

**EXPRESSION OF INTERLEUKIN 4, INTERLEUKIN 4
RECEPTOR AND IL-4 RECEPTOR RELATED
GP200-MR6 MOLECULE IN PTERYGIUM AND NORMAL
BULBAR CONJUNCTIVA TISSUE**

By

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DISCLAIMER

I hereby certify that the work in this dissertation is my own except for the quotations which have been duly acknowledged.

Dated November 30th 2001



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Dear Lord Buddha, only in Thou I've found my refuge! Sadhu!

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May God bless!

ABSTRACT

IL-4 has been shown to have anti-tumoural activity in many cell lines. IL-4 needs to bind with IL-4 Receptor to exert its action. Down-regulation of IL-4 associated gp200-MR6 expression has been associated with increasingly malignant tissues.

Fifty-one pterygium and forty-one superior bulbar conjunctiva tissues from patients with pterygium, along with fourteen conjunctiva samples from pterygium-free subjects are subjected to immunohistochemical analysis to determine the expression of IL-4, IL-4 receptor and IL-4 receptor associated gp200-MR6 molecule.

Analysis of paired pterygium and conjunctiva samples revealed a lower percentage of pterygium stained positive for IL-4 and MR6. This trend is reversed for the expression of IL-4 receptor. None of these findings are statistically significant.

This may represent a down-regulation of IL-4 and MR6 secondary to chronic actinic assaults. The up-regulation of IL-4R expression may be a compensatory mechanism to cope with less IL-4 concentration. The fact that differential expression of IL-4 and its receptors in pterygium and 'normal' conjunctiva tissue is not significant statistically points towards the field change sustained by conjunctiva when there is an actinic damage to the limbal stem cells from the very beginning.

The age of the patient and the size of pterygium tissues do not seem to influence the expression of IL-4, IL-4 receptor and IL-4 receptor associated gp-200 MR6 molecule in such tissues. Higher percentage of recurrent pterygium expressed IL-4 and MR6. Subjects with hypertension, diabetes and allergy disorders has less IL-4 and gp-200 MR6 but more IL-4R expression on the pterygium tissues. Smoker seems to have higher staining for IL-4. This may again be the reflection of our body's immune mechanism coping with various different forms of stress.

Higher percentage of conjunctiva of pterygium-free patients express IL-4R ($p < 0.05$). Similar trend is true for MR6 molecule but statistically not significant.

In the light of these evidences, more studies should be carried out to determine the presence of other cytokines or growth factors in ocular surface so that a composite picture of this possible autocrine loop that regulates the homeostasis of ocular surface can be elucidated.

ABSTRAK

IL-4 mempunyai aktiviti anti-ketumbuhan dalam sebilangan besar aliran sel yang telah dikaji. Untuk menjalankan fungsinya, IL-4 mesti bergabung dengan reseptor IL-4 (IL-4R). Penurunan regulasi ekspresi gp200-MR6 telah dikaitkan dengan peningkatan malignansi tisu.

Sejumlah lima puluh satu tisu pterygium dan empat puluh satu tisu bulbar konjunktiva telah diambil daripada pesakit-pesakit yang menjalani pembedahan pembuangan tisu pterygium. Sejumlah empat belas sampel konjunktiva juga diperolehi daripada subjek yang tidak mempunyai ketumbuhan pterygium. Analisa imunohistokimia telah dijalankan ke atas semua sampel yang terkumpul untuk menentukan ekspresi IL-4, IL-4R dan gp200-MR6.

Analisis yang dijalankan ke atas sampel berkembar tisu pterygium dan konjunktiva mendapati peratusan keputusan positif yang lebih rendah untuk IL-4 dan gp200-MR6 dalam tisu pterygium. Manakala tisu pterygium didapati mempunyai peratusan keputusan positif yang lebih tinggi untuk IL-4R berbanding tisu konjunktiva. Namun demikian, ia tidak signifikan (bererti) mengikut analisa statistik.

Keputusan ini mungkin mencerminkan penurunan regulasi ekspresi IL-4 dan MR6 disebabkan pendedahan aktinik yang kronik. Peningkatan regulasi ekspresi IL-4R

mungkin merupakan suatu mekanisme kompensatori tisu semasa menghadapi konsentrasi IL-4 yang berkurangan. Perbezaan keputusan ekspresi IL-4 serta reseptor-reseptornya dalam tisu pterygium dan tisu konjuntiva yang tidak signifikan mengikut tafsiran statistik besar kemungkinan disebabkan oleh perubahan peralihan medan berperingkat yang dihadapi tisu konjuntiva apabila sel induk limbusnya mengalami kerosakan actinik dari peringkat permulaan lagi.

