

U9PH
D5338



TRAIL MEDIATED APOPTOSIS IN ARTHRITIS

**ANAK AGUNG SAGUNG SRI KENCANA DHARMAPATNI
MD, MSc**

**DISCIPLINE OF PATHOLOGY SCHOOL OF
MEDICAL SCIENCES, THE UNIVERSITY OF
ADELAIDE, SOUTH AUSTRALIA**

**A thesis submitted to the University of Adelaide in the
fulfilment of the requirements for the Doctor of
Philosophy (Medicine)**

2007

TABLE OF CONTENTS

TITLE	i
DECLARATION OF ORIGINALITY	iii
ACKNOWLEDGEMENT	iv
PUBLICATIONS AND PRESENTATIONS	vi
ABBREVIATIONS	ix
TABLE OF CONTENTS.....	xiii
LIST OF FIGURES.....	xvi
LIST OF TABLES.....	xix
CHAPTER I : LITERATURE REVIEW.....	1
I.1 INTRODUCTION.....	1
I.2 SPONDYLOARTHROPATHIES (SpA).....	3
I.2.1 Disorders Included in SpA	4
I.2.2 Pathogenesis of SpA.....	8
I.3 OSTEOARTHRITIS (OA).....	8
I.3.1 Significance of Articular Cartilage.....	9
I.3.2 Pathology in OA.....	10
I.3.3 Histochemical Grading for OA cartilage.....	13
I.3.4 Synovitis in Osteoarthritis.....	13
I.3.5 Classification Criteria for Osteoarthritis	14
I.4 RHEUMATOID ARTHRITIS	15
I.4.1 Immuno- Pathogenesis of Rheumatoid Arthritis.....	16
I.4.2 Pathogenesis of Synovitis in Rheumatoid Arthritis	16
I.4.3 Changes in the Synovial Endothelial Cells in RA Synovitis.....	18
I.4.4 Expression of Proinflammatory Cytokines in RA Synovitis.....	19
I.4.5 Classification Criteria for Rheumatoid Arthritis	22
I.4.6 Histopathological Grading System Diagnostic Pathology in Chronic Synovitis	22
I.4.7 Comparison between RA, OA and SpA Synovitis.....	22
I.5 APOPTOSIS.....	25
I.5.1 Surface Molecules and Death Receptors (DR) that Mediate Apoptosis	25
I.5.2 Molecules that Inhibit Apoptosis.....	33
I.5.3 Apoptosis in the Pathogenesis of Rheumatoid Arthritis.....	35
I.5.4 Modulating Apoptosis in the Management of Arthritis	38
I.6 HYPOTHESIS.....	40
I.7 THESIS AIMS.....	41
CHAPTER II: TRAIL, TRAIL RECEPTORS AND APOPTOSIS IN INFLAMED AND NORMAL HUMAN SYNOVIAL TISSUES	42
II.1 SYNOPSIS	42
II.2 HYPOTHESIS	42
II.3 AIMS.....	42
II.4 INTRODUCTION.....	42
II.5 METHODS	45
II.5.1 Patient Demographics	45
II.5.2 Preparation of Frozen Tissues.....	53
II.5.3 Preparation of Paraffin Embedded Tissues.....	53
II.5.4 Routine Histology Staining (Hematoxylin Eosin staining).....	53
II.5.5 Immunohistochemistry (IHC).....	54

II.5.6	TUNEL Staining	59
II.5.7	Microscopic Analysis and Semiquantitative Scoring (SQA) of IHC and TUNEL Staining	60
II.5.8	Statistical Analysis of IHC and TUNEL Staining Results	60
II.6	RESULTS	61
II.6.1	Histological Features of the Synovial Membrane from Patients with Various Arthritides and Normal Subjects	61
II.6.2	TRAIL Expression in the Synovial Membrane from Patients with Various Types of Arthritides and Normal Control	64
II.6.3	TRAIL Receptor Expression	66
II.6.4	Correlation of Expression between TRAIL and TRAIL Receptors with Laboratory and Radiological Findings	78
II.6.5	Cells Expressing TRAIL and TRAIL Receptors	80
II.6.6	Apoptosis in Various Synovial Tissues	87
II.6.7	The Expression of IAP Family Proteins; Preliminary Study	93
II.7	DISCUSSION	100
CHAPTER III: REGULATION OF TRAIL AND TRAIL RECEPTOR EXPRESSION BY SYNOVIAL FIBROBLASTS OBTAINED FROM RHEUMATOID AND OSTEOARTHRITIC INDIVIDUALS.....		109
III.1	SYNOPSIS.....	109
III.2	HYPOTHESIS	109
III.3	AIMS	109
III.4	INTRODUCTION	110
III.5	METHODS	111
III.5.1	Isolation of Synovial Fibroblasts	111
III.5.2	Flow Cytometry	111
III.5.3	Immunofluorescence.....	113
III.5.4	Immunocytochemistry	113
III.5.5	Synovial Fibroblast Phenotyping.....	114
III.5.6	Reverse Transcription Polymerase Chain Reaction (RT PCR)	114
III.5.7	Pretreatment of Cells with Proinflammatory Cytokines TNF- α and IL-1 β	117
III.5.8	Analysis of Data.....	117
III.6	RESULTS	118
III.6.1	Expression of TRAIL and TRAIL Receptors in the TRAIL-Sensitive Human Breast Cancer Cell Line, MDA MB 231.....	118
III.6.2	Expression of TRAIL and TRAIL Receptors in RASF	122
III.6.3	Expression of TRAIL and TRAIL Receptors in OASF	127
III.6.4	Effect of TNF- α and IL-1 β Treatments on TRAIL and TRAIL Receptor Expression in RASF and OASF.....	129
III.6.5	Effect of TNF- α and IL-1 β Treatments on TRAIL and TRAIL Receptor mRNA.....	136
III.7	DISCUSSION	138
CHAPTER IV: THE SENSITIVITY OF RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS AND OSTEOARTHRITIS SYNOVIAL FIBROBLASTS TO APOPTOSIS INDUCED BY TRAIL, METHOTREXATE AND ACTINOMYCIN-D.....		143
IV.1	SYNOPSIS	143
IV.2	HYPOTHESIS.....	143
IV.3	AIMS	143

