Belvisi and Mitchell 2009). We have found significant increase in PPARγ expression in sputum cells, not only after tiotropium therapy, but also after formoterol/inhaled corticosteroids treatment (Holownia et al. 2008). Although in the allergen challenge of asthmatic patients, rosiglitazone was associated with only modest reduction in the late asthmatic reaction (Richards et al. 2010), our data suggest that combined therapy of tiotropium and rosiglitazone in COPD may be more effective.

The role of tiotropium in regulation of histone signaling is not clear. However, interactions of anticholinergic drugs and immune system are well established. It is well known that chronic lung diseases are related to *in utero* nicotine exposure (Miller and Marty 2010). In COPD there is higher acetylcholine release, increased vagal tone, airway inflammation and increased mucus production, all efficiently blocked by tiotropium (Gosens et al. 2006). It has been shown that the bronchodilatory activity of tiotropium against acetylcholine-induced bronchoconstriction is in the same dose range as the anti-inflammatory activity (Wollin and Pieper 2010). In asthma, tiotropium bromide significantly reduced airway inflammation and the T helper cytokine production in bronchoalveolar lavage fluid (Ohta et al. 2010). Our earlier data showed that tiotropium therapy involved pharmacodynamic changes in cholinergic M3 receptors and alterations in histone acetylation (Holownia et al. 2010). The present data suggest that the latter effect is most probably not mediated by alterations in CREB signaling.

Our results show that the mechanism whereby tiotropium reduces COPD exacerbations and ameliorates respiratory parameters is not only a result of persistent increase in airway functions and reduced hyperinflation caused by the drug, but also of its anti-inflammatory effects, involving increased PPARγ and decreased CREB signaling.

Conflicts of interest: The authors declare no conflicts of interest in relation to this article.

References

Belvisi, M. G., & Mitchell, J. A. (2009). Targeting PPAR receptors in the airway for the treatment of inflammatory lung disease. *British Journal of Pharmacology, 158*, 994–1003.

Casarosa, P., Kiechle, T., Sieger, P., Pieper, M., & Gantner, F. (2010). The constitutive activity of the human muscarinic M3 receptor unmasks differences in the pharmacology of anticholinergics. *Journal of Pharmacology and Experimental Therapeutics, 333*, 201–209.

Fogli, S., Pellegrini, S., Adinolfi, B., Mariotti, V., Melissari, E., Betti, L., Fabbrini, L., Giannaccini, G., Lucacchini, A., Bardelli, C., Stefanelli, F., Brunelleschi, S., & Breschi, M. C. (2011). Rosiglitazone reverses salbutamol-induced β(2)-adrenoceptor tolerance in airway smooth muscle. *British Journal of Pharmacology, 162*, 378–391.

Global Strategy for the Diagnosis, Management and Prevention of COPD. (2008). *Global Initiative for Chronic Obstructive Lung Disease* (GOLD). Available from: http://www.goldcopd.org

Gosens, R., Zaagsma, J., Meurs, H., & Halayko, A. J. (2006). Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respiratory Research, 7*, 73–88.

Grabiec, A. M., Tak, P. P., & Reedquist, K. A. (2008). Targeting histone deacetylase activity in rheumatoid arthritis and asthma as prototypes of inflammatory disease: Should we keep our HATs on? *Arthritis Research and Therapy, 10*, 226.

Holownia, A., Mroz, R. M., Noparlik, J., Chyczewska, E., & Braszko, J. J. (2008). Expression of CREB-binding protein and peroxisome proliferator-activated receptor gamma during formoterol or formoterol and corticosteroid therapy of chronic obstructive pulmonary disease. *Journal of Physiology and Pharmacology, 59*(Suppl. 6), 303–309.

Holownia, A., Mroz, R. M., Skopinski, T., Kielek, A., Kolodziejczyk, A., Chyczewska, E., & Braszko, J. J. (2010). Tiotropium increases cytosolic muscarinic M3 receptors and acetylated H3 histone proteins in induced sputum cells of COPD patients. *European Journal of Medical Research, 15*(Suppl. 2), 64–67.