Faktor umur subjek dan saiz tisu pterygium didapati tidak mempengaruhi ekspresi IL-4, IL-4R dan MR6 dalam tisu pterygium. Peratusan tisu pterygium yang rekuren (berulang) yang didapati positif terhadap IL-4 dan MR6 adalah lebih tinggi daripada tisu pterygium primer. Subjek yang mengalami penyakit darah tinggi, diabetis dan penyakit alergi didapati mempunyai ekspresi IL-4 dan MR6 yang kurang berbanding dengan subjek yang normal. Perokok pula mempunyai ekspresi IL-4 yang lebih tinggi. Semua ini mungkin menggambarkan mekanisme daya ketahanan tubuh kita apabila menghadapi tekanan yang pelbagai.

Peratusan lebih tinggi untuk tisu konjuntiva daripada subjek yang tidak mempunyai ketumbuhan pterygium menunjukkan ekspresi IL-4R yang lebih tinggi. Keputusan ini adalah signifikan mengikut analisa statistik.

Sesungguhnya, daripada kesimpulan keputusan-keputusan yang diperolehi dalam kajian ini, lebih banyak kajian perlu dijalankan untuk menentukan kehadiran serta peranan sebenar yang dimainkan oleh pelbagai sitokin dan faktor ketumbuhan dalam tisu permukaan okular. Ini diharapkan akan dapat memberi gambaran yang lebih jitu tentang kemungkinan kehadiran suatu lengkaran autokrin yang mengawal proses homeostasis pada permukaan tisu ocular.

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LIST OF ABBREVIATIONS

Ab(s)	Antibody(-ies)
Ag(s)	Antigen(s)
DAB	Diaminobenzedine
DH₂O	Distilled water
EGF	Epidermal growth factor
EGFR	epidermal growth factor receptor
H&E	Hematoxylin and eosin
Ig(s)	Immunoglobulin(s)
IL(s)	Interleukin(s)
IL-R(s)	Interleukin receptor(s)
KD	kilodalton
Mab(s)	Monoclonal antibody(ies)
NS	Not significant statistically
OCT	Optimum Cold Temperature
PBS	Phosphate-buffered saline
SPSS	Statistical Package for Social Science
SS	Statistically significant
TNF	Tumour necrosis factor

INTRODUCTION

1. INTRODUCTION

Pterygium is not just a degenerative disease, but may be a proliferative disorder of the ocular surface. This may explain the high incidence of recurrent rate after its primary excision. Studies have consistently shown that countries nearer the equator have higher rates of pterygium. A possible reason for this geographic variation is that the ultraviolet B irradiation may be one of the most important risk factors for the development of pterygium.

While many treatment modalities have been attempted to manage pterygium, it is equally important to try to elucidate the consequences of chronic UV light exposure on this tissue. This will provide us with valuable information regarding the changes in its micro-environment which may form the basis of possible new and more effective treatment modalities. In the light of better understanding of the epithelial biology of the ocular surface and availability of improved materials and methods in immunological research, potential plausible pathogenesis of this tissue can be identified.

This study involved the use of immunocytochemical technique to gauge the expression of IL-4, IL-4 Receptor and IL-4 Receptor related gp200-MR6 molecule, the presence of all of which has been investigated and proven to play a role in the pathogenesis of malignancy in other tissues and other cell lines. Their potential role in pterygium has not been explored thus far.

OBJECTIVES OF STUDY

2. OBJECTIVES OF STUDY

2.1 GENERAL OBJECTIVE

The aim of this study is to investigate the expression and postulate the role of IL-4 and its receptors in the pathogenesis of pterygium

2.2 SPECIFIC OBJECTIVES

- i. To compare the expression of IL-4, IL-4 receptor and MR6 molecule in pterygium and normal conjunctiva tissue unexposed to UV light in patients with pterygium.
- ii. To compare the expression of IL-4, IL-4 receptor and MR6 molecule in pterygium in relation to the age of the subjects, size of the pterygium and other clinical parameters.
- iii. To compare the expression of IL-4, IL-4 receptor and MR6 molecule in normal conjunctiva tissue unexposed to UV light in subjects with and without pterygium.

2.3 NULL HYPOTHESIS

- i. There is no significant difference of expression of IL-4, IL-4 receptor and IL-4 receptor related gp200-MR6 molecule in pterygium and normal conjunctiva tissue in patients with pterygium.
- ii. There is no significant difference of expression of IL-4, IL-4 receptor and IL-4 receptor related gp200-MR6 molecule in pterygium in relation to the age of the subjects, size of the pterygium and other clinical parameters.
- iii. There is no significant difference of expression of IL-4, IL-4 receptor and IL-4 receptor related gp200-MR6 molecule in normal conjunctiva tissue unexposed to UV light in subjects with and without pterygium.

