

IN VITRO STUDY OF NEUTROPHIL APOPTOSIS IN CHRONIC HEPATITIS C PATIENTS

Thesis

Submitted for Partial Fulfillment for M.D. Degree

In

(Clinical and Chemical Pathology)

By

Magda Ahmed Moustafa

M.B. B.Ch., M.Sc

Supervisors

Prof. Dr. Mervat M. El-Ansary

Professor of Clinical Pathology

Faculty of Medicine,

Cairo University

Prof. Dr. Faiza Essawy

Professor of Haematology

Theodor Bilharz Research

Institute

Prof. Dr. Maysa El-Razky

Professor of Tropical Medicine

Faculty of Medicine,

Cairo University

Faculty of Medicine

Cairo University

2008

Abstract

The aim of this study was to detect the role of neutrophil apoptosis as a mechanism of liver cell injury in chronic hepatitis C in order to determine its relation to shortened neutrophil survival in HCV patients, accounting in part for the mechanism of neutropenia.

Thirty patients with chronic HCV infection were selected for this study and divided into two groups, fifteen patients with chronic HCV without neutropenia (Group I) (8 males and 7 females) and fifteen patients with chronic HCV with neutropenia (Group II) (7 males and 8 females). In addition fifteen apparently healthy individuals served as control group (6 males and 9 females).

Key Words :

Adrenocorticotrophic hormone - Alanine amino transferase - Annexin A1

ACKNOWLEDGEMENT

First of all great thanks and merciful to *Allah*

I would like to express my greatest gratitude and respect to *Professor Dr. Mervat Saad El-Ansary, Professor of Clinical Pathology, Faculty of Medicine, Cairo University*, who sacrificed a good deal of her valuable time and experience to guide me throughout the whole work, it's a great honor, enjoyment to work under her guidance and supervision.

The words fail to express my grateful thanks, love and respect to *Professor Dr. Faiza El-Essawy, Professor of Haematology Department, Theodor Bilharz Research Institute*, for establishing the milestone of this research work, her instructive supervision guidance, patience, generous and meticulous cooperation to follow closely every step in this work.

I would like to express my deepest gratitude and sincere thanks to *Professor Dr. Maysa El-Razky, Professor of Tropical Medicine, Faculty of Medicine, Cairo University*, for her effective guidance, valuable suggestions, unlimited help and unfailing support.

I wish to express my deep appreciation and profound gratitude to *Professor Dr. Iman Abdel-Aziz, Assistant Professor of Haematology Department, Theodor Bilharz Research*

Institute, for her effective guidance, creative thinking, valuable suggestions and sincere initiating power.

I would like to send my grateful thanks and appreciation to *Professor Dr. Amira Helmy, Assistant Professor of Electron Microscopy Department, Theodor Bilharz Research Institute*, for her excellent guidance and expertise in supervising the Electron Microscopy section in this thesis, I have benefited much from her valuable suggestions.

In fact, I owe much to my colleagues and staff members of Haematology Departments, Theodor Bilharz Research Institute, for their Kind help and moral support.

Magda Ahmed Moustafa

2008

To my Husband,

Father, Mother and

My Children Mohamed, Yara and Sara

Who suffered a lot during preparation of this work

CONTENTS

Pages

❖ <i>Acknowledgements</i>	
❖ <i>List of Tables</i>	
❖ <i>List of Figures</i>	
❖ <i>List of Abbreviations</i>	
❖ <i>Introduction and Aim of the Work</i>	1
❖ <i>Review of Literature</i>	4
<u>Chronic Hepatitis C virus (HCV)</u>	4
➤ Introduction	4
➤ Modes of HCV Transmission	6
➤ Genomic Organization of Hepatitis C Virus	8
➤ Genotypes	12
➤ Diagnosis of Viral Hepatitis C	14
➤ Assessment of Liver Fibrosis (Non Invasive Indices)	22
➤ Haematologic Changes in HCV	23
➤ Treatment of HCV	24
<u>Apoptosis</u>	28
➤ The History and Biology of Apoptosis	28
➤ Intracellular Alterations During Apoptosis	31
➤ Inducers and Inhibitors of apoptosis	38
➤ The Mechanisms of Apoptosis Signalling Pathways	41
➤ Importance of Apoptosis	48
➤ The Regulatory Proteins for Apoptosis	51
➤ The Annexins (the early apoptotic markers)	66
➤ Normal Neutrophil Apoptosis	72
➤ Methods to Detect and Measure Apoptosis	80
(I) Techniques based on the morphological changes	80