Kato, M., Komamura, K., & Kitakaze, M. (2006). Tiotropium, a novel muscarinic M3 receptor antagonist, improved symptoms of chronic obstructive pulmonary disease complicated by chronic heart failure. *Circulation Journal, 70*, 1658–1660.

Kaur, M., Holden, N. S., Wilson, S. M., Sukkar, M. B., Chung, K. F., Barnes, P. J., Newton, R., & Giembycz, M. A. (2008). Effect of beta2-adrenoceptor agonists and other cAMP-elevating agents on inflammatory gene expression in human ASM cells: A role for protein kinase A. *American Journal of Physiology. Lung Cellular and Molecular Physiology, 295*, 505–514.

Khan, S. N., & Khan, A. U. (2010). Role of histone acetylation in cell physiology and diseases: An update. *Clinica Chimica Acta, 411*, 1401–1411.

Lim, E. J., Lu, T. X., Blanchard, C., & Rothenberg, M. E. (2011). Epigenetic regulation of the IL-13-induced human eotaxin-3 gene by CREB-binding protein-mediated histone 3 acetylation. *Journal of Biological Chemistry, 286*, 13193–13204.

Loh, L. C., Kanabar, V., D'Amato, M., Barnes, N. C., & O'Connor, B. J. (2005). Sputum induction in corticosteroid-dependant asthmatics: Risks and airway cellular profile. *Asian Pacific Journal of Allergy and Immunology, 23*, 189–196.

Meyer, T., Reitmeir, P., Brand, P., Herpich, C., Sommerer, K., Schulze, A., Scheuch, G., & Newman, S. (2011). Effects of formoterol and tiotropium bromide on mucus clearance in patients with COPD. *Respiratory Medicine, 105*, 900–906.

Miard, S., & Fajas, L. (2005). Atypical transcriptional regulators and cofactors of PPARγ. *International Journal of Obesity, 29*, 10–12.

Miller, M. D., & Marty, M. A. (2010). Impact of environmental chemicals on lung development. *Environmental Health Perspectives, 118*, 1155–1164.

Mroz, R. M., Holownia, A., Chyczewska, E., Drost, E. M., Braszko, J. J., Noparlik, J., Donaldson, K., & Macnee, W. (2007). Cytoplasm-nuclear trafficking of CREB and CREB phosphorylation at Ser133 during therapy of chronic obstructive pulmonary disease. *Journal of Physiology and Pharmacology, 58*, 437–444.

Ohta, S., Oda, N., Yokoe, T., Tanaka, A., Yamamoto, Y., Watanabe, Y., Minoguchi, K., Ohnishi, T., Hirose, T., Nagase, H., Ohta, K., & Adachi, M. (2010). Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma. *Clinical and Experimental Allergy, 40*, 1266–1275.

Richards, D. B., Bareille, P., Lindo, E. L., Quinn, D., & Farrow, S. N. (2010). Treatment with a peroxisomal proliferator activated receptor gamma agonist has a modest effect in the allergen challenge model in asthma: A randomised controlled trial. *Respiratory Medicine, 104*, 668–674.

Viegi, G., Pistelli, F., Sherrill, D. L., Maio, S., Baldacci, S., & Carrozzi, L. (2007). Definition, epidemiology and natural history of COPD. *European Respiratory Journal, 30*, 993–1013.

Wollin, L., & Pieper, M. P. (2010). Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD. *Pulmonary Pharmacology & Therapeutics, 23*, 345–354.