BACKGROUND

3. BACKGROUND

Ever since Susruta recorded the removal of a pterygium lesion in 1000 BC (Jaros PA *et al*, 1988), this triangular fold of vascularised bulbar conjunctiva still poses a challenge to the practising ophthalmologists and researchers alike. Its histology and the ultrastructure has been studied extensively (Duke-elder S, 1954; Spencer WH *et al*, 1985; Hogan MJ *et al*, 1967; Austin P *et al*, 1983) and hypothesis put forth suggesting its aetiopathogenesis (Moran DJ *et al*, 1984; Karai I *et al*, 1984; Wong WW, 1978; Pinkerton OD *et al*, 1984), hoping to find a more satisfactory management of this disease process as it has a high recurrent rate after primary excision.

Duke-Elder has defined pterygium as a degenerative and hyperplastic process (Duke-Elder S, 1954) where there is accumulations of amorphous, eosinophilic, hyalinised or granular-appearing material resembling degenerated collagen interspersed with coiled or fragmented fibres resembling abnormal elastic tissue (Spencer WH *et al*, 1985) which interestingly is resistant to the nonproteolytic enzyme elastase, hence the terms elastoid and elastotic degeneration (Cogan DG *et al*, 1959). It is often found that the stromal fibrocytes are increased in number as if proliferating in response to injury. Minor aggregates of proteinaceous substance, acid mucopolysaccharide and calcific concretions are also seen in older lesion.

Austin P, Jackobiec *et al* concluded that a large component of pingueculae and pterygia is the result of newly synthesised elastic fibre precursors with abnormal maturation (elastodysplasia) that undergo secondary degeneration (elastodystrophy) (Austin P *et al*, 1983). The histological features have uncanny resemblance to those found in pingueculae and in the dermis of sun-exposed skin. Clinically, pterygium differs from pinguecula by exhibiting progressive invasion of fibrovascular tissue into the cornea. This actinic elastosis are widely believed to arise from an abnormal elastogenesis secondary to fibroblastic activation by UV radiation. (Spencer WH *et al*, 1985; Ledoux-Corbusier M *et al* 1979)

Immunologic basis for the pathogenesis of pterygium has gained substantial momentum in the last decade. In addition to the early demonstration of plasma cell and lymphocytes infiltration in the stroma with the presence of IgG and IgE, which is absent in normal conjunctiva (Austin P. *et al*, 1983). Liu L *et al* have also demonstrated that most of the lymphocytes are T-cell (CD3+) and alteration of the helper-suppressor ratio from 1:2.7 in the normal conjunctiva to 1:1.5 in pterygium, which lead them to believe it to be a phenomenon of type 1,3 and 4 hypersensitivity (Liu L *et al*, 1993). Mast cells count in pterygium was found to be twice as high as in normal conjunctiva (Nakagami T *et al*, 1997, 1998).

In the quest of finding growth factors in pterygium, Kria *et al* has demonstrated with immunohistochemical and ELISA analysis that basic fibroblast growth factor (b-FGF) is strongly expressed in tissue cultures from recurrent pterygium as the platelet-derived growth factor (PDGF) in primary pterygium. There is however only a weak expression of both transforming growth factor beta (TGF- β) and tumour necrosis factor alpha (TNF

α). All four growth factors are expressed strongly in pterygium tissue *in vivo*. All these GFs were however sparse in normal conjunctival fibroblasts (Kria L *et al*, 1998). These findings provide evidence that b-FGF may participate in recurrence and rapid growth of pterygium by its angiogenic activity. TGF- β , being a fibrogenic factor may enhance the fibrotic process. (Kria L *et al*, 1998).

TGF- β family contains prototypic and potent cytokines involved in fibrosis (scarring) during wound repair processes (Border WA *et al*, 1992; Roberts AB *et al*, 1993; Grande JP *et al*, 1997). They consist of 3 different isoforms and they exert their actions via binding with three respective receptors.

Establishment of the limbal location of corneal epithelial stem cells has brought about the concept of limbal stem cell deficiency. (Schermer A *et al*, 1986; Tseng SCG *et al*, 1989; Tseng SCG *et al*, 1996). In normal subjects, the epithelial mass formed at the limbus constitutes a growth pressure and acts as a junctional barrier to prevent conjunctival epithelial migration onto the corneal surface in the event of a total corneal epithelial defect. When there is an insult to this region, a spectrum of abnormal corneal surfaces is produced (Chen JJY *et al*, 1991). This can manifest as conjunctivalization with vascularization, chronic inflammation, destruction of the basement membrane and fibrous ingrowth.

