Research Report 2010–2012

THE ROYAL COLLEGE OF SURGEONS OF EDINBURGH

FROM HERE, HEALTH

Contents

Design: Omnis Partners, Cumbernauld

Foreword

David A Tolley, President, RCSEd

Recognising and supporting cutting-edge surgical research has a key part to play in achieving the College's central aim: the pursuit of excellence and advancement in surgical practice. I am therefore delighted that, despite the continuing difficult economic climate, we have maintained and expanded the research activities supported by this College, and that we are continuing to attract a high number of quality applications for the awards and bursaries we provide.

The College's Research Strategy Committee has contributed to our ongoing success in this area through providing strategic direction to these activities and assisting in identifying new opportunities. I am also grateful to the Research Allocation Committee for their continuing expert assessment of the applications we receive. It is this thorough assessment which ensures that the research projects supported are of a consistently high quality, innovative and relevant; the enclosed reports are testament to this process.

Finally, I would also like to extend my thanks to our many benefactors, both organisational and individual, whose generous contributions ensure the continued success of these awards and allow such research to continue to contribute to the expansion of surgical knowledge.

Introduction

Professor Kenneth Fearon, Chairman, RCSEd Research Allocation Committee

The pace at which biomedical science continues to move forwards is simply breathtaking. Equally, the evident limitation of financial resources for health services within 'developed nations' is becoming all too familiar. In such circumstances, it is particularly important that research is focused on unmet clinical need and that there is a clear route whereby basic research can be readily translated to the bedside. Due to its craft-based nature, surgery (and thereby surgical research) has mostly had a strong clinical component or at least an element that is readily translateable into the clinical environment. The Research Strategy Committee and Research Allocation Committee continue to support this view.

The development of an academic track in postgraduate medical training provides a well-funded and well-supervised environment in which future academics can grow until capable of gaining independent funding for research. It is evident, however, that for surgeons this model has to be welded carefully to sufficient clinical training and mentoring so that academic excellence is combined with clinical competence. The College seeks to support individuals wishing to embark on this route by providing a range of Fellowship schemes and small grants. The individual who seeks research training and experience but who does not wish to become a full-time clinical academic is valued equally.

It is always a pleasure to nurture academically minded medical students, and the summer bursary scheme continues to grow and flourish. The Syme Medal has been the subject of intense competition, with the quality of applicants being outstanding. The King James IV Professorship continues to be our most prestigious award to mark a lifetime of academic achievement. The Ophthalmology Sub-committee continues to provide a high level of grant support and fund projects of great distinction.

The activity of the Committee and the number of awards made is dependent on resources, and we are extremely grateful to the Donors of Research Funds who help sustain such vital activities. It is hoped that current fundraising efforts within The College will add to the resources available and embelish further our extensive programme of research funding.

Research Allocation Committee

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Professor C M Steel, Retired

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Mr C Widgerowitz, Senior Lecturer in Orthopaedics, Division of Surgery & Oncology, Ninewells Hospital and Medical School, Dundee

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Mr D A Tolley/Mr J L Duncan, Honorary Treasurer, Royal College of Surgeons of Edinburgh

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Professor Andrew Dick, Department of Ophthalmology, Cellular and Molecular Medicine, University of Bristol, School of Medical Sciences, Bristol

Dr B W Fleck, Consultant Ophthalmologist, Princess Alexandra Eye Pavilion, Edinburgh

Professor J V Forrester, Consultant Ophthalmologist and Head of Department of Ophthalmology, University of Aberdeen Medical School

Professor B Dhillon, Consultant Ophthalmic Surgeon, Princess Alexandra Eye Pavilion, Edinburgh

Mr G Dutton, Consultant Ophthalmologist

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Royal College of Surgeons in Ireland
Royal College of Physicians and Surgeons of Glasgow

The College and the Research Allocation Committee gratefully acknowledge the donations from numerous Fellows of the College in the UK and overseas.

Grants and Awards

Small Research Grants

Mr Thomas Madura, Specialty Registrar/Academic Clinical Fellow, Plastic and Reconstructive Surgery Research, University of Manchester (£7,000)
Autotransplantation of BDNF-expressing tissue-engineered Schwann cells to enhance post-traumatic regeneration of the peripheral nervous system

Mr Ramsey Cutress, Senior Lecturer/Consultant Surgeon, Cancer Research UK Centre, Southampton (£7,000)
Novel small molecule inhibitors of the BAG1–HSC70 interaction

Mr Mark Duxbury, Clinician Scientist and Honorary Consultant Surgeon, Cancer Research Centre, University of Edinburgh (£6,960)
Microfluidic entrapment and functional interrogation of circulating pancreatic cancer cells based on biorheological characteristics

Mr Douglas Morran, Clinical Research Fellow, Beatson Institute for Cancer Research, Glasgow (£7,000)
Investigating personalized therapies in a mouse model of pancreatic cancer

Dr Thomas Flannery, Senior Lectuer/Consultant, Centre for Cancer Research and Cell Biology, Belfast (£7,000)
Investigation of the effect of cathepsin inhibitors as an anti-invasive strategy in an orthotopic mouse glioma model

Mr Siong-Seng Liau, Clinical Lecturer and Honorary Specialist, Department of Surgery, University of Cambridge (£7,000)
Dissecting the roles of the high-mobility group A1 pathway in pancreatic adenocarcinoma progression through *in vivo* monitoring of cancer dynamics in a conditional transgenic mouse model

Miss Rachel Guest, Clinical Research Fellow, MRC Centre for Inflammation Research, University of Edinburgh (£7,000)
The role of notch signalling in the tumour-stroma microenvironment of cholangiocarcinoma

Mr Alexander Leeper, Research Fellow, Breakthrough Research Unit, University of Edinburgh (£6,848)
A novel three-dimensional assay to predict an individualised response to drug therapy in breast cancer

Mr Innes Smith, Clinical Research Fellow, Department of Trauma & Orthopaedics, University of Edinburgh (£6,980)
Effect of *Staphylococcus aureus* alpha and gamma toxins on *in situ* bovine and human chondrocytes

Mr George Tse, Core Surgery Training 2, Queen's Medical Research Institute, University of Edinburgh (£7,000)
B-Cell phenotypes in chronic injury following renal transplantation

Miss Jenny Richards, Surgical Registrar, Clinical Surgery and Centre for Cardiovascular Research, University of Edinburgh (£6,997)
Effects of heme arginate induction of heme oxygnase-1 activity on endothelial function in a healthy volunteer model of ischaemia–reperfusion injury

Miss Shauna Culshaw, Clinical Lecturer/Honorary Specialist Registrar, Glasgow Dental Hospital, University of Glasgow (£6,900)
What is the role of interleukin 33 in periodontal disease?

Mr Mohammed Sufian Miah, Specialist Registrar in ENT and Head and Neck Surgery, University of Dundee (£6,900)
Links between human papilloma virus, ERM (ezrin, radixin, moesin) proteins, the suppressor of metastasis NDPK and their associated proteins in the pathogenesis of squamous carcinomas of the head and neck

Mr Adam Frampton, Clinical Research Fellow, HPB Surgery Unit, Imperial College London (£6,809)
Revealing new microRNAs involved in epithelial-to-mesenchymal transition (EMT) in pancreatic cancer

Miss Sarah Bache, Burns Research Fellow, Burn Unit, Canniesburn Plastic Surgery Unit, University of Strathclyde (£4,700)
Clinical evaluation of a visible light source (HINS-light EDS) for continuous decontamination of the burn unit environment

Mr Arfon Powell, Research Fellow, Department of Surgery, Glasgow Royal Infirmary (£6,500)
The role of the Src kinase family in colorectal cancer

Mr Neil Johns, Clinical Research Fellow, School of Clinical Sciences & Community Health, University of Edinburgh (£5,000)
Genetic markers for sarcopenia/muscle wasting in cancer

Mr Stephen O'Neill, Clinical Research Fellow, MRC Centre for Inflammation Research, University of Edinburgh (£6,050)
Role of heat shock protein 90 inhibitors in modulating ischaemia–reperfusion injury in kidney transplantation

Dr Colin Watts, Consultant Neurosurgeon, Department of Neurosurgery, University of Cambridge (£7,000)
Evaluation of the EIAV-vector endoangio-GT platform as a surgical tool to target tumour competent cell populations in glioblastoma

Miss Olivia McBride, Surgical Registrar (ST3), Clinical Surgery and Centre for Cardiovascular Research, University of Edinburgh (£6,996)
Strategies to improve haemostasis: evaluation of lyophilized human platelets and tranexamic acid

Mr Richard Stevenson, Clinical Research Fellow, Beatson Institute for Cancer Research (£6,061)
Targeting the actin cytoskeletal protein fascin as a novel modulator of intestinal epithelial cell proliferation and colon cancer

Dr Matthew Bedford, Clinical Research Fellow, School of Cancer Sciences, University of Birmingham (£6,900)
A role for deferasirox as an anti-tumour and chemosensitising agent in oesophageal adenocarcinoma

Bursaries for Undergraduate Elective or Vacation Awards

Miss Abigail Campbell, Institute of Infection, Immunity and Inflammation, University of Glasgow (£1,500)
Mast cells and human tendinopathy: a critical partnership

Mr Munim Moiz, Department of Craniofacial Development, Kings College London (£1,500)
Regulation of bone cell differentiation from pluripotent stem cells

Mr Sam Inman, Queen's Medical Research Institute MRC/UoE Centre for Inflammation Research (£1,050)
Defining the role of macrophage phagocytosis in liver fibrosis remodeling and hepatic regeneration

Fellowship Awards

The Robertson Trust Research Fellowship

Mr Alexander Laird, Specialty Registrar in Urological Surgery, Breakthrough Research Unit, University of Edinburgh (£45,000)
Employing a high-throughput, quantifiable proteomic approach to the development of a panel of markers predictive of outcome from sunitnib treatment in patients with metastatic renal cell cancer

Kidney cancer accounts for 2.5% of all adult cancers and is the most deadly of all urological malignancies. Metastases to other organs affects one-third of patients with kidney cancer at the initial diagnosis, with a further 30–40% of patients eventually developing metastases. Newer targeted drugs such as sunitinib are the mainstay of treatment. Despite the improved survival seen with sunitinib, it is an expensive medication that not all patients respond to, with some suffering significant side effects. There is no reliable method for determining which patients will benefit from receiving sunitinib and which will not. We propose that, by studying kidney cancer tissue before and after treatment with sunitinib, we will be able to identify molecular changes in the tissue that indicates likely response to treatment. We can then assess if these potential biomarkers are predictive of clinical improvement by studying kidney tissue from patients who have been administered sunitinib, and correlating this with known outcomes. We hope that these biomarkers can be developed into a clinical/pathology test to prevent the administration of ineffective treatment with harmful side effects to terminally ill patients. Development of such a test could have an appreciable impact on patients and save money for healthcare providers.

Maurice Wohl Research Fellowship in Surgery/Dental Surgery

Mr Stephen O'Neill, Clinical Research Fellow, MRC Centre for Inflammation Research, University of Edinburgh (£45,000)
Role of heat shock protein 90 inhibitors in modulating ischaemia–reperfusion injury in kidney transplantation

Kidney transplantation is the 'gold standard' treatment for established renal failure. However, there is an increasing disparity between the number of patients awaiting transplantation and the availability of donor organs. One strategy to redress this balance is the use of donation-after-cardiac death (DCD) donors (now 30% of deceased kidney donors). DCD kidneys are an important resource but suffer greater ischaemia–reperfusion injury (IRI) than conventional brain-dead donors as a result of the donation procedure. IRI contributes to a delay in graft function which is associated with poorer graft survival. Our research team has shown a reduction in renal IRI using the Hsp90 inhibitor geldanamycin, but this drug has significant toxicities. We have identified a novel, small-molecule Hsp90 inhibitor with activity at nanomolar concen-

trations and a low toxicity profile in phase-II studies. The mechanism of protection by Hsp90 inhibition is unknown and the efficacy of such protection in preclinical models remains to be tested. If administered to donors or directly to the kidney, this agent could significantly improve outcomes after kidney transplantation. There is currently no active pharmacological agent used at the time of organ donation to reduce IRI. Use of this drug in a clinical setting would therefore be unique.