Chapter 3
Expression of Ki-67, Bcl-2, Survivin and p53 Proteins in Patients with Pulmonary Carcinoma

E. Halasova, M. Adamkov, T. Matakova, D. Vybohova, M. Antosova, M. Janickova, A. Singliar, D. Dobrota, and V. Jakusova

Abstract Apoptosis is the fundamental process necessary for eliminating damaged or mutated cells. Alterations in the apoptotic pathway appear to be key events in cancer development and progression. Bcl-2 is the key member of the Bcl-2 family of apoptosis regulator proteins with anti-apoptotic effects. Survivin acts as an inhibitor of apoptosis as well and has been implicated in both inhibition of apoptosis and mitosis regulation. p53 is one of the tumor suppressor proteins, prevents tumor formation through cell cycle blocking and eliminates damaged cells *via* the activation of apoptosis. The Ki-67 protein is a cellular marker for proliferation. To investigate the possible interactions of the aforementioned proteins, we examined their expression in 76 patients with diagnosed lung cancer using immunohistochemical visualisation. Ki-67 protein was expressed in the cancer cells of all patients with small cell lung cancer (SCLC). We found a negative correlation

E. Halasova
Department of Medical Biology, Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin, Martin, Slovakia

M. Adamkov (✉) • D. Dobrota
Institute of Histology and Embryology, Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin, Mala hora 4, Martin SK-03754, Slovakia
e-mail: adamkov@jfmed.uniba.sk

T. Matakova
Department of Medical Biochemistry, Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin, Martin, Slovakia

D. Vybohova
Department of Anatomy, Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin, Martin, Slovakia

M. Antosova
Department of Pharmacology, Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin, Martin, Slovakia

M. Janickova
Clinics of Stomatology and Maxillofacial Surgery, Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin and Faculty Hospital in Martin, Martin, Slovakia

A. Singliar
Department of Pathology, Hospital in Dolny Kubin, Dolby Kubin, Slovakia

V. Jakusova
Department of Public Health, Jessenius Faculty of Medicine in Martin, Martin, Slovakia

M. Pokorski (ed.), *Respiratory Regulation - The Molecular Approach*,
Advances in Experimental Medicine and Biology 756, DOI 10.1007/978-94-007-4549-0_3,
© Springer Science+Business Media Dordrecht 2013

between survivin and p53 expression. A decreased intensity of survivin expression and fewer cells positive for survivin (66.7%) in SCLC in comparison with other lung cancer types (98.0%) was detected. Reversely, expression of Bcl-2 was found in more than 90% of cases with SCLC. We hypothesize that high expression and intensity of Bcl-2 protein could be a factor behind a bad prognosis in SCLC.

Keywords Lung cancer • Ki-67 protein • Bcl-2 protein • Survivin • p53 protein • Immunohistochemistry

3.1 Introduction

Lung cancer has now become the leading cause of cancer deaths in both men and women worldwide (Ferlay et al. 2010; Krug et al. 2008). Small cell lung cancer (SCLC) is an aggressive and highly metastatic disease, and it accounts for about one-quarter of all lung cancer cases. Although SCLC tumors are initially responsive to chemotherapy, multidrug resistance tends to develop after relapse, and the majority of SCLC patients die of their disease within 2 years (Tsao and Glisson 2006).

Carcinogenesis is a multi step process involving changes in expression of many genes especially related to regulation of cell cycle, differentiation, and apoptosis. Apoptosis or programmed cell death is needed for maintenance of cell homeostasis and to destroy cells that represent a threat to the integrity of organism. Apoptosis can be induced by either specific extracellular or internal signals. The molecular mechanisms involved in apoptotic enzymatic pathways have been sufficiently reviewed (Jinz and El-Deiry 2005). Protein p53 plays an important role in apoptosis induction. It acts as a transcription factor which in humans is encoded by the TP53 gene (Matlashewski et al. 1984; Isobe et al. 1986). p53 is activated by various stress signals such as radiation (UV, gamma, or X), carcinogens (polycyclic aromatic carbohydrates, heavy metals), oxidative stress, hypoxia, oncogene activation, telomere shortening, and others (Pluquet and Hainaut 2001). Many genes are involved in p53-dependent apoptotic pathways (Moll and Zaika 2001; Schuler and Green 2001; Pietenpol and Stewardt 2002; Yu and Zhang 2005). Expression and activity of p53 is precisely regulated at several levels (Coutts and Thangue 2006). p53 prevents tumor formation through cell cycle blocking and eliminates damaged cells. Mutations or inactivation of p53 are the most frequent changes in tumor cells (Deng and Wu 2000).