Kwork LS *et al* has demonstrated in his animal model for pterygium, that an albedo (indirect) light projected from the temporal sclera is focused and concentrated at the nasal limbus, suggesting that UV light might cause focal alteration of the limbal tissue thus causing pterygium formation (Kwork LS *et al*, 1994). This can perhaps explained

the nasal bulbar conjunctiva predilection of pterygium and the intrinsic abnormalities in DNA repair as a result of UV irradiation as evident by a high incidence of microsatellite instability and loss of heterozygosity. (Spandidos DA *et al*, 1997).

Pterygium can indeed be locally invasive, and the pterygium epithelium exhibits varying degrees of abnormality, ranging from mild dysplasia to carcinoma in-situ (Clear AS *et al*, 1979). Tan DTH *et al* and Dushku *et al* have independently in their immunohistochemical study with p53 monoclonal antibody found that there is increased nuclear p53 gene product in the limbal epithelium of pterygium but with little or no apoptosis. This, they concluded, is consistent with an activating mutation of p53 gene secondary to UV irradiation (Tan DTH *et al*, 1997; Dushku N *et al*, 1997). p53 is a tumour suppressor gene, it is the most common marker of human neoplastic growth. It is thought to act as a transcription factor that activates or represses the expression of growth-controlling genes and is abnormally expressed in a variety of human cancer (Greenblatt MS *et al*, 1994) as well as in actinic skin lesions (Sim CS *et al*, 1992).

Dushku *et al* also demonstrated the expression of vimentin in limbal epithelium of pterygium, which is normally found in mesenchymal cells and migrating epithelial cells. (Dushku N *et al*, 1994). In the related development, Kennedy M *et al* has demonstrated that ultraviolet B light (UV B) does induce the production of multiple cytokines (IL-1, IL-6, IL-8 and TNF α) by the corneal stromal cells. (Kennedy M *et al*, 1997).

Interleukins (IL) as a part of the cytokines family were first described as a group of signalling polypeptides, controlling the activity of lymphoid and haemopoietic cells (O' Garra A, 1989a; 1989b). Various human tumours have been shown to express receptors

for many different ILs (McMillan *et al*, 1995; Topp *et al*, 1995; Moore *et al*, 1999). Exposure of these cells to variety of ILs leads to positive or negative receptor-mediated regulation of cell growth. In the recent years, however, emphasis has been on the possibility that human tumour cells of non-lymphoid origin may also be capable of producing and / or responding to some of these cytokines. For example, IL-1 (α & β) and IL-6 has been shown to stimulate the proliferation of certain tumour cell lines in vitro (Tsai S.C. *et al*, 1987; Howard M *et al* 1982).

IL of interest in this study is IL-4, a member of the cytokine family with multifunctional activities on a variety of cell lines. It is a glycoprotein of 129 amino acids with a molecular weight of 15 000 - 20 000 Dalton. IL-4 is glycosylated at two arginine residues (position 38 and 105) and contains six cysteine residues involved in disulfide bond formation, which is essential for its biological activity.

IL-4 is secreted by activated CD4 T cell of Th 2 subset, also produced by mast cells and basophils. Initially described as a B-cell growth factor (Howard M *et al* 1982), it has subsequently shown to have a wide spectrum of activities on T lymphocytes, macrophages, granulocytes and epithelial cells (O'Hara J *et al*, 1987; Monroe JG *et al* 1988; Thornhill MH *et al*, 1990; Toi M *et al* 1991). Being a prototypic type 2 immunoregulatory cytokine (Brown MA *et al*, 1997; Chomarat P *et al* 1997), it modulates the production of cytokine by endothelial cells, monocytes and macrophages, also reducing inflammation by stimulating the production of monocytes IL-1 Receptor antagonist (Chomarat P *et al* 1995) and soluble 'decoy' IL-1 receptor (Calotta F *et al*, 1993).

IL-4 has been reported to exert anti-proliferative effect on some cancer cells. Hoon *et al* have demonstrated that IL-4 alone or in combination with other cytokines could modulate cell surface Ag expression and inhibited growth of human melanoma cells in a dose-dependent manner, its anti-proliferative effect synergises with IFN- γ . IL-4 also enhanced the anti-proliferative effect of TNF- α and the additive effect was significantly more potent than the individual cytokines or IL-4 plus IFN- γ (Hoon *et al* 1991).