	<i>Pages</i>
(II) Techniques based on biochemical changes	82
<u>Apoptosis in HCV</u>	85
➤ Apoptosis in the Pathophysiology of HCV Infections	88
❖ <i>Subjects and Methods</i>	95
❖ <i>Results</i>	124
❖ <i>Discussion</i>	159
❖ <i>Summary</i>	179
❖ <i>Conclusion</i>	184
❖ <i>Recommendations</i>	185
❖ <i>References</i>	186
❖ <i>Appendix</i>	
❖ <i>Arabic Summary</i>	

LIST OF FIGURES

	<i>Pages</i>
<u>Review of Literature</u>	
<i>Figure (1):</i> Diagramatic structure of HCV	9
<i>Figure (2):</i> The different regions of the HCV genome	11
<i>Figure (3):</i> The effect of different rates of cell death on homeostasis	29
<i>Figure (4):</i> Sequence of ultrastructural changes in apoptosis and necrosis	30
<i>Figure (5):</i> An apoptotic cell showing multiple apoptotic bodies	32
<i>Figure (6):</i> The extrinsic (death receptor) pathway of apoptosis	43
<i>Figure (7):</i> The intrinsic pathway of apoptosis	45
<i>Figure (8):</i> The extrinsic and intrinsic pathways of apoptosis	46
<i>Figure (9):</i> The BCL-2 family contains members that are both pro and antiapoptotic	52
<i>Figure (10):</i> The function of the different types of P53	55
<i>Figure (11):</i> The central role of P53 in cell-cycle arrest, DNA repair and apoptosis following UV irradiation	56

<i>Figure (12):</i>		
Human Caspase family		58
<i>Figure (13):</i>		
One method by which cytotoxic T-cells induce their targets (e.g. virus-infected cells) to commit suicide (apoptosis)		66
<u>Results:</u>		
<i>Figure (1):</i>		
Sex ratio in the different groups		127
<i>Figure (2):</i>		
CBC in the diseased groups		130
<i>Figure (3):</i>		
Results of Annexin V by flow cytometry in different groups		139
<i>Figure (4):</i>		
The results of Annexin V by flow cytometry showing the gating areas		139
<i>Figure (5):</i>		
Mean value of apoptotic cells in the different groups		143
<i>Figure (6):</i>		
The nuclei with fragmented DNA were labeled green with fluorescein-12 –dUTP and the cells were counterstained with propidium iodide in red		145
<i>Figure (7):</i>		
Mean value of sFas test in the different groups		147
<i>Figure (8):</i>		
Results of light Microscope.		148-150
<i>Figure (9):</i>		
Normal mature neutrophil.		152
<i>Figure (10):</i>		

PMN cell in HCV patient showing cell surface membrane ruffles and blebs	152
<i>Figure (11):</i> PMN cell in HCV patient showing early apoptotic morphology.	153
<i>Figure (12):</i> PMN cell in HCV patient showing fragmented nucleus.	153
<i>Figure (13):</i> Ultrathin section of PMA cell in HCV patient showing late apoptotic morphology	154
<i>Figure (14):</i> Electron micrograph of PMN cell in HCV patient showing late apoptotic morphology.	154
<i>Figure (15):</i> Ultrathin section of PMN cell in HCV patient showing the late apoptotic morphology	155
<i>Figure (16):</i> High magnification of the released apoptotic bodies from the cells	155
<i>Figure (17):</i> A significant negative correlation between absolute neutrophils count and dual cells by flow cytometry in chronic HCV group with neutropenia	156
<i>Figure (18):</i> A significant positive correlation between ALT and cells taken PI dye by flow cytometry in chronic HCV group with neutropenia	157
<i>Figure (19):</i> A significant positive correlation between apoptotic cells by TUNEL Test and ALT level in chronic HCV group	158