On the other hand, cancer cells overexpress proteins which can block the apoptosis. Survivin is a member of the apoptosis inhibitors (IAP) gene family, which has been implicated in both apoptosis inhibition and mitosis regulation (Altieri 2003). Survivin upregulates genes in tumor tissues (Velculescu et al. 1999). High survivin expression is related to poor prognosis in many cancer types (Kawasaki et al. 1998; Yamashita et al. 2007). There are many studies showing that p53 leads to repression of survivin expression in non-small lung cancers (see e.g., Mirza et al. 2002). However, the studies on the expression of the aforementioned proteins in SCLC are sparse (Nakano et al. 2005; Akyürek et al. 2006; Jin et al. 2006). The Bcl-2 proto-oncogene functions as a suppressor of apoptotic death as well. The protein contributes to cell survival also by diminishing the rate of cell proliferation (Borner 1996). It has been estimated that up to 90% of SCLC overexpress Bcl-2 (Higashiyama et al. 1995). Ectopic expression of Bcl-2 in a SCLC cell line increases resistance to apoptosis induced by chemotherapy (Shert et al. 2008). The main characteristic of cancer cells is their ability to proliferate. The Ki-67 protein is used as a cellular marker of proliferation. Studies point to a prognostic value of Ki-67 in cancer, but the results are inconsistent (Soomro et al. 1998; Han et al. 2009; Skov et al. 2010).

In this study, we focused on the expression and correlation of selected pro- and anti-apoptotic proteins in different types of lung carcinoma.

3.2 Methods

A hundred operative samples from pulmonary carcinoma patients were evaluated for survivin, Bcl-2, Ki-67, and p53 expression. Seventy six of them were enrolled into this study. The remaining cases had to be excluded because of specimen damage. The hematoxylin and eosin stained slides from each case were independently reviewed by two pathologists to ascertain the diagnosis based on morphological and immunohistochemical parameters and correlated with clinical data. Three sections, 4 μm thick, from each paraffin block were stained for p53 and survivin proteins. To achieve greater adherence of the sections to the glass surface, silanized slides (DAKO, Denmark) were used, baked for 2 h at 56°C before use. Then, the sections were deparaffinized in xylene for 20 min, rehydrated in a series of descending ethanol concentrations and washed with phosphate-buffered saline (PBS). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigen unmasking was achieved by heating the sections immersed in a target solution (DAKO) in water bath at 96°C for 45 min. Immunohistochemical staining was performed using monoclonal mouse anti-p53 (Clone DO-7), anti-survivin (Clone12C4), anti-Ki-67 antibody (Clone MIB-1), and anti-Bcl2 (Clone 124) antibodies; all diluted 1:50, obtained from DAKO. After overnight incubation, the p53 and survivin antigens were visualized by means of the LSAB Visualization System (DAKO) using 3,3'-diaminobenzidine chromogen as substrate, according to the manufacturer's instructions. All sections were counterstained with Mayer's hematoxylin (DAKO). Negative controls were obtained by omitting the primary antibodies.

3.2.1 Evaluation of Immunochemical Staining

Survivin, p53, Bcl-2, and Ki-67 antibody stained sections were observed in a light microscope. In each case, the following features were assessed: (1) intensity of staining, (2) relative number of positively stained cells, and (3) subcellular localization of p53 and survivin antigens.

The positivity of cytoplasmic (C), nuclear (N), or a combination of both was determined. Survivin and p53 expressions were scored semi-quantitatively as follows:

1. The intensity of staining:
   a. negative
   b. weak (+)
   c. moderate (++)
   d. strong (+++)
2. Number of positively stained cells
   a. more than 10% (≥10%) per field of view
   b. less than 10% (≤10%) per field of view
3. Subcellular localization of staining
   a. nuclear (N) only
   b. cytoplasmic (C) only
   c. combined nuclear and cytoplasmic (NC)

3.2.2 Statistical Analysis

Chi-square (χ^2) test or Fischer's exact test was used for comparison between survivin and p53 immunoreactivity. Spearman's coefficient was used to estimate the correlation between parameters. All statistical calculations were performed using MedCalc v.5 software for Windows.