Accumulating data from transfection assays with the IL-4 gene or treatment of cell lines with IL-4 show that IL-4 has a potent anti-tumoural activity e.g. B.16 melanoma cell line in mice, mammary adenocarcinoma and Lewis Lung carcinoma (Zaloom Y *et al*, 1993; Tepper RI *et al*, 1989). Providing the evidence that IL-4 gene -transfected CA cells can be used for CA immunotherapy (Ohira T *et al* 1992). Synergism between IL-4 production and other growth inhibitors has also been described (Thornhill MH *et al*, 1990). Expression of IL-4 can be studied using an IL-4 antibody with immunohistochemistry technique. (Figure 3.1)

To enable IL-4 to exert its biological effects, it needs to bind with a specific IL-4 receptor. The human IL-4 receptor has an extracellular domain of 207 amino acids, a transmembrane domain of 24 residues, and a large intracellular domain of 569 amino acids. The IL-4 receptor is a complex consisting of two chains. One chain is high affinity IL-4 binding (p140, α chain, CD 124) chain and the other chain is the γ chain. The high affinity IL-4 binding chain belongs to the cytokine receptor superfamily. The cytoplasmic domain contains Ser/Pro-rich regions similar to those present in the IL-2 receptor and GM-CSF receptor β -chains. (Callard R *et al*, 1994).

The evidence of such vital marriage between the IL-4 and IL-4 receptor is elucidated in the study of proliferation of a number of breast cancer cell lines including anchorage-dependent and independent breast cancer cell lines MCF-7 and MDA-MB-231 were inhibited by IL-4, which exerts its effect through IL-4Rs expressed on these cells. The mechanism of IL-4-induced growth inhibition in human breast cancer was thought to be the induction of apoptosis (Gooch *et al*, 1998).

Further support of IL-4 role in the immunological surveillance of tumour comes from the studies on the IL-4 receptor related gp200-MR6 molecule, using antibody for gp200-MR6 (Figure 3.2). The facts that gp200-MR6 expression is lost with increasing malignancy in lung and colonic CA, does suggest its role in tumour suppression (Tungekar G *et al*, 1991; Kaklamanis L *et al*, 1992). In breast Ca however, both upregulation and downregulation have been reported (Mat IB *et al*, 1993; Al Jabaari B *et al*, 1989). Lorenzen *et al* suggested that the loss of IL-4R related gp200-MR6 represent a mechanism of tumour escape from immune surveillance. To the best of our knowledge, the expression of IL-4 and IL-4 receptor and IL-4 receptor associated gp200-MR6 molecule on the ocular tissue has never been studied before. Thus, this study was conducted to establish the presence of these cytokines on the ocular surface.