Travelling Fellowship Awards

Cutner Travelling Fellowship in Orthopaedics
Sponsored by the Cutner Bequest Fund

Mr Lukman Khan, SpR Trauma and Orthopaedic Surgery, Royal Infirmary of Edinburgh (£3,000)
Clinical and Research Fellowship in Shoulder and Upper Limb Surgery, Australia.
Report: see page 69

Mr Andrew Carrothers, Senior Arthroplasty Registrar (£3,000)
Pelvic Trauma and Lower Limb Reconstruction/Complex Arthroplasty Clinical Fellowship, Canada.

John Steyn Travelling Fellowship in Urology
Sponsored by the family of Mr John H Steyn

Dr Keng Siang Png, Registrar, Advanced Specialist Training, Department of Urology, Singapore (£900)
Fellowship in Minimally Invasive and Robotic Urology and Medical Stone Prevention, Indiana, USA.

Syme Medal Awards

Mr Mark Duxbury, Hepatobiliary and Pancreatic Surgery Unit, University and Royal Infirmary of Edinburgh
Characterisation of CEACAM6: function, signalling interactions and clinical significance in pancreatic cancer

Miss Cynthia-Michelle Borg, Queen's Hospital, Romford
Gut hormones and bariatric surgery

Dr Mandeep Singh Sagoo, Institute of Cancer, Queen Mary University of London
Custom-designed plaque radiotherapy for juxtapapillary choroidal melanoma

Miss Beatrix Elsberger, ST3 in General Surgery, NHS Tayside
Role of Scr kinase and Src kinase family members in breast cancer

Mr Wasim Khan, Clinical Lecturer, University College London
Stem cells in the synovium and fat pad and their potential for cartilage repair

Mr Andrew Robertson, Specialty Registrar, Royal Infirmary of Edinburgh
Gastro-oesophageal reflux, aspiring and anti-reflux surgery in a human lung transplant population

Mr George K C Wong, Consultant, Department of Surgery, Chinese University of Hong Kong
Magnesium sulphate infusion for patients with aneurysmal subarachnoid haemorrhage

Ophthalmology Awards

Funded by the Royal Blind and Scottish War Blinded

Small Research Grants

Professor Stephen Kaye, Consultant Ophthalmologist, Department of Eye and Vision Science, University of Liverpool (£9,000)
Infections of the eye – bacterial keratitis

Major Project Grants

Mr Robert MacLaren, Senior Clinical Research Fellow, Nuffield Laboratory of Ophthalmology, University of Oxford (£50,000)
Preclinical testing a new gene therapy vector for Stargardt disease

Professor Andrew Dick, Professor of Ophthalmology, Bristol Eye Hospital, University of Bristol (£47,456)
Refining conventional immunotherapy: targeting the differential response of Th1 and Th17 cells to corticosteroids and calcineurin inhibitors in a novel experimental model of uveitis

Dr Brian Fleck, Consultant Ophthalmologist, Princess Alexandra Eye Pavilion, Edinburgh (£38,723)
An analysis of retinal digital image abnormalities seen in acute retinopathy of prematurity – is reduced oxygen therapy protective? The benefits Of Oxygen Saturation Targeting Trial II UK Retinal Image Digital Analysis (BOOST-II UK RIDA) Study.
Report: see page 80

King James IV Profesorship Awards

Surgical

Professor C H Kau, University of Alabama
Imaging the maxillofacial region –creation of the virtual human patient and its implications

Mr T Bunker, Consultant Trauma and Orthopaedics, Royal Devon and Exeter Hospital
Rotator cuff repair: beyond arthroscopic repair
Report: see page 115

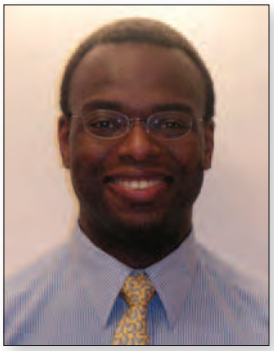
Professor H Kerr Graham, Professor of Orthopaedic Surgery, Royal Children's Hospital, Melbourne
Single-event, multilevel surgery for children with spastic diplegia: progress and future directions

Dental

Professor Lakshman P. Samaranayake, Dean of Dentistry, University of Hong Kong
Oral *Candida* in health and disease

Fellowship Reports

Role of CD154-positive macrophages in promoting chronic liver inflammation and apoptosis using a primary cell co-culture model



Edward Biobele Alabraba, Liver Research Group, Infection and Immunology, Institute of Biomedical Research, University of Birmingham, Birmingham

Medical Research Council/RCSEd Clinical Research Training Fellowship (1 June 2006–31 May 2009)

Lay summary

The vital functions of the liver are removal of blood toxins, protein production, syntheses of bile acids, iron storage and gluconeogenesis. Toxic injury may cause the liver to undergo episodes of inflammation, which usually resolve. The persistence of inflammation causes permanent tissue damage, leading to cirrhosis, liver failure and, occasionally, malignant disease. The only treatment for end-stage liver disease (ESLD) is transplantation. This has significant impact on quality of life and constitutes a major economic burden to the NHS. Some ESLDs are characterised by destruction of hepatocytes and the bile ducts. The liver contains various inflammatory cells which are resident or derived from the circulation. Lymphocytes, macrophages and Kupffer cells constitute the largest populations of cells within the liver and their numbers increase during inflammation. Macrophages clear unwanted cellular debris resulting from tissue damage. Theoretically, they may also promote inflammation if activated inappropriately. My preliminary work has demonstrated that macrophages can destroy the biliary epithelial cells that form the bile ducts that transport bile within the liver but the mechanisms responsible remain unclear. It is of crucial importance to dissect the mechanism of cell destruction to design more effective therapies for the treatment of chronic inflammatory liver disease.

Grant report

Kupffer cells induce the death of biliary epithelial cells. Targeting the mechanism of destruction of bile ducts may help in the development of therapy in immune-mediated liver diseases that are associated with such destruction.

Problems encountered and steps taken to overcome them

Firstly, antagonising CD154 using siRNA resulted in only moderate knockdown of CD154. Therefore, I obtained an antagonistic anti-CD154 antibody from a commercial source. The use of this antibody provided greater CD154 antagonism. Secondly, transferring Kupffer cells from the culture substrate was a major problem owing to their adherence. Therefore, I cultured the cells on plastic coverslips (which allowed transfer of monolayers of Kupffer cells between culture vessels) and used specialised culture dishes (which helped to release cells from the culture substrate in response to low temperatures).

Collaboration established

Pangenetics BV (Utrecht, the Netherlands). I obtained anti-CD154 antibody from this organisation.

Publications

1 Alabraba E, Nightingale P, Gunson B, et al. A re-evaluation of the risk factors for the recurrence of primary sclerosing cholangitis in liver allografts. *Liver Transpl* 2009; 15: 330–340.

2 Alabraba EB, Lai V, Boon L, Wigmore SJ, Adams DH, Afford SC. Coculture of human liver macrophages and cholangiocytes leads to CD40-dependent apoptosis and cytokine secretion. *Hepatology* 2008; 47: 552–562.

3 Alabraba EB, Curbishley SM, Lai WK, Wigmore SJ, Adams DH, Afford SC. A new approach to isolation and culture of human Kupffer cells. *J Immunol Methods* 2007; 326: 139–144.

4 Lai WK, Curbishley SM, Goddard S, et al. Hepatitis C is associated with perturbation of intrahepatic myeloid and plasmacytoid dendritic cell function. *J Hepatol* 2007; 47: 338–347.

5 Alabraba EB, Taniere P, Reynolds GM, Stewart PM, Wigmore SJ, Bramhall SR. Expression and functional consequences of oestrogen and progesterone receptors in human insulinotas. *Endocr Relat Cancer* 2007; 14: 1081–1088.

Presentations

1 European Society of Organ Transplantation, Prague, September 2007.

2 New Key Opinion Leader, Transplantation Society, Barcelona, September 2006.

3 International Liver Transplant Society, Milan, May 2006.

4 British Transplant Society, Edinburgh, March, 2006.

5 Surgical Academic Research Society, Edinburgh, January, 2006.

6 British Society of Immunology, Harrogate, December 2005.

7 American Association for the Study of Liver Disease, San Francisco, November 2005.

8 European Society of Organ Transplantation, Geneva, October 2005.

Prize

Best scientific presentation, European Society of Organ Transplantation Fellows Workshop, Warsaw, 2004.

Higher degree

PhD Medicine, University of Birmingham, 2009.

Acknowledgements

Medical Research Council

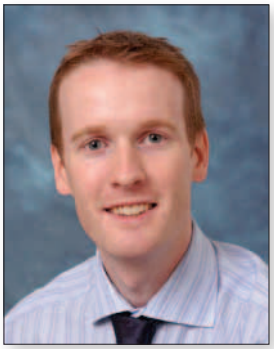
RCSEd

Professor DH Adams

Professor SJ Wigmore

Dr SC Afford

Role of interleukin-33 in tendon disease



Neal L Millar, Division of Immunology, Infection and Inflammation, University of Glasgow, Glasgow
RCSEd/Cutner Orthopaedic Research Fellowship (August 2009–August 2010)

Lay summary

Soft-tissue disorders represent the third most common orthopaedic condition in the UK, with an incidence of 18 cases per 1,000 individuals. These disorders primarily affect tendons. The most commonly affected tendons are those in the shoulder, elbow ('tennis elbow' and 'golf elbow'), knee and ankle.

Key inflammatory mediators are found at significantly higher levels in and around painful tendons. However, the role of inflammation in the cascade of tendon injury is not known. Cytokines are small signalling proteins which are critical for mounting an immune response and play a key part in inflammatory disorders such as rheumatoid arthritis. We have demonstrated increased production of cytokines in injured tendons from patients undergoing tendon repair surgery of the shoulder.

We found that cytokines have a significant role in tissue repair in tendon injuries. We also identified that inflammation has a key role in early tendon disease in humans. Our studies have provided better understanding of the role of cytokines in tendon disease. The the ultimate aim of our work is to improve/accelerate tendon healing in humans.

Grant report

Identification of a significant role for inflammation in early tendon disease

To characterize the subtypes of inflammatory cells in early human tendinopathy, we explored the phenotype and quantification of inflammatory cells in samples of torn tendons and control tendons.

Samples of torn supraspinatus tendons and matched intact subscapularis tendons were collected from 20 patients undergoing arthroscopic shoulder surgery. Control samples of subscapularis tendons were collected from 10 patients undergoing arthroscopic stabilization surgery. Tendon biopsies were evaluated by immunohistochemical means by counting the number of macrophages (CD68 and CD206), T cells (CD3), mast cells (mast cell tryptase) and vascular endothelium (CD34).

Biopsies of subscapularis tendons obtained from patients with torn supraspinatus tendons exhibited significantly greater numbers of macrophages, mast cells and T cells compared with samples of torn supraspinatus tissue or control subscapularis-derived tissue ($p<0.01$). Inflammatory cell infiltrates were correlated inversely ($r=0.5$, $p<0.01$) with rotator cuff tear size, with larger tears correlating with a marked reduction in all cell lineages. There was a modest (but significant) correlation between the number of mast cells and CD34 expression ($r=0.4$, $p<0.01$) in matched subscapularis tendons from shoulders with supraspinatus ruptures.

We provided evidence for inflammatory cell infiltrates in early mild/moderate human tendinopathy. In particular, we demonstrated significant infiltration of mast cells and macrophages. This finding suggested a role for innate immune pathways in the events that mediate early tendinopathy.

Further mechanistic studies to evaluate the net contribution of these cell lineages and their downstream processes may reveal novel therapeutic approaches to the management of early tendinopathy.

Interleukin-33 (IL-33) in tendon disease

In preliminary studies, we detected expression of several inflammatory cytokines (e.g. IL-18, tumour necrosis factor (TNF) in rodent and human models of tendonopathy. These experiments were extended to tendon samples from patients undergoing rotator cuff repair. These experiments demonstrated overproduction of key cytokines and apoptotic pathway-related molecules in samples of torn supraspinatus and intact matched subscapularis tendons. Immunohistochemical analyses of samples of torn and matched control rotator cuff tendons provided the first evidence that expressions of IL-33 and ST2 are upregulated in damaged tendons.