IV.4 INTRODUCTION	144
IV.5 METHODS	147
IV.5.1 Isolation of Synovial Fibroblasts	147
IV.5.2 Induction of Apoptosis with Soluble TRAIL, MTX and Actinomycin-D	148
IV.5.3 Induction of Apoptosis with TRAIL, TRAIL R1 mAb and TRAIL R2 mAb	148
IV.5.4 Cell Viability Assay (Crystal Violet).....	149
IV.5.5 DAPI Staining of Nuclei.....	149
IV.5.6 Annexin V-FITC and PI staining.....	149
IV.5.7 TUNEL Immunofluorescence in Cultured Cells	150
IV.5.8 Blocking of Decoy Receptors	150
IV.5.9 Blocking of Death Receptors	150
IV.5.10 Combination Treatment.....	151
IV.5.11 Effect of Actinomycin-D Pretreatment on TRAIL Induced Apoptosis of RASF	151
IV.5.12 Effect of Pretreatment of Cells with TNF- α and IL-1 β on the Sensitivity of RASF to TRAIL, MTX and Actinomycin-D Induced Apoptosis	152
IV.5.13 Caspase -3 Colorimetric Assay.....	152
IV.5.14 RT-PCR	152
IV.5.15 Statistical Analysis	152
IV.6 RESULTS	154
IV.6.1 Sensitivity of RASF and OASF to TRAIL, MTX and Actinomycin-D Induced Apoptosis	154
IV.6.2 Effect of Blocking Decoy and Death Receptors on TRAIL-Induced Cell Death	161
IV.6.3 The Effect of the Combination Treatments of TRAIL/MTX and TRAIL/Actinomycin-D on RASF Cell Death	163
IV.6.4 Effect of Actinomycin-D Pretreatment on TRAIL Induced Cell Death of RASF	165
IV.6.5 Effect of TNF- α and IL-1 β Pretreatment on TRAIL, MTX and Actinomycin-D Induced Cell Death	166
IV.6.6 Change in mRNA Expression of TRAIL, TRAIL Receptors and Caspases after Treatment with TRAIL, MTX or Actinomycin-D	169
IV.6.7 Change in the Expression of TRAIL R2 in RASF after Treatment with TRAIL, MTX and Actinomycin-D; FACS Analysis.....	171
IV.6.8 Caspase-3 Activation in RASF after Treatment with TRAIL, MTX and Actinomycin-D	174
IV. 7 DISCUSSION.....	175
CHAPTER V: CONCLUSION.....	179
REFERENCES	185
APPENDIX 1 CHEMICAL USED IN THE STUDIES.....	210
APPENDIX 2 STATISTICAL ANALYSIS	215
APPENDIX 3 FLOW CYTOMETRY ANALYSIS EFFECT TNF- α AND IL-1 β ON TRAIL, TRAIL R1, TRAIL, R3, TRAIL R4 EXPRESSION IN RASF AND OASF.....	225

CHAPTER I : LITERATURE REVIEW

I.1 INTRODUCTION

Arthritis is a term commonly used for diseases that target the joint. Diseases such as, Rheumatoid arthritis (RA), Osteoarthritis (OA), and spondyloarthropathy (SpA) are major inflammatory joint disorders found in the community and are the subject of this thesis.