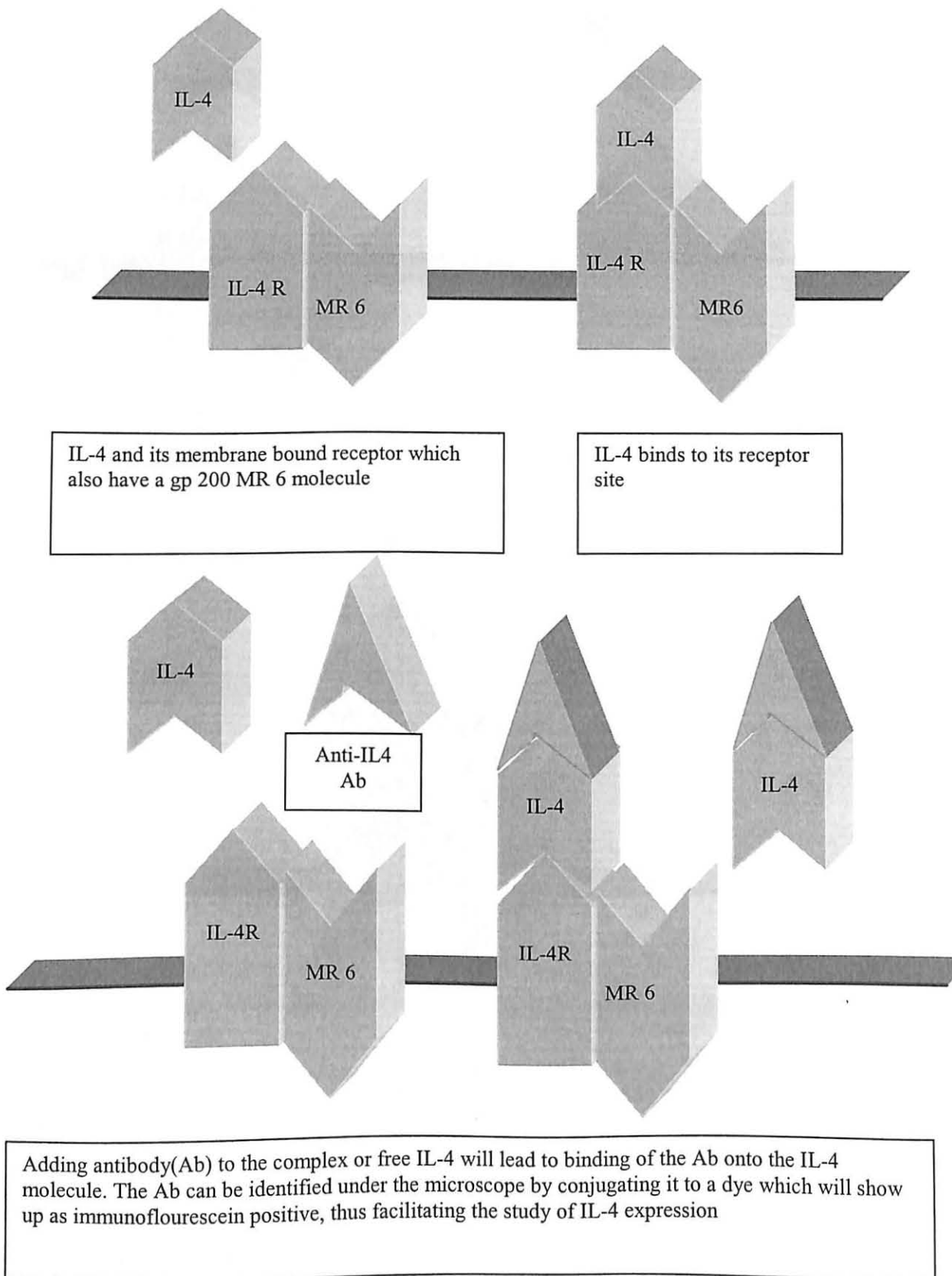
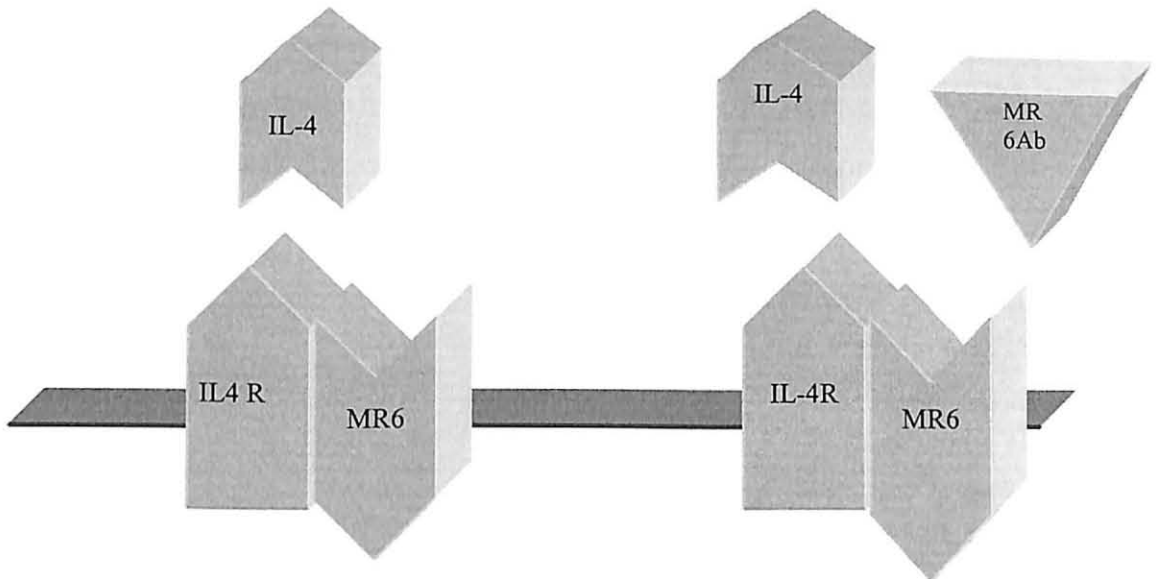
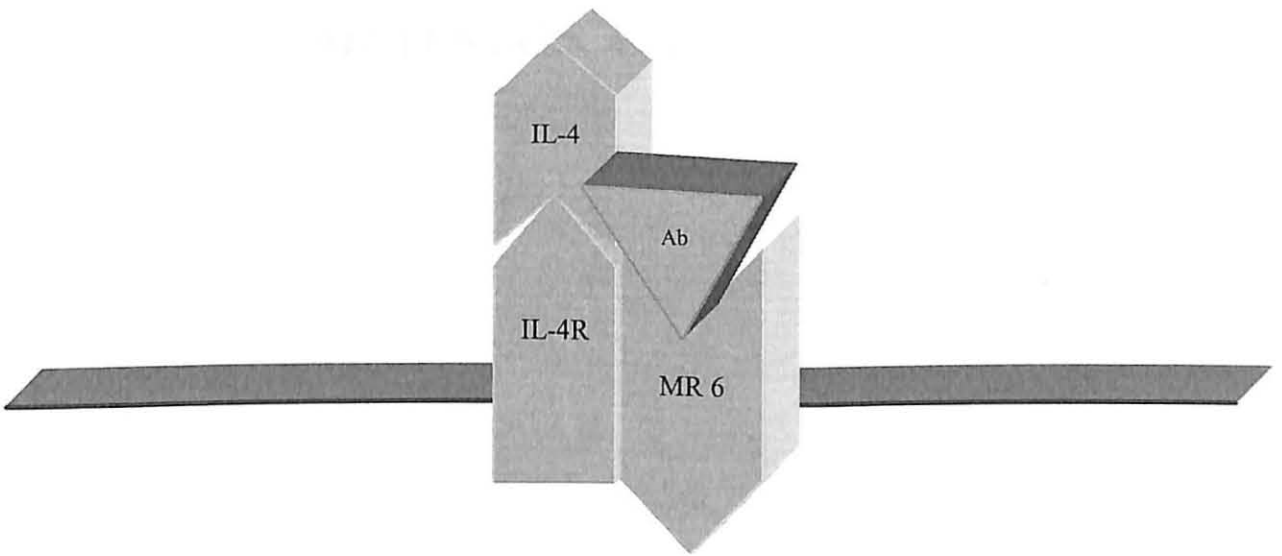