Quantification of protein expression revealed significantly higher levels of IL-33 and ST2 in samples of human tendons. Early mechanistic studies using human explanted tenocytes demonstrated significant upregulation of IL-33 in TNF- and IL-1β-stimulated tenocytes. Based on evidence of the key role of fibroblast-derived IL-33 in inflammatory disease and investigations showing increased numbers of mast cells in torn rotator cuff tendons, we hypothesize that IL-33 and ST2 are overexpressed in damaged human fibroblast-like tenocytes. Moreover, we propose that IL-33 plays a key part in the recruitment and activation of leucocytes in damaged tendons. This action results in disruption of matrix regulation with particular reference to the production of collagen and matrix metalloproteinases. Thus, IL-33 could represent a novel damage-associated alarmin which could influence remodelling of tendon matrices.

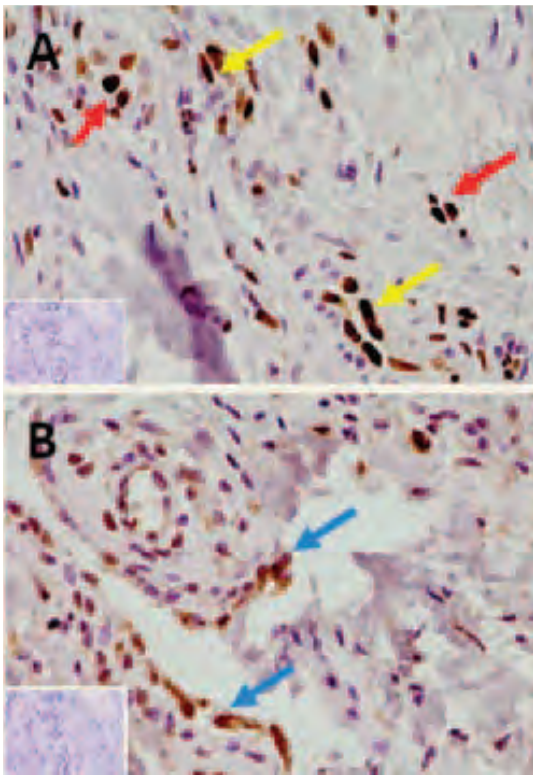


Fig 1 (A) IL-33-positive staining in torn rotator cuff tendon (x400). Red arrows highlight nuclear staining for IL-33 in tenoblasts while yellow indicates nuclear staining in tenocytes. Control tendon shown in corner photograph with no positive staining and (B) ST2-positive staining in rotator cuff tendon (x400) highlighted by blue arrows.

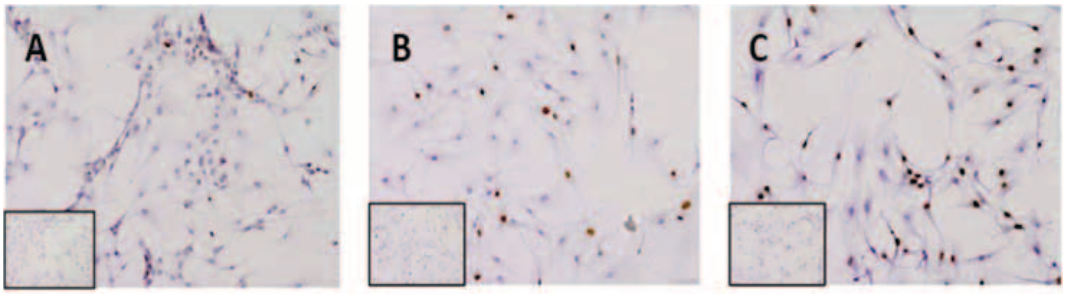


Fig 2 Immunohistochemical staining for IL-33 in explanted human tenocytes stimulated with TNFα and IL-1β. (A) Control tenocytes showing minimal positive staining for IL-33, (B) stimulation with 10 ng/ml of TNFα showing moderate upregulation of IL-33 expression and (C) stimulation with 10 ng/ml of TNFα and 1 ng/ml of IL-1β showing marked upregulation of IL-33 expression. The isotype control is shown in the corner photograph.

We have further investigated the role of the addition of IL-33 to tendon cells *in vitro*. We found that IL-33 affects the synthesis of pro-apoptotic molecules and the balance of syntheses of type-I and type-III collagen, which may lead to altered biomechanical properties. This work is ongoing and shall be completed under the arc/RCSEd grant.

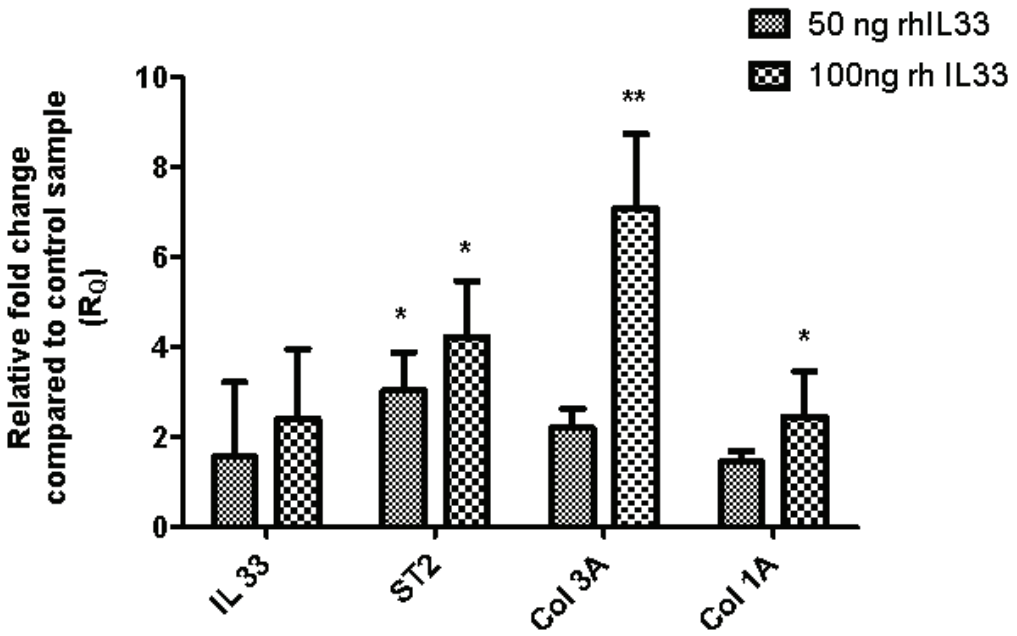


Fig 3 Real-time PCR data showing fold increases of genes upon the addition of 50 ng/100 ng of recombinant IL-33.

Problems encountered and steps taken to overcome them
Further funding has been secured for a doctoral project from the arc/RCSEd grant.

Collaborations established

Professor George AC Murrell, Professor of Orthopaedic Surgery, Chief Sports Surgery, St George Hospital, Kogarah, Sydney, Australia.

Publication

Millar NL, Heuber AJ, Reilly JH, et al. Inflammation is present in early human tendinopathy. *Am J Sports Medicine* 2010; 38: 2085–2091.

Presentations

Shortlisted as a NIRA Finalist, Orthopaedic Research Society Annual Meeting, New Orleans, March 2010.

Acknowledgements

I thank the Research Committee for awarding me this prestigious fellowship. It has allowed vital investigation of inflammatory pathways in tendon disease at a world-renowned immunological laboratory. The project has been further funded by the arc/RCSEd Orthopaedic Clinical Research Fellowship and has provided me with key resources to complete my doctoral studies.

LRH-1 as a key regulator of estrogen responses in breast cancer cells

Paul T R Thiruchelvam, Department of Oncology, Imperial College, London

Cancer Research UK/RCS Joint Fellowship (December 2005–May 2009)

Lay summary

Liver receptor homolog-1 (LRH-1) has been linked to several key developmental, metabolic and proliferative processes. It is also known to play an important part in the regulation of cholesterol biosynthesis, lipid homeostasis, and the control of steroid aromatisation. In this respect, LRH-1 has a key role in breast cancer because it regulates aromatase activity, leading to the local production of estrogen. We identified LRH-1 to be an estrogen-responsive gene that may be important in the estrogen-regulated growth of breast cancer cells.

We went on to further study the role of LRH-1 in the estrogen response in breast cancer cells. Using estrogen receptor-positive breast cancer cell lines, we confirmed that LRH-1 levels increase in response to estrogen, and are inhibited by anti-estrogens (tamoxifen and ICI 182,780). Using RNA interference experiments directed against LRH-1, we identified ERα as an important LRH-1-regulated gene. These results suggest that LRH-1 is an estrogen-regulated transcription factor which can potentially act in a positive feedback loop to regulate ERα expression, in tumour cells. We propose that LRH-1 has a central role in regulating estrogen responses in breast cancer. Hence, LRH-1 could be an important new target for the treatment of breast cancer.

Grant report

LRH-1 has been reported to be an estrogen-responsive gene that may be important in the estrogen-regulated growth of breast cancer cells. Using the MCF-7 breast cancer cell line, we confirmed that LRH-1 is regulated with stimulation by estrogen at 2 h and reaches maximal expression at 4–6 h. Treatment of MCF-7 cells with the anti-estrogens tamoxifen and ICI 182,780 reduced the expression of LRH-1 at the protein level. We showed that two genes known to be regulated by LRH-1, i.e., INHA and CCND1, are also regulated by estrogen. Their greatest expression was seen when LRH-1 expression was maximal. Further confirmation that LRH-1 is estrogen-regulated was demonstrated by a reduction in LRH-1 expression after knockdown of ERα in MCF-7 cells using a siRNA to ERα.

We showed that LRH-1 is highly expressed in tissues derived from the gut endoderm (e.g., small intestine, colon, liver) as well as in the testes, ovary and breast. In a panel of cancer cell lines, LRH-1 was highly expressed in the liver cell line HepG2 and in ERα-positive breast cancer cell lines, but was minimally expressed in those breast cancer cell lines which do not express ERα. We also showed that a homologue of LRH-1, SF-1, was minimally expressed in breast tissue, but highly expressed in the ovary and small intestine. SF-1 expression was shown to be undetectable in nearly all breast cancer cell lines tested. Expression of the LRH-1 repressors DAX-1 and SHP was very low in breast tissue and breast cancer cell lines.

Using 5'RLM-RACE, we identified three novel LRH-1 variant transcripts, two of which, LRH-1 289 and LRH-1 326, were shown to be estrogen-regulated. LRH-1 289 and LRH-1 326 were expressed in LRH-1-containing tissues and LRH-1-positive breast cancer cell lines. LRH-1 289 was also shown to be a major form of LRH-1 expressed in the breast.

Using siRNA-mediated gene knockdown, we showed that, by inhibiting LRH-1 expression, the growth of ERα/LRH-1-positive MCF-7, T47D and ZR-75-1 breast cancer cell lines was abrogated. No effect was seen in the ERα+/LRH-1-negative cell line BT474 nor the ERα/LRH-1-negative cell line MDA-MB-231. Confirmation of LRH-1 knockdown using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) employing several LRH-1 siRNAs demonstrated a reduction of LRH-1 expression in MCF-7 cells of ≈50–90%. This was confirmed at the protein level using immunoblotting. Expression of the LRH-1-regulated gene INHA was seen after a reduction in LRH-1 expression and, interestingly, the expression of ERα was reduced after a reduction in LRH-1 siRNA expression. The reduction in ERα expression mirrored the reduction in LRH-1 expression. This effect was confirmed in two other ERα/LRH-1-positive breast cancer cell lines (T47D and ZR-75-1) but not in the ER+α/LRH-1-negative cell line BT474. Overexpression of LRH-1 resulted in an increase in ERα expression. In contrast, transfection of the LRH-1 repressor SHP into MCF-7 cells reduced ERα expression, confirming that LRH-1 regulates ERα expression. Further evidence that LRH-1 regulates the growth of breast cancer cells through ERα regulation was obtained from the use of the 'Whitby' small-molecule compounds 5a, 5b and 5L. These small-molecule activators of LRH-1 stimulated the growth of ERα/LRH-1-positive cell lines in a dose-dependent manner, but had no effect in the ERα/LRH-1-negative line MDA-MB-231. These compounds also potently activated LRH-1 activity and ERα expression. Using fluorescence-activated cell sorting (FACS) we showed that LRH-1 siRNA in MCF-7 cells resulted in a 25% increase in the number of apoptotic cells, which may (at least in part) explain the reduction in the number of cells seen in the growth assays.