LIST OF TABLES

	<i>Pages</i>
<u>Review of Literature</u>	
<i>Table (1):</i> Death receptors and their ligands	42
<u>Subjects and methods:</u>	
<i>Table (1):</i> Preparation of TdT incubation buffer for experimental and positive and negative control reactions	103
<i>Table (2):</i> Protocol overview for use of the apoptosis detection system, fluorescent microscopy of attached cells	106
<u>Results:</u>	
<i>Table (1):</i> Summary of clinical data in the different groups	126
<i>Table (2):</i> The results of CBC between the two patients groups	129
<i>Table (3):</i> Absolute neutrophil count in different groups	131
<i>Table (4):</i> The results of blood chemistry of different groups	134
<i>Table (5):</i> Annexin V by flow cytometry in the control group per 100 cells	136
<i>Table (6):</i> Annexin V by flow cytometry in the control hepatitis C group per 100 cells	137

Table (7):

Annexin V by flow cytometry in the chronic hepatitis C with neutropenia group per 100 cells	138
--	-----

Table (8):

Annexin V in patients groups in comparison to control group	140
--	-----

Table (9):

Annexin V between the two patients groups	141
---	-----

Table (10):

The results of sum of Annexin and dual cells in comparison to control group	141
--	-----

Table (11):

The number of apoptotic cells by TUNEL test in the different groups	143
---	-----

Table (12):

The results of apoptotic cells by TUNEL test	144
--	-----

Table (13):

The results of sum of apoptotic cells in the two diseased groups in comparison to the control group by TUNEL test	144
--	-----

Table (14):

The results of sFas in the different groups	146
---	-----

Table (15):

sFas results in the patients groups to the control group	147
--	-----

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AIF	Apoptosis inducing factors
ALT	Alanine amino transferase
ANXA1	Annexin A1
Apaf-1	Apoptotic protease activating factor-1
APRI	Aspartate amino transferase (<u>A</u> ST) to p <u>l</u> atelet <u>r</u> atio <u>i</u> ndex
AST	Aspartate amino transferase
ATP	Adenosine triphosphate
Bad	Bcl-xL/Bcl-2 associated death promoter
Bak	Bcl-2 antagonist killer
Bax	Bcl-2 associated x protein
BcL-2	B-cell lymphoma 2
Bid	BH-3 interacting death domain protein
Bik	Bcl-2 interacting Killer
BIR	Baculovirus IAP repeat domain
CARD	Caspase activation and recruitment domain
Caspase	Cysteiny ^l aspartic acid protease
CAT	Catalase

CGD	Chronic granulomatous disease
CHC	Chronic hepatitis C
CTL	Cytotoxic T lymphocyte
Cyt-c	Cytochrome c
DBD	DNA-binding core domain
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low PI
DIAP1	Drosophila inhibitor of apoptosis protein1
DISC	Death inducing signaling complex
EC	Extracellular domain
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
FADD	Fas associated death domain
Fas	Factor of apoptotic signal
Fas-L	Fas ligand
FHF	Fulminant hepatic failure
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1 converting enzyme
FLIPs	FLICE inhibitory proteins

G-CSF	Granulocyte colony stimulating factor
HBV	Hepatitis B virus
HCL	Hepatoma cell lines
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HIV	Human immunodeficiency virus
HVR	Hypervariable region
IAPs	Inhibitors of apoptosis proteins
IC	Intracytoplasmic domain
ICE	Interleukin-1 β converting enzyme
IFN-α	Interferon alpha
IGF-BP3	Insulin like growth factor binding protein-3
IRES	Internal ribosome entry site
ISEL	In situ end labelling
KDa	Kilo Dalton
LAD-1	Leucocyte adhesion deficiency-1
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAC	Membrane attack complex