Figure 3.1 Diagrammatic representation of IL-4, IL-4 receptor and IL-4 receptor associated gp-200 MR6 molecule with anti-IL-4 Ab



IL-4 and its membrane bound receptor which also have a gp 200 MR 6 molecule

IL-4 binds to its receptor site
Addition of MR6 antibody



The Ab to MR6 will bind to the IL-4 receptor-related gp200 MR6 molecule . The Ab can then be identified under the microscope by conjugating it to a dye which will show up as immunoflourescent positive, thus facilitating the study the expression of IL-4 Receptor.

Figure 3.2 Diagramatic representation of IL-4, IL-4 receptor and MR6 with anti-MR6 Ab.

MATERIALS AND METHODS

4. MATERIAL AND METHODS

4.1 STUDY DESIGN

This is a laboratory-based comparative study. All the laboratory work is done in the laboratory of Department of pathology and Department of Immunology.

4.2.1 POPULATION, TIME AND PLACE OF STUDY

Study population: Patients attending eye clinic in Hospital USM and General Hospital Kota Bharu who have consented for pterygium excision.

Patients undergoing extracapsular cataract extraction in Hospital USM.

Period of study: 1st January 2000 to 30th November 2001

Place of study: Department of Ophthalmology, School of Medical Sciences, USM.

Department of Ophthalmology, Hospital Kota Bharu.

Immunohistochemistry laboratory, Department of Pathology, USM

4.3 SAMPLING AND SAMPLE SIZE

A convenient sampling size was taken as there was no previous study on similar subject.

4.4 SELECTION CRITERIA

4.4.1 INCLUSION CRITERIA

All patients who underwent pterygium excision or cataract extraction with otherwise normal eye were included in the study.

4.4.2 EXCLUSION CRITERIA

The following cases were excluded from the study:

- a) Patients on long term (in the last six months) or current use of topical medicated preparations (all preparation except eye lubricants without any active ingredients), nasal spray (e.g. steroid etc.).
- b) Patients on systemic steroid therapy or any other systemic immunomodulating medication (e.g. systemic cyclosporin A) or any form of chemotherapy.
- c) Patients with history of trauma or insult to the eye within the last 6 months. (postoperative, subconjunctival haemorrhage etc).
- d) Patients with hisoty of any form of conjunctivitis or inflammation in the eyes (uveitis etc).
- e) Contact lens wearers.

- f) Patients who has any form of keratoplasty performed.
- g) Patients with a known history of autoimmune disorders. (rheumatoid arthritis, SLE etc.)

4.5 CONSENT

All participating patients were explained in-depth regarding the nature and objectives of this study and the potential complications associated with tissue harvesting. This was done in patients' native language or dialects with the aid of diagrammatic representations of the procedures. Written informed consent is then obtained from the willing subjects. (Appendix C)

4.6 VARIABLES OBSERVED

The units of variables recorded include demographic datas e.g. the age, sex and race of the patients involved. The laterality of the pterygium or conjunctiva excised is duly recorded. Also recorded is the status of the pterygium, whether it is a primary or recurrent lesion. The size of the pterygium is also measured with slitlamp in millimetre (from the limbus of the cornea perpendicularly to the head of the pterygium). Also recorded is the presence of systemic illness like diabetes, hypertension, evidence of allergy or asthma and smoking.

(Data collection sheet - Appendix A and B)

4.7 SPECIMEN

Excision of tissue for the specimen is done with written consent (**Appendix C**).

Three groups of tissues are obtained.

Group 1:

Pterygium proper

Group 2:

Superior or inferior bulbar conjunctival tissue from patient with pterygium who undergone cataract extraction or patient undergoing pterygium excision with conjunctival transposition and consented for bulbar conjunctival biopsy.