Using qRT-PCR, we also assessed ERα gene promoter usage in MCF-7, T47D, ZR-75-1 and BT474 breast cancer cell lines. Interestingly, all isoforms of ERα were shown to be reduced after LRH-1 knockdown. We mapped ten potential LRH-1 binding sites in the ERα promoter. Generation of a reporter gene encoding the region of the ERα gene 7 kb upstream of promoter A and transfecting this into MCF-7 cells with LRH-1 resulted in a significant increase in ERα activity. Transfection of a mutated form of LRH-1 (LRHRE1) resulted in a 40% reduction in ERα reporter activity compared with the wild-type reporter, suggesting that this site is required for LRH-1 binding to the ERα gene promoter.

Taken together, these studies showed that LRH-1 has a significant role in regulating responses in ERα-positive breast cancer cells, and this occurs (at least in part) through direct regulation of ER expression. Importantly, these findings identified LRH-1 as a potentially important new target for the treatment of breast cancer.

Problems encountered and steps taken to overcome them

Validating an antibody to detect endogenous LRH-1 expression in breast cancer cell lines. This was overcome by determining the forms of LRH-1 expressed in breast cancer cell lines by 5'RACE and finding an antibody that would detect these forms.

Previously described forms of LRH-1 were not detectable in breast cancer cells. We had to discover new isoforms of LRH-1 and determine their relative expression. This was undertaken using 5'RACE as well as cloning and sequencing DNA.

Collaborations established

1 A Hurtado and JS Carroll, Cancer Research UK, Cambridge Research Institute, Cambridge.

2 AC Spivey, Department of Chemistry, Imperial College London (South Kensington Campus), London.

3 RJ Whitby, School of Chemistry, University of Southampton, Southampton.

4 W Hudson and EA Ortlund, Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, USA.

Publications

1 Tolhurst RS, Thomas RS, Kyle FJ, et al. Transient over-expression of estrogen receptor-alpha in breast cancer cells promotes cell survival and estrogen-independent growth. *Breast Cancer Res Treat* 2011; 128: 357–368.

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Higher degree

PhD, Imperial College London: awarded August 2010.

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Differential expression and function of 15 prostaglandin dehydrogenase in colorectal cancer liver metastases

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Joint RCSEd/Cancer Research UK Clinical Research Training Fellowship (1 August 2007–30 September 2009)

Lay summary

Colorectal cancer (CRC) is the second leading cancer killer in the UK. In the vast majority of cases, death is due to metastatic disease. The epithelial–mesenchymal transition (EMT) is a key embryologic process which is thought to be adopted by certain tumour cells to provide them with the capabilities to metastasise. Prostaglandins (PGs) are signalling molecules which promote cancer progression at various stages.

We showed that PGE2 promoted the EMT in a cell model of CRC. We found that PGE2 levels varied significantly within CRC liver metastases, with higher levels seen towards the tumour centre. Next, the enzymes that control PGE2 levels were investigated. The enzyme which breaks down PGE2 was shown to work poorly in the more hypoxic conditions seen in the central regions of CRC liver metastases. This effect was shown to correlate with the promotion of the EMT in these areas in tissue and cell models.

We proposed a new relationship whereby the hypoxic environment seen in the central regions of tumours modifies enzyme function. This action results in increased PGE2 levels, which subsequently promotes the EMT and the development of metastases. This mechanism may explain how hypoxia causes tumours to evolve to promote metastases.

Grant report

The EMT has emerged as a key process which allows tumour cells to acquire the capabilities to metastasise. The EMT was studied in the context of PG metabolism *in vivo* using colorectal cancer liver metastases (CRCLM) and backed-up by carrying out similar studies using appropriate *in vitro* models

We took a cell model of EMT, LIM 1863 cells, and showed that these cells demonstrated the key features of the EMT. We then developed and validated an assay to study the EMT in this model. This subsequently showed that PGE2 and hypoxia promoted development of the EMT in LIM 1863 cells.

Tissue was retrieved from a series of large CRCLM to allow comparison of tissue taken from distinct central and peripheral regions of tumours. A previous Research Fellow had shown that central regions of CRCLM were more hypoxic than peripheral regions. Hence, one of the aims of this work was to compare the effect of a more hypoxic environment on PG metabolism in CRCLM by comparing the tissue from central and peripheral regions of tumours.

We demonstrated significant regional variation in PGE2 levels within CRCLM, with significantly higher levels of PGE2 found in tissue from central regions of tumours. The enzymes responsible for the control of PGE2 levels were investigated using immunohistochemical analyses. Regional variation in the levels of the enzyme controlling PGE2 synthesis (COX-2) were not observed but, paradoxically, there were significantly higher levels of the enzyme controlling PGE2 catabolism (NAD+-dependent 15-PGDH)

in central regions of tumours. This finding was confirmed using a radioactive enzyme activity assay which also showed significantly higher levels of viable 15-PGDH protein in central regions of tumours. These results were explained by showing that NAD+ (the essential cofactor required to allow 15-PGDH to work) was significantly depleted in central regions of tumours.

These findings led to the hypothesis that that, in hypoxic regions of tumours, NAD+ levels are depleted. This will impair the activity of the 15-PGDH enzyme, leading to the accumulation of PGE2 in hypoxic regions of tumours.

The data from CRCLM tissue were reinforced by carrying out similar studies using *in vitro* models. The hypothesis was proven to be correct using an *in vitro* model and modifying the NAD+ concentration. By carrying out the enzyme activity assay we could demonstrate that the activity of the 15-PGDH enzyme was dependent upon cofactor concentration. This was the first study to show that the tumour microenvironment modifies enzyme function to affect the level of a signalling molecule such as PGE2.

The significance of this finding was in showing that increased PGE2 levels promoted the EMT. This was shown directly using the LIM 1863 model of the EMT in which PGE2 promoted the EMT in a dose-dependent manner. This was also shown indirectly by showing a changing relationship between 15-PGDH and EMT markers with oxygen tension *in vivo* and *in vitro*. Since the onset of this work, other research teams have also shown that PGE2 promotes the EMT.

In summary, we demonstrated that there was significant regional variation in PGE2 levels within CRCLM. A new hypothesis was proposed and proven to explain this finding whereby PGE2 catabolism is impaired in hypoxic conditions due to depletion of the essential cofactor NAD+.

Problems encountered and steps taken to overcome them

Heterogeneous tumours The EMT is not a generalised phenomenon within tumours: only certain cells within the tumour are thought to undergo the EMT. This created a problem in studying the EMT within a whole tumour. By adhering to a meticulous sample-collection protocol and recruiting tissue from large tumours, samples of tissue from central and peripheral regions within a tumour were retrieved. Such a strict protocol of tissue collection has not been used previously. However, to adhere to this collection protocol, only tumours of sufficient size could be included. This meant that the period of tissue collection lasted >12 months. This yielded surprisingly consistent regional variations in the assays carried out. This has implications for future studies using tumour tissue because the location from which the tissue is retrieved is another variable that must be considered due to intratumoural variations in the tumour microenvironment.

Collaborations established

1 Dr Tsuyoshi Igami, Associate Professor of Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan. For studies on hilar cholangiocarcinoma.

2 Dr Eva Morris, University of Leeds, Northern and Yorkshire Cancer Registry and Information Service, Leeds. To assess for inequalities in access to liver resection for colorectal cancer liver metastases through a large prospective multicentre audit.

3 Professor PJ Robinson, J Ward and Dr D Wilson, Departments of Radiology and Medical Physics, University of Leeds, Leeds. Assessing how MRI can be exploited to improve patient assessment before major resection of the liver.

4 Dr Darren Treanor, Department of Histopathology, University of Leeds, Leeds. How to utilise computer software for objective scoring of immunohistochemistry.

Publications

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7 Gomez D, Farid S, Malik HZ, et al. Preoperative neutrophil-to-lymphocyte ratio as a prognostic predictor after curative resection for hepatocellular carcinoma. *World J Surg* 2008; 32:1757–1762.

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9 Young AL, Rajagenashan R, Asthana S, et al. The role of MELD and sodium as predictors of outcome in potential liver transplant recipients. *Transplant Int* 2007; 20: 331–337.

Manuscripts under review

1 Young AL, Igami T, Senda Y, et al. Surgical resection for hilar cholangiocarcinoma evolution: of results in a Western centre.

2 Young AL, Adair RA, Prasad KR, Toogood GJ, Lodge JPA. Resection of hepatocellular carcinoma in non-cirrhotic, non-fibrotic, seronegative liver.

Manuscripts in preparation

1 Differential expression and function of 15-PGDH in colorectal cancer liver metastases.

2 Redo liver resections for colorectal liver metastases.

3 Physiology of wound healing (invited article).

4 Quantification of hepatic steatosis and future liver remnant volume in predicting hepatic dysfunction following liver resection.

Book chapters

1 Adair R, Young AL, Toogood GJ. Updates in hepatobiliary Surgery. Current Critical Care and Anaesthesia. Elsevier (in press).

2 Adair R, Young AL, Toogood GJ. Surgery for metastatic disease of the liver. Oxford Handbook of Oncology (colorectal section). Oxford University Press (in press).

Oral presentations

1 Hypoxic depletion of NAD+ promotes development of colorectal liver metastases. Presented to the Association of Upper Gastrointestinal Surgeons of Great Britain and Ireland (AUGIS), Oxford, September 2010.

2 Hypoxic depletion of NAD+ promotes development of colorectal liver metastases. Presented in the Young Investigators Prize session at International Hepato-Pancreato-Biliary Association (IHPBA), Buenos Aires, April 2010.

3 Index admission laparoscopic cholecystectomy for patients with acute biliary symptoms: results from a specialist centre. Presented at IHPBA, Buenos Aires, 2010.

4 NAD+ depletion in hypoxia may promote colorectal liver metastases. Presented to the Society of Academic and Research Surgery, London, January 2010.

5 Regional variation in expression and function of 15-PGDH in colorectal liver metastases. Presented to the Annual Clinical Fellows meeting, Cancer Research UK, London, December 2009.

6 Specialist Centre management of patients with acute biliary symptoms. Presented to the Association of Surgeons of Great Britain and Ireland (ASGBI), Glasgow, May 2009.

7 Prostaglandin metabolism in advanced colorectal cancer. Presented to the Yorkshire Gut Club, Leeds, September 2008.

8 Prostaglandin metabolism in advanced colorectal cancer. Presented to the Leeds Institute of Molecular Medicine, Leeds, June 2008.

9 Resection for hepatocellular carcinoma: pre-operative biopsy increases tumour recurrence. Presented to the IHPBA, Mumbai, 2008.

10 Large hepatocellular carcinomas: time to stop preoperative biopsy. Presented to the Association of Upper Gastrointestinal Surgeons, Cardiff, September 2007.

Poster presentations

1 Redo resection for colorectal cancer liver metastases. Presented to AUGIS, Oxford, September 2010.

2 Intratumoral variability in prostaglandin E2 levels in human colorectal cancer liver metastases is associated with differences in NAD+ levels and expression of NAD+-dependent 15-prostaglandin dehydrogenase. Presented to Digestive Diseases Week, New Orleans, May 2010.

3 Same admission laparoscopic cholecystectomy for patients admitted with acute biliary symptoms. Presented to IHPBA, Buenos Aires, April 2010.

4 Predictors of microvascular invasion following potentially curative resection for HCC. Presented to IHPBA, Buenos Aires, April 2010.

5 Liver resection for hepatocellular carcinoma arising in a non-cirrhotic, non-fibrotic seronegative liver. Presented to IHPBA, Buenos Aires, April 2010.

6 Quantification of hepatic steatosis and volume in predicting hepatic dysfunction and complications after liver resection of colorectal metastases. Presented to IHPBA, Buenos Aires, April 2010.