RA is a common form of arthritis in the community with approximately 1% of all people in Western societies being affected (Emery *et al.*, 2001). The prevalence of rheumatoid arthritis has been reported to vary between 0.3 and 2% of the population in many studies. Some studies reported an estimated prevalence of 0.5% in Spain (Carmona *et al.*, 2002; Dubska *et al.*, 2005) while Symmons et al reported the prevalence of RA was 1.16% in women and 0.44% in men in the United Kingdom (Symmons *et al.*, 2002). In the United States Rasch et al (2003) reported the prevalence of RA to be about 2% of the population (Rasch *et al.*, 2003). In Asian populations, the prevalence varies from below 0.4% in China, Indonesia and Philipines to 0.75% in India (Mijiyawa, 1995). Health care resource expenditure is over 3 times higher in patients with RA compared to people without RA (Sorensen, 2004). There is also an increased mortality rate in RA patients compared with normal subjects due to coronary artery atherosclerosis resulting from the disease (Van Doornum *et al.*, 2002). For example, a study has shown that mortality is nearly two times higher in RA patients in the Spanish population (Martinez *et al.*, 2001).

Rheumatoid arthritis affects females more than males (Pietschmann, 2001) by two to three times. In addition, females tend to develop the first clinical features earlier than males with a median age of about 45 years for women compared to 50 in males (Goemaere *et al.*, 1990). These findings indicate that RA is an autoimmune disease associated with hormonal factors, however, other genetic factors, such as human leucocyte antigens-DR (HLA-DR) genotypes, are also likely to be important (Feldmann *et al.*, 1996).

Synovitis in RA is caused by hyperplasia of the synovial membrane due to a migration of cells from the vasculature as well as proliferation of the residential cells in the synovial membrane. This leads to the formation of pannus which damages cartilage and bone. Reduction in the accumulation of cells in RA synovial membrane is associated with

reduced severity of the disease. The main focus of this investigation is to determine if this increase in cell number is caused by a defect in programmed cell death (apoptosis) (Firestein *et al.*, 1995; Rabinovich, 2000).

Osteoarthritis (OA) is the most common form of arthritis (Peat *et al.*, 2001) and is characterized by focal and slow progressive degeneration of articular cartilage, sclerosis of the subchondral bone, and formation of osteophytes at the joint margins. The pathogenesis involved in OA is probably multifactorial (Felson & Zhang, 1998). OA can be classified as primary (idiopathic) and secondary depending upon the presence or absence of underlying conditions. Primary OA occurs mainly in the middle age or in the elderly, without obvious association with other diseases although there are recognised risk factors, such as obesity, (Felson *et al.*, 2004; Sharma *et al.*, 2000), abnormalities of joints and malnutrition that may predispose individuals to OA (Lorenz & Richter, 2006). Other underlying conditions include genetic (as in some primary OA), biochemical (as in metabolic disease), biomechanical (as in many form of secondary OA) and hormonal factors (Benito *et al.*, 2005; Goldring, 2000; Sandell & Aigner, 2001). Secondary OA may develop at any age in a joint damaged by trauma, disease, or deformity. The histopathological features of OA include depletion of matrix proteoglycans, cartilage fibrillation, cloned or clusters of chondrocytes, and chondrocyte death. In addition, osteophytes formation is a hallmark of OA.

Cartilage and bone degradation in both OA and RA usually leads to chronic pain and disability which is costly to treat. Surgery, such as total knee or hip arthroplasty, may often eventually be required because the joint has limited ability to repair itself. As a result, the medical cost of treating end stage OA and RA patients is high due to the added expense of surgical intervention. Preventing or inhibiting the cartilage damage will not only reduce the disability associated with OA and RA but will also reduce the cost to the community. One factor that contributes to the progressive nature of cartilage degradation in OA and RA is the limited ability of chondrocytes to regenerate. Although OA is not usually associated with inflammation it is possible that a dysregulation of cell death may be involved in the pathogenesis. Stimulation of chondrocyte death observed in OA (Heraud *et al.*, 2000; Kapitonova & Mansor, 2003; Sharif *et al.*, 2004) may be due to apoptosis. It may therefore, be beneficial to inhibit apoptosis of the chondrocyte population and subsequently prevent the cartilage degradation observed in OA or RA.

While the histopathology in RA occurs predominantly in the synovial membrane, histopathological features in OA occur predominantly in the cartilage with minimal changes in the synovial membrane. The synovial membrane in OA may show fibrosis but minimal inflammatory changes are observed compared with RA. However, synovitis can be common in advanced OA (Myers *et al.*, 1990).

Spondyloarthropathy (SpA) describes a group of related rheumatic disorders that have clinically features that are subtly distinct from RA. SpA is a chronic inflammatory disease of the joints, which have varying degrees of involvement of the spine and peripheral joint. SpA disorders are variably associated with the expression of the class I antigen, HLA B27 (Khan, 2002; Pavy *et al.*, 2005), with particularly strong linkage to ankylosing spondylitis (AS) as well as having an association with Reactive arthritis. SpA usually affects the sacroiliac joints with inflammation prevalent in the synovium and the entheses (enthesitis). Entheses are the regions of bone where the ligaments, tendon, joint capsule and fascia attach. Unlike RA in which synovitis is the initial primary pathology seen, synovitis in SpA occurs secondary to the effects on the enthesitis (McGonagle *et al.*, 1998). The synovitis of SpA is also the subject of this thesis in comparison with OA and RA.