3.3 Results

The results of expression profiling are summarized in Table 3.1 and illustrated in Fig. 3.1a–d. Survivin was predominantly expressed in both nucleus and cytoplasm in 58 cases (96.7%) (Fig. 3.1a), whereas p53 was expressed in the nucleus only in 64 (90.1%) (Fig. 3.1b). Expression of Ki-67 was found in nucleus (Fig. 3.1c) and Bcl-2 was in the cytoplasm only (Fig. 3.1d). In the majority of cases (17; 100% in SCLC and 49; 92% in NSCLC), there was >10% of positively stained cells per field of view for p53. All cases of SCLC showed the expression of Ki-67, but only 13 (78%) had >10% of positively stained cells per field; in NSCLC it was 48 (88%); the difference was insignificant. Survivin was positively stained in 12 (66%) of cases in SCLC and in 48 (98%) cases in NSCLC. There was a negative correlation ($r = -0.72$) between survivin and p53 expression. It seems, therefore, that p53 down-regulated survivin expression.

Comparison of SCLC and NSCLC for survivin expression showed a significant decrease in intensity and number of positive stained cells in the former type (χ^2; 15.30, $P<0.001$ and 8.43, $P<0.05$, respectively). There were no significant differences in intensity and number of positive cells for p53 between SCLC and NSCLC. A different trend was found in Bcl-2 expression, where 13 cases (92%) in SCLC and only 44 cases (75%) in NSCLC expressed the protein, although the difference did not assume statistical significance (χ^2; 1.97, $P=0.15$) . No correlations between Bcl-2 and p53 or survivin were observed.

3.4 Discussion

Lung tumorigenesis proceeds in multiple steps and is due to interrelated genetic events. p53 is a multifunctional protein that regulates cell division and activates apoptosis. On the other hand, survivin can act as an apoptosis inhibitor which is overexpressed in malignancies, including lung carcinoma.

Table 3.1 Expression of survivin, p53, Bcl-2, and Ki-67 in biopsies from patients with lung cancer

		I				%		SL		
		Negative	+	++	+++	<10	>10	N	C	NC
Survivin	SCLC	6	2	8	2	1	11	0	0	12
	NSCLC	1	19	24	5	14	34	1	1	46
p53	SCLC	1	2	10	5	0	17	15	0	2
	NSCLC	4	15	20	19	5	49	49	1	4
Bcl-2	SCLC	1	1	5	7	1	12	0	13	0
	NSCLC	14	18	21	5	13	21	0	44	0
Ki-67	SCLC	0	1	6	7	3	11	12	0	0
	NSCLC	6	0	24	30	6	48	49	0	0

I intensity of immunoreactivity: + weak, ++ moderate, +++ strong, % of labeled cells; *SL*: subcellular localization of survivin and p53 positivity: *N* nuclear, *C* cytoplasmic, *NC* nuclear and cytoplasmic