7 Size of tumour predicts outcome in redo resection for colorectal liver metastases. Presented to IHPBA, Buenos Aires, April 2010.

8 Evolution of results for resection of hilar cholangiocarcinoma in a Western centre. Presented to IHPBA, Buenos Aires, April 2010.

9 Intratumoral variability in prostaglandin E2 levels in human colorectal cancer liver metastases is associated with differences in NAD+ levels and expression of NAD+-dependent 15-prostaglandin dehydrogenase. Presented to the British Society of Gastroenterology, Liverpool, March 2010.

10 Resection of hepatocellular carcinoma in non-cirrhotic, non-fibrotic, seronegative liver.Distinction poster presented to AUGIS, Nottingham, September 2009.

11 Specialist Centre management of patients with acute biliary symptoms. Distinction poster presented to AUGIS, Nottingham, September 2009.

12 Epithelial-mesenchymal transition of human colorectal cancer cells is associated with reduced 15-prostaglandin dehydrogenase protein levels. Presented to ASGBI, Glasgow, May 2009.

13 Small adult patients wait longer for liver transplantation. A comparison of UK and USA data. Presented to ASGBI, Glasgow, May 2009.

14 Epithelial–mesenchymal transition of human colorectal cancer cells is associated with reduced 15-prostaglandin dehydrogenase protein levels. Presented to the Annual Fellows Meeting of Cancer Research UK, London, November 2008.

15 Epithelial–mesenchymal transition of human colorectal cancer cells is associated with reduced 15-prostaglandin dehydrogenase protein levels. Presented to the National Cancer Research Institute, Birmingham, October 2008.

17 Specialist Centre management of patients with acute biliary symptoms. Presented to the Association of Upper Gastrointestinal Surgeons, Liverpool, September 2008.

18 Portal vein arterialisation: a salvage technique in left hepatic trisectionectomy for hilar cholangiocarcinoma. Presented to IHPBA, Mumbai, February 2008.

Higher degree

Differential expression and function of 15-prostaglandin dehydrogenase in colorectal cancer liver metastases. PhD, University of Leeds, December 2010.

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Quantifying remnant volume and steatosis in liver resection patients. Yorkshire Cancer Research Grant: £13,482.

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Free tissue transfer of a transduced flap as a vehicle for gene and virus therapy of cancer

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Royal College of Surgeons of Edinburgh and Ireland Joint Fellowship RCSEd/RCSI Research Fellowship for 2009 (June 2009–August 2010)

Lay summary

The basis of this research project was to determine the feasibility of using free flaps as a therapeutic aid in administering localised gene therapy. The animal model was set up successfully using adult male Fischer 344 rats, and the flap under investigation was the superficial inferior epigastric artery (SIEA) free flap. Initial studies were carried out to characterise the feasibility of treatment passing across the flap onto the tumour bed. Histological analyses (haematoxylin and eosin (H&E)) and immunohistochemical (IHC) stains were used to successfully characterise the flap–tumour bed interface. The results were useful for determining when and which type of therapy could be possible. The next set of experiments aimed to establish a therapeutic model using viral vectors to transfer the required therapeutic genes into the target tissue. As a proof of principle, the flaps were raised and vector and tumour introduced into the flap: tumours were injected subcutaneously into the flap and virus was transduced through intra-arterial cannulation of the SIEA once the flap had been disconnected. A significant delay in tumour growth was noted (P=0.004). This led to the next therapeutic experiment, in which tumours were implanted subcutaneously into the abdomen. After reaching 1 cm^3, tumours were excised and 0.1 cm^3 was left to imitate microscopic residual disease. Free tissue flaps were then raised and transduced with viral vectors and placed over the tumour beds. A significant delay in tumour growth was seen between controls and therapeutic samples, with one animal showing no tumour growth during the experimental period (P=0.0005). We successfully established a tumour model and assessed therapy across the flap–tumour bed interface.

Grant report

An animal model was established to assess the growth and regression of tumours using Fischer 344 rats; adenoviral vector with a thymidine kinase therapeutic gene; and rat glioma cells as the tumour cell group. Two-hundred rats underwent surgery. The anastomotic procedure was technically demanding and was the most important component of this project. Therefore, surgical modifications were made to optimise the conditions and maximise success. The success rate was 64%. Surgically dependent complications (necrotic flap after failed anastomosis, haematoma, sepsis, incarcerated hernia) comprised 19% of all procedures carried out; necrotic flaps made up 14.1% of this group. Non-surgically dependent complications (animals found dead in the cage with no apparent cause, autophagia, breathing difficulties immediately post-operatively, excessive loss of weight post-operatively) comprised 17% of all surgical cases.

The efficiency of transduction with an adenoviral vector and plasmid virus was also assessed. Intravascular plasmid and viral transduction of the tissue was shown quantitatively with X-Gal staining and qualitatively using an *in vivo* imaging system. Adenoviral

expression was found to be more diffuse and gave a greater duration of expression when compared with plasmid transduction. Intravascular flap transduction with viral vectors was the most effective, with biodistribution being limited to flap tissue.

With respect to the flap–tumour bed interface, flaps were assessed at days 3, 5, 7, 10, 14, 28. For each time point, three flaps were harvested. Within each flap, three sections were examined and counts were made at three points per section. For H&E staining, neutrophils and fibroblasts could be clearly differentiated and were counted (162 counts per cell type). The number of blood vessels and the thickness of the interface were determined. The counts were then repeated, after IHC staining, to assess the numbers of neutrophils, macrophages, fibroblasts and CD3+ T cells. A total of 684 counts were made (162 per cell type).

Healing characteristics at the flap–bed interface were assessed using a combination of H&E and IHC staining. Although healing at the flap–bed interface was shown to follow the normal cascade of events seen with other wound-healing models, our results were useful for determining the optimum times to institute the various cancer gene therapies. The degree of wound-healing, the numbers of inflammatory cells present, and the thickness of this interface were important and have not been characterised previously. Wound healing at this interface identified the most appropriate time points to establish 'suicide gene therapy', immunotherapy, or radioisotopic gene therapy. The results showed that radioisotopic therapy would be feasible in the first 5 days after tumour resection and flap insetting, and subsequently after day 10. At these time points, the thickness within the interface was maintained between 0.5 mm and 0.8 mm. Between days 5 to 10, the thickness varied between 0.8 mm and 1.2 mm. These results were significant (P=0.0369).

The number of inflammatory cells (neutrophils, macrophages, CD3+ T cells, and fibroblasts) within the flap–bed interface was analysed over a 28-day period. At each time point, the harvested tissue was assessed macroscopically and microscopically. Wound healing followed the expected sequence, and was shown macroscopically with initial swelling of the flap on days 3–7 and a subsequent reduction in flap size as the tissue healed. By day 14 the wound edges were healed and there was minimal scarring. Microscopically, greater numbers of neutrophils were seen early, with little collagen formation and organisation of the wound. As wound healing progressed, there was a reduction in the number neutrophils after day 5 to zero, which remained consistent through to day 28 (P=0.0006). The number of fibroblasts increased over the same time period, peaking at day 10 and then reducing in number by day 28 (P=0.0006). Applying the Bonferroni correction at all time points assessed, the effect of day on cell type was shown to be highly significant (P=0.0001). Similarly, when assessing the relationship between cell types on the day was highly significant (P=0.0001). The distribution of these inflammatory cells was confirmed using IHC stains and cell counts showed a similar pattern.

The number of T cells and macrophages was determined by counting cells that were positively stained with CD3 and CD68, respectively. The number of macrophages peaked at day 7 and reduced to near zero by day 28. CD3+ cells followed a similar pattern to fibroblasts and peaked at day 10, after which the levels again reached baseline by day 28. The controls all showed negligible amounts of inflammatory cells.

The distribution of all inflammatory cells over the 28-day period, as determined by positive staining with CD3, CD68, neutrophil elastase and P4H, was significant when compared with the control populations (P=0.005). At all time points, the variation in cells was dependent on the day (P=0.0001). By day 3, this was shown in all specimens

and was significantly different when compared with the control population. This was confirmed on H&E and IHC staining. The degree of inflammation could be characterised by the level of leucocytosis and by a greater number of leucocytes within blood vessels. There were more lymphocytes and macrophages from day 5 onwards. By day 7, the level of T cells increased to ≈75% of the maximum number seen, reaching peak levels by day 10; the number of macrophages reached their peak by day 7 and began to reduce in number thereafter. This was useful for determining the feasibility of suicide gene therapy and immunotherapy. Immunotherapy aims to restore the host immune system to recognise and kill tumour cells with fewer side effects than with other therapies. Many cancer cells display tumor-associated antigens (TAAs) on their surface, which can be recognised by humoral and cellular limbs of the immune system. Nevertheless, the immune system rarely mounts an effective anti-tumour response. Evidence points towards evasion of immune surveillance by reducing tumour immunogenicity and inhibiting the ability of the immune system to respond effectively. Immunogenic therapy would be most effective during the first 2 weeks after insetting of the flap. During this time, more macrophages and lymphocytes were present at the flap–bed interface. Using suicide gene therapy initially to initiate lysis of tumour cells would result in tumour cells and antigens being ingested by antigen-presenting cells (APCs) and macrophages present within the interface. These cells would subsequently present to CD4+ helper T cells, which in turn would release cytokines to activate CD8+ T cells. APCs could also directly activate co-stimulatory cells using B7 co-stimulators (which provide secondary signals for the differentiation of CD8+ T cells). This process would result in differentiation of tumour-specific T cells and potentiation of further tumour lysis through direct killing of tumour cells.

In therapeutic model 1 (i.e., tumours implanted within the SIEA flap and VDEPT administered within the flap), adult male Fischer 344 rats (250–300 g) were used in this cohort (n = 18). Tumours were implanted into the SIEA flap. The flaps were disconnected at the level of the femoral artery and femoral vein. Three groups were used in this cohort. The effects of viral multiplicity of infection (MOI) on the magnitude and duration of therapeutic gene expression in tumour cell lines implanted within the flap tissue were determined using: phosphate-buffered saline (PBS; control) (n=6); adenovirus at a ratio of 10 viral particles per cell (10 MOI) (n=6); and adenovirus at 50 viral particles per cell (50 MOI) (n=6). Ganciclovir was administered every day at 50 mg/kg. Animals were assessed for growth or regression of tumours over a 30-day period using hand-held calipers.

There was a significant delay in growth up until day 31 in control *versus* Adtk therapy at 10 MOI; and similarly a significant delay in growth up to day 24 in the control *versus* the 50 MOI group. There was a significant difference in survival between the control and each of the therapeutic groups (P=0.004). Assessing each therapeutic group individually with the controls, the survival of the therapeutic groups was significantly greater than the control (control *versus* 10 MOI: P=0.0007 P=0.015); (control *versus* 50 MOI: P=0.015 and P=0.0007). However, there was no overall survival difference between the 10 and 50 MOI groups (P=0.3247 and P=0.8504). The treatment groups were therefore combined, and the overall survival between the combined treatment group and controls analysed. A significantly greater survival in the therapeutic group (P<0.0001 and P<0.0001) was observed.

In therapeutic model 2 (i.e., tumours implanted outside of the SIEA flap territory and VDEPT administered within the flap), tumours were implanted subcutaneously into the upper abdomen. After reaching 1 cm^3, tumours were resected and 0.1 cm^3 of tumour

left to mimic microscopic residual disease. Free flaps were used to reconstruct the defects left by tumour resection and the flaps armed with adenoviral vectors at 50 MOI (therapeutic arm) or PBS alone (control arm). Twelve adult male F344 rats (280–300 g) were used. Ganciclovir was administered every day at 50 mg/kg. Rats were then assessed for the growth or regression of tumours over a 30-day period using hand-held callipers.