Group 3:

Superior bulbar conjunctival tissue from patient who has undergone cataract extraction and no evidence of pterygium.

4.7.1 STORAGE

Specimens collected will be immediately put into a cryo-vial and snap freezed in a portable nitrogen tank before being transferred to nitrogen tank for storage.



Figure 4.1 Tissues stored in the cryovials while awaiting sectioning.



Figure 4.2 Cryovials are stacked-up in a nitrogen tank preserving the tissues at -70°C.

4.8 DEFINITION OF TERMS:

- a. Diabetes Mellitus- records of diabetic treatment or newly diagnosed diabetes with a fasting whole blood concentration of over 6.7mmol/l, or a random value exceeding 10mmol/l.
- b. Hypertension- clear records of treatment for hypertension or newly diagnosed hypertension defined as blood pressure recording of systolic more than 140mmHg and/or diastolic of more than 90mmHg sustained over three consecutive recordings at least 6 hours apart.
- c. Allergy- clear history and/or record of treatment of allergy disorders. Clear history or clinical sign and symptom of allergy illness, eg. acute exacerbation of bronchial asthma, allergic conjunctivitis, allergic rhinitis, history of urticaria or rashes after food or drug ingestion etc.
- d. Smoking- Clear history of active smoking or long duration of cigarette smoking (e.g. more than 6 months).
- e. Superior bulbar conjunctiva-- a small 3mm X 3mm conjunctival tissue harvested anywhere from eleven to one O'clock positions.
- f. Inferior bulbar conjunctiva—a small 3mm X 3mm conjunctival tissue harvested anywhere from five to seven O'clock positions.

4.9 ETHICAL APPROVAL

This Research protocol and methodology has been approved by the Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia.

Reference: USM/PPSP®/Ethics Com./2001[54.4(4)]

4.10 IMMUNOHISTOCHEMISTRY – MATERIALS AND METHOD

4.10.1 TISSUE SECTIONING

Frozen pterygium tissue was embedded in Optimum Cold Temperature (OCT) compound and allowed to set on a chuck inside the cryotome before sectioning. The temperature inside the cryotome is maintained at -28°C at all time. Longitudinal sections of the tissue was then performed with cryotome (Leica CM 1850) to achieve a thickness of $5\mu\text{m}$.



Figure 4.3 Tissue is sectioned at a thickness of $5\mu\text{m}$ with the cryostats.

4.10.2 STAINING

All specimens will be subjected to routine eosin-hematoxylin staining for better morphology interpretation. Standard immunohistochemical study is performed using Strept.Avidin-biotin complex/HRP procedure with specificity controls.

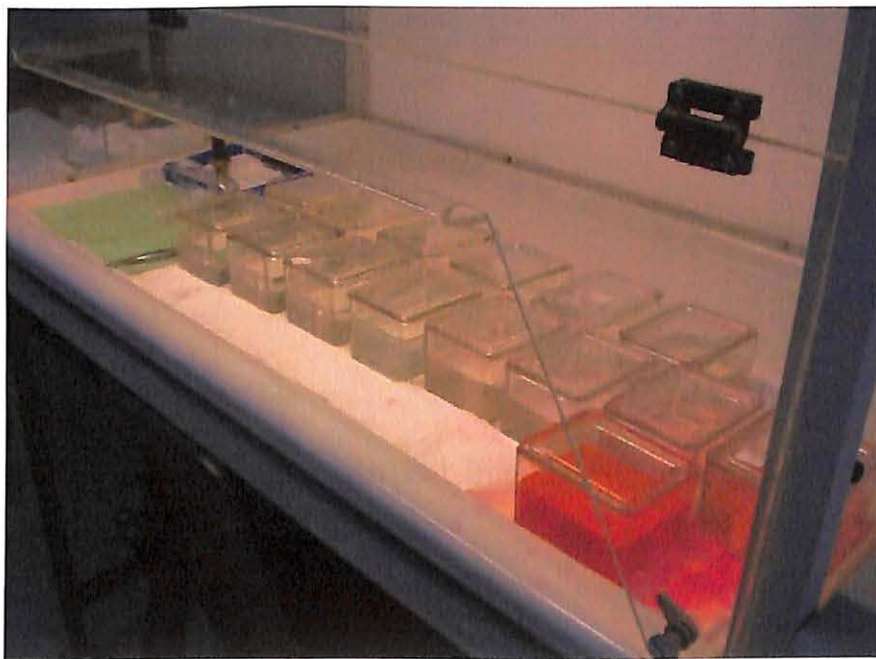


Figure 4.4 Staining station where the tissue sections are subjected to H&E staining and dehydration process