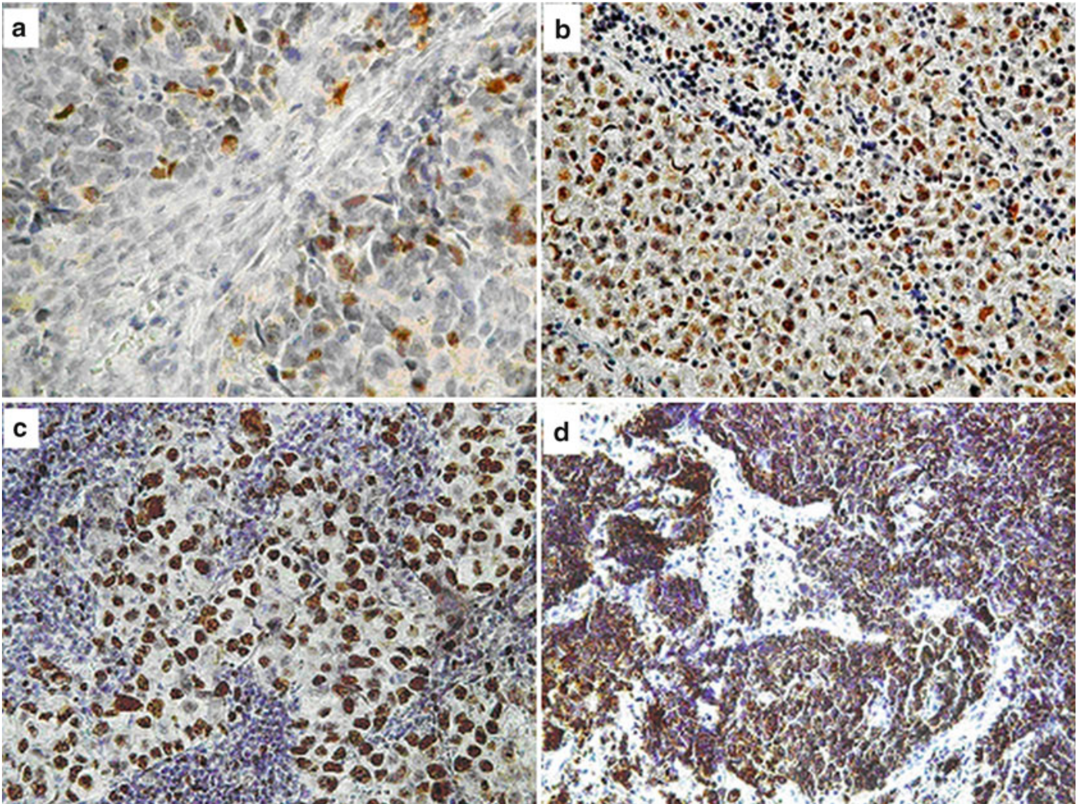

Fig. 3.1 Immunoreactions in tumor cells. (**a**) Combined cytoplasmic and nuclear survivin expression; (**b**) Nuclear p53 expression; (**c**) Ki-67 nuclear expression; (**d**) Bcl-2 cytoplasmic expression. Original magnifications: ×400 in panel (**a**) and ×200 in the remaining panels

A lot of studies have focused on the relationship between survivin and p53 expression, but the results have been quite controversial. Jin et al. (2006) suggested that survivin expression is negatively regulated by p53. Nakano et al. (2005) investigated survivin and p53 expression in specimens from 140 NSCLC patients. The authors found significant differences in survivin expression between squamous cell carcinomas and adenocarcinomas. Furthermore, survivin expression in tumors with mutant p53 was significantly higher than that in tumors with wild-type p53. They concluded that survivin gene expression is negatively regulated by p53 in NSCLC, and that survivin could inhibit apoptosis and accelerate tumor proliferation to produce more aggressive carcinomas. These findings are consistent with our results. We found a negative correlation between p53 and survivin expressions that possibly confirms a relationship between these two opposite acting proteins. The opposite results were published by Akyürek et al. (2006). The authors investigated the role of survivin in the early steps of lung carcinogenesis, in NSCLC, and its relationship to the expression of p53. They found no correlation between survivin and p53 expression; however, the patients with the expression of survivin had clearly worse prognosis.

Dysregulation of the apoptotic process has been implicated in both tumorigenesis and therapeutic resistance. Key regulators of this process, which are overexpressed in SCLC pathology, include Bcl-2 and TP53; the proteins which can synergize with classical oncogenes (Kitada et al. 1994; Higashiyama et al. 1995; Jiang et al. 1995; Schmitt et al. 2000; Wistuba et al. 2001). In the present study, a high incidence of Bcl-2 expression was noted in SCLC which is in accord with the literature outlined above, but surprisingly this expression also was highly elevated in NSCLC.