In the control population, the tumours grew from the resected tumour bed and were incorporated into the flap. All the tumours grew from directly underneath the flap. In the therapeutic group, the tumours initially grew outside of the flap margins and then eventually grew to incorporate the flap tissue. In one rat in the therapeutic group, tumour growth was not exhibited. The control cohort demonstrated measureable tumour growth, on average 13.27 days earlier than the therapeutic group (average time to measureable tumour in control *versus* therapeutic 6.33 days *versus* 19.6 days P=0.0001). Comparing the control and therapeutic cohorts, overall tumour growth was significantly different up until days 16–19, with the most significant difference in growth between days 16 and 19. All rats in the control population were killed by day 18–19 because the tumour size had reached the maximum tolerable level. In the therapeutic group: 2 animals were killed at day 22; 1 animal at day 27; 1 animal at day 33; 1 animal at day 40; and 1 animal did not exhibit tumour growth after resection.

In summary, we developed an animal model to assess suicide gene therapy and showed significant differences in delay in tumour growth and overall survival in this therapy compared with untreated control populations.

Problems encountered and steps taken to overcome them

Animal-related issues Of 200 flaps that underwent surgery, only 28 became necrotic. In the first 100 flaps, 15 were necrotic compared with only 8 in the second 100 flaps. A horizontal mattress interrupted suture was used to anastomose the vein in the second cohort of animals, and this possibly resulted in fewer necrotic flaps. Also, the experience gained from operating would result in fewer failed flaps.

Autophagia of the flap was encountered in 1 rat. To prevent this from occurring, several modifications were made. A 6-0 vircyl rapide suture was used to close the wound. This stopped the rats attacking the flap because it would be difficult to distinguish the fine sutures from the rats own hair (as compared with black silk/nylon sutures). Also, a 'rat jacket' was developed out of felt and masking tape to stop the animals reaching the SIEA flap and eating through it.

Animals that weighed ›300 g invariably had an adverse post-operative outcome. Most successful procedures were seen in rats that weighed ‹300 g. Most problems were respiratory arrests (intra- or post-operatively).

Gene-related issues Luciferase and LacZ reporter genes were used to track the growth and regression of tumours over the experimental period. Once tissue was transduced with luciferase, after administration of luciferin, light would be released in the affected tissue or cells. At the time of killing, flaps were harvested and stained for LacZ to qualitatively express the growth or regression of tumours. Initial experiments showed promising results with respect to these reporter genes, and tumour growth could be tracked up to 3 weeks in most animals. However, subsequent therapeutic experiments began to show a reduction in bioluminescence from 2 weeks onwards, and a reduction in positive staining for LacZ was seen. We felt that the immunocompetent rats were developing an immune reaction against these reporter proteins because control and

therapeutic animals were showing a reduction in bioluminescence. Therefore, parental cell lines were used without the reporter genes and the growth and regression of tumours assessed using visual measurement (hand-held calipers).

Collaborations established

1 Mr T Pencavel, Targeted Therapy Team, ICR, Surrey. ILP model for treatment of sarcoma.

2 Dr Guy Simpson, Dept of Oncology, Postgraduate Medical School, University of Surrey, Surrey. Bladder tumour model.

3 Mr A Khan, Targeted Therapy team, ICR, Surrey. Radioprotective flap model.

Publications

Currently in the process of submitting the thesis to attain my doctorate. MPhil was awarded for the first year of work.

A manuscript is being submitted to *Lancet Oncology* as an invited article on therapeutic gene therapy in plastic surgery.

Manuscripts are being prepared based on: (a) a review of rat flaps that can be used in experimental surgery; (b) the characterisation of the flap–bed interface to determine the feasibility of gene therapy across it; (c) the study of VDEPT therapy within the flap and across the flap–bed interface.

1 Pencavel T, Seth R, A Hayes, A Melcher, H Pandha, R Vile and K J Harrington. Locoregional intravascular viral therapy of cancer: precision guidance for Paris' arrow? *Gene Ther* 2010; 17: 949–960.

2 Agrawal V K, Copeland K M, Barbachano Y, et al. Microvascular free tissue transfer for gene delivery: *in vivo* evaluation of different routes of plasmid and adenoviral delivery. *Gene Ther* 2009; 16: 78–92.

Abstracts

1 Seth R, Pencavel T, Agrawal V, Thway K, Harris P, Nutting C, Harrington K. Free tissue flaps as a vehicle to transfer gene therapy directly to the tumour bed post-resection. Fifteenth World Congress of International Confederation for Plastic, Reconstructive and Aesthetic Surgery. Abstract Book 2009; 334.

2 Seth R, Copeland K, Agrawal V, Harris P, Harrington K. Free Tissue flaps as a vehicle to deliver cancer gene therapy specifically to the tumour site with minimal systemic toxicity. Institute of Cancer Research Centenary Conference. Cancer Genes: Discovery and Exploitation 2009; 74.

3 Pencavel T, Seth R, Harrington K, Hayes A. Isolated limb perfusion and viral therapy: precision guidance for Paris' arrow? BSG Conference, London, February 2010.

Poster presentations

1 Seth R, Pencavel T, Agrawal V, Thway K, Harris P, Nutting C, Harrington K. Free tissue flaps as a vehicle to transfer gene therapy directly to the tumour bed post-resection. Poster presentation. Fifteenth World Congress of International Confederation for Plastic, Reconstructive and Aesthetic Surgery, New Delhi, November/December 2009.

2 Seth R, Copeland K, Agrawal V, Harris P, Harrington K. Free tissue flaps as a vehicle to deliver cancer gene therapy specifically to the tumour site with minimal systemic toxicity. Poster Presentation. Institute of Cancer Research Centenary Conference, London, June 2009.

Oral presentations

1 Pencavel T, Seth R, Hayes AJ, Harrington KJ. Oncolytic virotherapy in an *in vivo* model of isolated limb perfusion for advanced extremity sarcoma. SARS Annual Meeting, Dublin, January 2011.

2 Pencavel T, Seth R, Hayes AJ, Harrington KJ. Oncolytic virotherapy in an *in vivo* model of isolated limb perfusion for advanced extremity sarcoma. Imperial College Surgical Symposium, London, 2010.

3 Pencavel T, Seth R, Hayes A, Harrington K. Isolated limb perfusion and viral therapy: precision guidance for Paris' arrow? British Sarcoma Group Annual Conference, London, 2010.

4 Pencavel T, Seth R, Hayes A, Harrington K. Development of an *in vivo* model of isolated limb perfusion for oncolytic viral therapy in advanced extremity sarcoma and melanoma. Imperial College Surgical Symposium, London, 2009.

Acknowledgements

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Mr Paul Harris, clinical and educational supervisor.

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Role of the Src kinase family in breast cancer

Beatrix Cornelia Elsberger, Institute of Cancer, College of Medical, Veterinary and Life Sciences, University of Glasgow, Western Infirmary, Glasgow
Joint RCSEd/RCPSG Davies Fund Research Fellowship (5 August 2009–3 August 2011)

Lay summary

This project demonstrated that Src and Src kinase family (SFK) members have a definitive role in breast cancer. These proteins belong to a family of non-receptor tyrosine kinases and have important roles in cell signalling. Due to the paucity of translational studies, we investigated if SFK members are expressed in human breast tissue. Eight SFK members were present with distinct gene expression patterns in normal, non-malignant and malignant breast tissue. Immunohistochemistry was employed to investigate protein expression and activation of Src and SFK members. Survival analyses revealed that Src and its activation site Y419 were associated with worse outcome (confirming current *in vitro* literature), whereas a different activation site of Src (Y215) and expression of Lck was associated with improved outcome. Dasatinib (an inhibitor of Src) is currently being used in clinical trials. This inhibitor was employed in breast cancer cell lines to establish its effect on those activation sites. Decreased expression of Src and Src at Y419 was observed, whereas Src expression at Y215 remain unchanged, thereby providing a *rationale* for using this Src kinase inhibitor in clinical trials. Further investigations are necessary to identify the biomarkers to which patients are most likely respond to.

Grant report

Translational studies indicating a role of Src kinase and SFK members in breast cancer are lacking. We aimed to assess the expression and activation of Src and SFK members in large patient cohorts with full clinical data and follow-up. We also wished to investigate the effects of the Src kinase inhibitor dasatinib on Src and its various activation sites by utilising fresh frozen tissue and tissue microarrays in conjunction with *in vitro* functional studies.

Using reverse transcription-polymerase chain reaction (RT-PCR) we determined mRNA expression levels for all SFK members in specimens of human breast tissue. Src and other SFK members were expressed at different levels in normal, non-malignant and malignant breast tissue. Src and Lyn were the most highly expressed SFK members in non-malignant and malignant tissue. Lck was expressed more in oestrogen receptor (ER)-negative tumours than in ER-positive tumours. This varied significantly from the expression profile of normal breast tissue specimens, in which Fyn was the most highly expressed SFK member, followed by Src and Lyn.

Having identified which SFK members were the most noteworthy in breast tumours, the role of Src kinase, Lyn and Lck protein expression was investigated by immunohistochemistry in an expanded cohort of breast cancer specimens. Four separated Src phosphorylation/activation sites were also incorporated to explore their clinical relevance and impact on outcome. Additionally, Ki67 expression was examined to determine if increased tumour proliferation was linked to the expression and activation of SFK members.

Increased cytoplasmic expression of c-Src kinase was significantly associated with decreased disease-specific survival. These results were in accordance with cell line studies, demonstrating that c-Src is associated with more aggressive growth and poor outcome. c-Src expression was also correlated with increased tumour grade, ER-negativity and human epidermal growth factor receptor (HER)2-positivity. No significant associations with survival were detected with Lyn at any cellular location. However, membrane expression of Lck was significantly associated with longer survival in breast cancer patients. Even so, none of the patients expressing Lck at the cellular membrane died of breast cancer-related causes.

When Src kinase was activated at the classical site Y419 and located in the cellular membrane, it was associated with shorter disease-specific survival, increased grade, tumour size, ER-negativity and HER2-positivity. Our results with Y419 support the role of activation of Src kinase described in the literature, but we found contradictory results with the alternative activation site Y215. Phosphorylation at this site was strongly associated with improved survival and independent of other known clinical parameters on multivariate analyses. It remains unclear why Y419Src and Y215Src are associated with different outcome measures. These contrasting roles may be due to phosphorylation at Y215 and Y419 residing in different SH protein domains. Phosphorylation in different domains may result in varying protein configurations, which might enable activation of other downstream signalling pathways. An alternative explanation of these results may be that the antibodies detect phosphorylation of other SFK members (e.g., Lyn, Fyn, Yes) in addition or in preference to c-Src because the phosphorylated regions are highly conserved. However, we could not establish a link with the SFK member Lck, which also showed improved outcome.

Examining ER-, progesterone receptor (PgR)- and HER2-negative breast tumours as a representation of the triple negative group of breast tumours, we found that high cytoplasmic expression of Y215 Src kinase resulted in a significant survival advantage.

Based on our findings in clinical specimens we moved our attention to cell line studies. Four breast cancer cell lines, each representing one of the breast cancer subgroups, were studied to observe the effects of the Src kinase inhibitor dasatinib on the various Src phosphorylation sites, the SFK member Lck, and the downstream substrate Y861FAK. Membrane expression at the phosphorylation site Y419Src (which was associated with decreased survival in the IHC study) was significantly reduced in all cell lines, yet almost abolished in the triple negative cell line. Expression of the downstream substrate Y861FAK (which in this study was functioning as a biomarker for activation and inhibition of Src) was also diminished, whereas expression of the activation site Y215 (associated with improved outcome in breast cancer patients) remained unchanged in all cellular compartments.

This comprehensive study on the expression and activation of Src and SFK members and their response to the Src kinase inhibitor dasatinib fills a gap in the literature. It strengthens the role of Src in breast cancer, potentially provides a diagnostic method for the identification of patients that would respond to Src inhibitors, and justifies the use of Src inhibitors.

Problems encountered and steps taken to overcome them

In the initial application we intended to investigate if the site of phosphorylation in breast cancer cell lines varies depending on ER and HER2 status. The panel of the proposed cell lines represented the different subgroups of breast cancer. Through a simple steroid depletion experiment we found that the MDAMB-453 cell line (ER-neg-

ative and HER2-positive) did not express Src kinase in measurable mRNA and protein concentrations. Furthermore, we discovered that when the cell line MCF7 was transfected with HER2, ER was downregulated, which made ER silencing unfeasible. However, based on our findings in clinical specimens and the knowledge that Src inhibitors (e.g., dasatinib) were undergoing clinical trials, it seemed an essential requirement to establish which phosphorylation site they targeted. Therefore, we altered our research question to verify the effect dasatinib has on those different Src phosphorylation sites and downstream targets to ensure the correct subgroup of breast cancer patients was targeted.

Collaborations established

1 Dr Valerie Brunton, Edinburgh Cancer Research Centre, Edinburgh, Scotland. Actions of Src kinase.

2 Professor Susan Pyne and Professor Nigel Pyne, University of Strathclyde, Strathclyde, Scotland. Actions of sphingosine kinase.

3 Bristol Squibb Myers. Provided us with dasatinib for our cell line studies.

Publications

Articles

1 Elsberger B, Fullerton R, Zino R, et al. Breast cancer patients' clinical outcome measures are associated with Src kinase family member expression. *Br J Cancer* 2010; 103: 899–909.

2 Elsberger B, Tan BA, Mallon EA, Brunton VG, Edwards J. Is there an association with phosphorylation and dephosphorylation of Src kinase at tyrosine 530 and breast cancer patient disease-specific survival? *Br J Cancer* 2010; 103: 1831–1834.

3 Elsberger B, Stewart B, Tartarov O, Edwards J. Is Src a viable target for treating solid tumours? *CCDT* 2010; 10: 683–694.

4 Brown SBF, Mallon EA, Edwards J, et al. Is the biology of breast cancer changing? A study of hormone receptor status 1984-86 and 1996-1997. *Br J Cancer* 2009; 100: 807–810.

5 Elsberger B, Tan BA, Brown S, et al. Expressions of total and activated c-Src correlate differently with patient survival in ER and HER2 negative breast cancer patients. Implication for use of Src kinase inhibitors in triple negative patients? *Am J Path* 2009; 175: 1389–1397.

Abstracts

1 Elsberger B, Tan BA, Brown SFB, Mallon EA, Edwards J. Disease specific survival of breast cancer patients is not associated with expression of inactive or even partially activated Src kinase. *Cancer Res* 2010.

2 Elsberger B, Fullerton R, Zino S, Mitchell TG, Brunton V, Shiels P, Edwards J. Src kinase family members expression in human breast tissue and their association to clinical outcome of breast cancer patients. *Cancer Res* 2009; 69 (Suppl): 2.

3 Elsberger B, Tovey SM, Tan BA, Brown S, Brunton V, Mallon E, Cooke TC, Edwards J. Role of Src family members in breast cancer-dependent on site of phosphorylation. *Cancer Res* 2009; 69 (Suppl): 2.

4 Elsberger B, Tovey SM, Tan BA, Brown S, Brunton V, Mallon E, Cooke TC, Edwards J. Phosphorylated c-Src predicts clinical outcome in triple negative breast cancers. *Cancer Res* 2009; 69 (Suppl): 2.

5 Elsberger B, Fullerton R, Tan BA, Mitchell TJ, Edwards J. High expression of Src kinase family members in breast cancer specimens and their association to patients' clinical outcome. *EJSO* 2009; 35: 1237.

6 Elsberger B, Brown SFB, Mitchell TJ, Edwards J. PR status of invasive breast cancer in relation to Src kinase expression. EJSO 2009; 35: 1237.

7 Elsberger B, Zino S, Fullerton R, Mitchell TJ, Shiels P, Edwards J. Are expression levels of Src kinase family members in human breast tissue related to the clinical outcome of breast cancer patients? *EJC* 2009; 7: 93.

8 Elsberger B, Fullerton R, Tan BA, Mitchell TG, Edwards J. Survival analysis of most expressed Src kinase family members in human breast carcinoma. *J Virchows Archiv* 2009; 455 (Supp 1): S33.

9 Elsberger B, Samer S, Jordan F, Shiels P, Edwards J. Are Src kinase family members expressed in human breast cancer? *J Virchows Archiv* 2009; 455 (Supp 1).

10 Elsberger, B, Tan C.A, Brown, S., Tovey, S., Cooke, T., Edwards, J. Does expression and activation of Src kinase influence the outcome of breast cancer patients? *EJSO* 2008; 34: 1162.

11 Elsberger B, Brown S, Tovey S, Cooke T, Edwards J, Tan C.A. Activated c-Src215 kinase expression predicts relapse on tamoxifen in human breast cancer. *EJSO* 2008; 34: 1159.

12 Elsberger B, Tan CA, Brown S, Tovey S, Cooke T, Edwards J. Src kinase expression and localisation in human breast cancer. *EJSO* 2008; 34: 1029.

Poster presentations

1 Elsberger B, Tan BA, Brown SFB, Mallon EA, Edwards J. Disease specific survival of breast cancer patients is not associated with expression of inactive or even partially activated Src kinase. San Antonio Breast Cancer Symposium, Texas, USA, December 2010.

2 Elsberger B, Shepherd S, Edwards J, McMillan D. Do common solid tumours express C-reactive protein? ASGBI, Liverpool, May 2010.

3 Elsberger B, Fullerton R, Zino S, Mitchell TG, Brunton V, Shiels P, Edwards J. Src kinase family members expression in human breast tissue and their association to clinical outcome of breast cancer patients. San Antonio Breast Cancer Symposium, Texas, USA, December 2009.

4 Elsberger B, Fullerton R, Tan BA, Mitchell TJ, Edwards J. High expression of Src kinase family members in breast cancer specimens and their association with patient's clinical outcome. BASO–American Cancer Society (ACS) and Cancer Genetics Group Joint Scientific Conference, London, November 2009.

5 Elsberger B, Brown SFB, Mitchell TJ, Edwards J. PR status of invasive breast cancer in relation to Src kinase expression. BASO–ACS and Cancer Genetics Group Joint Scientific Conference, London, November 2009.

6 Shepherd STC, Edwards J, McMillan D, Elsberger B. C-reactive protein expression in solid tumours.

7 Scottish Clinical Cancer Conference, Stirling, October 2009.

8 Elsberger B, Zino S, Jordan F, Shiels P, Edwards J. Expression levels of Src kinase family members in human breast tissue. National Cancer Research Institute (NCRI) Conference, Birmingham, October 2009.

9 Elsberger B, Zino S, Fullerton R, Mitchell TJ, Shiels P, Edwards J. Are expression levels of Src kinase family members in human breast tissue related to clinical outcome of breast cancer patients?

10 European Cancer Association 15/European Society for Medical Oncology 34 (ECCO 15/ESMO 34) Conference, Berlin, September 2009.

11 Fullerton R, Tan BA, Edwards J, Elsberger B. Lck:-does it have a role in predicting clinical outcome of breast cancer patients? International Symposium on Translational and Pre-clinical Cancer Research, Beatson Institute for Cancer Research, Glasgow, June 2009.

12 Elsberger B, Zino S, Fullerton R, Mitchell TJ, Shiels P, Edwards J. Do expression levels of Src kinase family members (SKFMs) in human breast tissue relate to clinical outcome? International Symposium on Translational and Pre-clinical Cancer Research, Beatson Institute for Cancer Research, Glasgow, June 2009.

13 Elsberger B., Tan BA, Mitchell TJ, Brown SBF, Edwards J. Is Src kinase expression/ activation in invasive breast cancers linked with PR status? International Symposium on Translational and Pre-clinical Cancer Research, Beatson Institute for Cancer Research, Glasgow, June 2009.

14 Elsberger B, Zino S, Shiels, P, Edwards J. Expression of Src kinase family (SFK) members in breast tissue. ASGBI, Glasgow, May 2009.

15 Elsberger B, Tovey SM, Tan BA, Brown S, Brunton V, Mallon E, Cooke TC, Edwards J. Role of Src family members in breast cancer- dependent on site of phosphorylation. San Antonio Breast Cancer Symposium, Texas, December 2008.

16 Elsberger B, Tovey SM, Tan BA, Brown S, Brunton V, Mallon E, Cooke TC, Edwards J. Phosphorylated c-Src predicts clinical outcome in triple negative breast cancers. San Antonio Breast Cancer Symposium, Texas, December 2008.

17 Elsberger B, Tan BA, Brown SBF, Tovey SM, Cooke TG, Edwards, J. Is clinical outcome of breast cancer patients affected by Src kinase expression and/ or activation? Scottish Breast Cancer Network and Clinical Trial Meeting, Dundee, November 2008.

Oral presentations

1 Are Src kinase family members expressed in human breast cancer? Twenty-second European Conference of Pathology, Florence, Italy, September 2009.

2 Survival analysis of most expressed Src kinase family members in human breast carcinoma. Twenty-second European Conference of Pathology, Florence, Italy, September 2009.

3 Does expression and activation of Src kinase influence the outcome of breast cancer patients? BASO and ABS Joint Scientific Conference, London, November 2008.

4 Src kinase expression and localisation in human breast cancer. Fourteenth Congress of the European Society of Surgical Oncology, Den Haag, the Netherlands, September 2008.

Awards and prizes

1 American Association for Cancer Research (AACR) Translational Research Scholarship Award for a meritorious proffered paper on translational breast cancer research, December 2009. A total of £1,200 was given to attend and present data at the San Antonio Breast Cancer Symposium in Texas, USA.

2 University of Glasgow/Robertson Travelling Fellowship, July 2009. To present data at the European Pathology Congress in Florence, Italy.

3 Poster Prize: Do expression levels of Src kinase family members in human breast tissue relate to clinical outcome? Clinical Lecturer Day, Glasgow, June 2009.

4 AACR-AstraZeneca International Scholar-in-Training Award for exemplary contribution to translational breast research, December 2008. A total of £1,500 was given to me to attend and present at the San Antonio Breast Cancer Symposium in Texas, USA.

5 Poster Prize: The role of Src kinase expression and localisation in human breast cancer. International Cancer Symposium, Hong Kong, May 2007.

Higher degree

PhD awarded by the University of Glasgow, June 2011

Acknowledgements

Dr Joanne Edwards for her scientific support and supervision.

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Role of the SLC2A9 gene in the pathogenesis of hyperuricaemia and gout: final report

Philip L Riches, Rheumatology Department, Institute of Genetics and Molecular Medicine, Edinburgh
Lorna Smith Research Fellowship (1 July 2009–31 June 2011)

Lay summary

Gout affects 1 in 20 men and 1 in 100 women. The risk of developing gout is influenced by certain foods and drinking too much alcohol, but also by the genes we inherit. Previously, we found variations in a gene called SLC2A9 that were strongly associated with the risk of developing gout. Patients with gout usually have high levels of uric acid in the body and we showed that this gene is responsible for making a channel that is present in the kidney and which affects the transport of uric acid.

We gathered blood samples from ›300 patients with gout. By studying these patients we identified several new variations of the SLC2A9 gene that may be important in causing gout. We also showed that these changes influenced the amount of the channel that is produced and identified important areas in the gene that control how much of the channel is made. We also demonstrated that this channel is expressed in the lining of joints. Ultimately, we hope that better understanding of how gout is caused will lead to improvements in the care of patients with gout.

Grant report

SLC2A9 expresses a novel urate transporter that has consistently been identified as an important regulator of serum levels of urate and gout. Although non-synonymous coding polymorphisms of SLC2A9 have been reported, these have typically shown fewer significant associations with serum urate levels than non-coding variants, suggesting that the disease causing the mutation remains to be identified. It is known that SLC2A9 is expressed in the kidney on the basolateral and apical membranes. Given the importance of renal handling to serum levels of uric acid, it is a reasonable assumption that this is the main site of action. Slc2A9 has been shown to be a voltage-dependent transporter of urate favouring efflux of urate from the cell. Non-functioning mutations of SLC2A9 are associated with hypouricaemia. This indicates that the net effect of SLC2A9 (alongside other urate transporters) is reabsorption of urinary urate. The role of apical Slc2A9 remains incompletely understood. We set out to investigate further the role of the SLC2A9 gene in the pathogenesis of hyperuricaemia and gout.

Results

Haplotypes of SLC2A9 are associated with marked differential expression There is recent evidence that the level of SLC2A9 expression has a greater impact on urate levels than variation within the gene itself. The coding polymorphism rs16890979 was selected as a haplotype tagging marker known to be in strong linkage disequilibrium with intronic single nucleotide polymorphisms (SNPs) identified from genome-wide association studies of serum urate. To further investigate whether this polymorphism tagged variants within the gene that regulates gene expression, we extracted RNA from peripheral blood lymphocytes as well as from renal tissue obtained from nephrectomy specimens. We screened these samples to find those that were heterozygous at rs16890979 in whom relative levels of each allele could be measured by quantitative PCR using custom TaqMan genotyping probes recognising either allele.

We observed approximately 3–10-fold increased expression of SLC2A9 associated with the minor 'A' allele in the peripheral blood lymphocytes of gout patients. This result must be interpreted with caution because SLC2A9 is expressed at very low levels in peripheral blood. However, when we repeated this experiment looking at renal tissue in which SLC2A9 was strongly expressed, we observed the same trend. In these samples, expression of the minor 'A' allele was associated with approximately sixfold enhanced expression relative to that of the major 'G' allele. Given that the major allele is over-represented in gout, this suggests that lower overall levels of expression of SLC2A9 are associated with the development of gout.

Promotor activity in SLC2A9 is seen predominantly from an intronic region In view of the results of expression studies in peripheral blood and the kidney, we investigated the regulatory elements associated with SLC2A9. Several splice variants have been described which result in a 'short' or 'long' variant of the gene. We developed promoter-reporter assays to assess putative promoter regions of the gene. Fragments that were 2-kb upstream of exon 1 of the short and long splice variants of SLC2A9 were cloned as well as a 2-kb region immediately upstream of exon2 which was predicted to have regulatory potential. Promotor constructs were transfected into HEK293 cells using a pGL3 basic vector with expression levels determined by measurement of firefly luciferase. Succesful cloning and transfection of 2-kb fragments from all regions into HEK 293 cells have been conducted with minimal increase in luciferase activity from the regions upstream of each variant. Approximately twofold increased expression was associated with the exon 2 intronic region, suggesting that SLC2A9 had a weak internal promoter. Although HEK293 cells are derived from embryonic kidney cells, there is some dispute as to their true origin. Therefore, we repeated these results in a differentiated human kidney cell line (HK cells): we observed similar results.

Screening gout patients reveals possible new disease-causing mutations Over 300 gout patients were recruited locally in the study, well above the initial target of 250 subjects. These patients were phenotyped extensively for environmental factors known to predispose to gout and gave DNA samples. In the course of this action, significant failings in the routine care of gout patients were identified. These failings were fed back directly to clinicians through presentations at the Scottish Society for Rheumatology and local general practitioner (GP) practices. A 'discovery cohort' of 32 patients with a positive family history of gout was identified from these recruits in whom sequencing had been done in all SLC2A9 exons, the immediately surrounding introns, and putative promoter/enhancer regions of the gene. Two novel non-synonymous coding variants and three putative promoter/enhancer variants were discovered. Bioinformatic analyses suggested that protein folding/ binding of transcription factors could be affected by these variants. In addition, sequencing of SLC2A9 from 3 patients with hyperuricaemia led to the identification of an entirely novel coding polymorphism in exon 9. Sequencing of the novel coding variants was carried out in 250 cases as well as age- and sex-matched controls. These preliminary results showed the novel variants to be significantly over-represented in gout patients, and showed a trend toward more severe disease. These results will need to be replicated in a larger cohort to establish their relevance given the extensive linkage disequilibrium within SLC2A9.

Extra-renal effects of SLC2A9 The primary site of action of SLC2A9 is widely assumed to be the renal tubule. From this it would follow that variation in SLC2A9 is more closely associated with the fractional excretion of uric acid than the serum level of urate – an assumption challenged recently. To clarify this issue, data from an independent, genome-wide association scan of quantitative traits (including serum urate and fractional

uric acid excretion) from 885 individuals from the Croatian island of Korcula were analysed (data kindly provided by Caroline Hayward, MRC Human Genetics Unit, London). Eighteen likely candidate genes were selected and the strength of association tested against uric acid and fractional excretion of uric acid. Unsurprisingly, SLC2A9 remained the strongest candidate gene for regulation of both traits, but the strength of association was much higher with respect to serum urate than the fractional excretion of uric acid. A novel candidate gene for the regulation of uric acid was identified in this analysis: NHERF (SLC9A3R1). This encodes a scaffold protein that builds a raft of urate transporters in the renal tubule and would also be expected to influence the fractional excretion of uric acid more strongly than serum urate. Once again the strongest association was seen with serum urate (P=0.0002). This result suggested that the expression and regulation of SLC2A9 at other sites may be at least as important as renal expression.

Though hyperuricaemia is of great scientific interest, the most important clinical manifestation remains urate deposition within the joint. We extracted RNA from synovial tissue obtained from patients undergoing joint replacement surgery and RT-PCR identified SLC2A9 expression within this tissue. The expression seen was equivalent to that in placental tissue or renal tissue (in which SLC2A9 is known to be highly expressed). Synovial samples were obtained from patients with rheumatoid arthritis (n=6) as well as osteoarthritis (n=6) to look for evidence of altered regulation of SLC2A9 in the context of inflammatory disease. However, clear trends were not evident.

Future directions

The city of Edinburgh participated in a clinical gout study that started Autumn 2011 which aimed to recruit ›5,000 gout patients. Agreement was given by the management committee of Generation Scotland to provide all necessary age- and sex-matched controls. This allowed definitive assessment of the contribution to gout risk made by the variants identified from this work. It also provided an invaluable resource for the future analysis of the responses to treatment in gout. We established western blot techniques for measuring SLC2A9 protein directly from kidney lysates, as well as IHC techniques to stain for SLC2A9 in kidney sections. We also intend to investigate a putative enhancer of SLC2A9 within intron 6.

Conclusions

Our results suggested that a common haplotype associated with gout causes hyperuricaemia through reduced expression of SLC2A9. Though unexpected, this finding is biologically plausible and suggests that the role of apical SLC2A9 is to secrete urate into the renal lumen. This would be in accordance with its role as a voltage-driven transporter and suggests that apical expression of SLC2A9 has a dominant effect over basal expression (which is also plausible given that the apical membrane is estimated to have an ≈20-fold greater surface area). Our results also highlight the importance of considering the extra-renal effects of urate transporters (e.g., in the gut where transport into the intestine may allow degradation of urate by bacterial uricase and in synovial tissue). The latter finding raises the possibility of developing novel therapeutics that could block SLC2A9-mediated urate transport into the joint. This advantage could be negated by the effect of blockade in the gut and kidney. This remains an important area of research that I am keen to pursue.

Problems encountered and steps taken to overcome them

Several technical challenges arose in the course of this work which involved learning a wide variety of laboratory techniques. I am grateful to my many colleagues in the Molecular Medicine Centre for guiding me through them!

Collaborations established

1 Resource Management and Development Committee of Generation Scotland, Glasgow. Use of stored DNA samples.

2 Tissue Committee of the Edinburgh Experimental Medicine Cancer Centre, Edinburgh. Use of renal tissue obtained from nephrectomy samples.

3 Professor Breusch, Department of Orthopaedics, Royal Infirmary of Edinburgh, Edinburgh. To obtain samples of synovial tissue.

Publications

1 Riches PL, Ralston SH. Recent insights into the biology of bone turnover *J R Coll Physicians Edinb*, 2010; 40: 66–69.

2 Riches PL, Wright AF, Ralston SH. Recent insights into the pathogenesis of hyperuricaemia and gout. *Hum Mole Gen* 2009; 18: R177–R184.

3 Riches PL, McRorie E, Fraser WD, Determann C, van't Hof R, Ralston SH. Osteoporosis associated with neutralising autoantibodies to osteoprotegerin. *New Engl J Med* 2009; 361: 1459–1465.

Presentations

1 Tan A, Thomson J, Watters H, Riches PL, Assessing the impact of BSR Gout Guidelines on current practice. Scottish Society for Rheumatology Annual Conference, Edinburgh, June 2011.

2 Riches PL, Gray S, Albagha O, Ralston SH. SLC2A9 Gene expression is associated with a haplotype tagging polymorphism. American College of Rheumatology Annual Conference, Prague, November 2011.

Prizes

1 European Calcified Tissue (ECTS) Society Young Investigator Award, 2010.

2 British Society for Rheumatology Innovations in Rheumatology Award 2010 (category 2).

Further funding obtained

1 ECTS/Amgen Bone Biology Fellowship (March 2010): €100,000 over 3 years.

2 Chief Scientist Office (CSO) Research Grant (March 2010): £280,302 over 2 years.

Higher degree

This work was recognised as contributing to a doctorate.

Acknowledgements

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Studies on the recruitment and activation of lymphocytes in hepatic ischaemia–reperfusion injury

James Richards, Centre for Inflammation Research, University of Edinburgh, Edinburgh
Maurice Wohl Fellowship (August 2010–August 2011)

Lay summary

The prevalence of liver failure and the subsequent need for liver transplantation in the UK is increasing. Transplantation is the only effective treatment for end-stage disease, providing (on average) 22 years of additional life. Liver transplantation requires a liver to be taken from a donor and reimplanted into the recipient. During this process the liver becomes injured (ischaemia–reperfusion injury (IRI)). IRI may cause the transplant to fail. Certain donated livers ('marginal organs') are particularly susceptible to IRI and have to be discarded, thereby compounding donor shortages.

Initially liver cells are injured by a lack of oxygen (ischemia), then activation of the immune system causes reperfusion injury. Authors have shown that depletion of T cells (a type of white blood cell) reduces IRI. In this project, the isolation and characterisation of T cells from the liver was refined and improved. In IRI, these cells do not appear to be activated in the conventional (antigen-specific) manner, as suggested previously. Furthermore, a failure of suppressive mechanisms (signalling through programmed death-1 (PD-1)) in these cells leads to worse injury; PD-1 is a potential therapeutic target.

Grant report

IRI is a model of sterile inflammation and is known to be T cell-dependent; in the absence of T cells, the extent of injury is significantly reduced. Some authors have suggested that the accumulation of CD4+ T cells in the liver occurs early after reperfusion.

PD-1 is a transmembrane receptor which acts as a negative regulator of activated T and B cells. It has two known ligands: PD-L1 and PD-L2. Some authors demonstrated, in a hepatic model of IRI, that blockade of signalling through PD-1 led to worse injury.

Models and methods

Surgery Wild type C57B6 and PD-$1^{-/-}$ (on a C57B6 background) mice from the University of Edinburgh Animal House were used. Under inhalational general anaesthesia, a midline laparotomy was carried out. Using a surgical microscope, the vascular pedicle of the left lobe of the liver was isolated. This was then clamped with a micro-serrefine clamp. At a pre-determined time point, the clamp was removed and the left lobe allowed to reperfuse. Haemostasis was confirmed and the wound closed in layers. The core body temperature was maintained at 36°C throughout the procedure using a homeothermic blanket attached to a rectal thermometer to minimise experimental noise.

The animal was killed after a pre-determined period of reperfusion. Blood was taken by cardiac puncture. If the liver was being taken for flow cytometric analyses, it was flushed under pulsed low pressure *via* the portal vein with 5–10 ml PBS containing Ca^{2+}/Mg^{2+} (PAA) through a 27-G needle; this procedure was not carried out if the liver was being taken for histology. The ischaemic left lobe, non-ischemic lobes (NILs) and spleen were taken for further analyses.

Injury The extent of injury was evaluated by measuring the level of the biochemical marker alanine transaminase (ALT) in serum and/or histological scoring. Serum levels of creatinine as well as renal histopathology were used as proxies of systemic injury.

Single-cell preparations The liver was processed by a combination of mechanical (using a dissociator) and enzymatic (2 mg/ml collagenase D) digestion. This was then passed through a 70-µm filter and centrifuged slowly to remove hepatocytes. Red cells were lysed (Red Cell Lysis Buffer). Cells were then filtered and immune cells separated by a gradient (Lympholyte-M) or by using CD45+ MicroBead AutoMACS separation. Cells were then washed and centrifuged, and the pellet resuspended in FACS buffer or complete medium.

Some research teams found that enzymatic digestion led to the cleavage of cell-surface markers (CD3, CD4, CD8, NK1.1, $\alpha\beta$ and $\gamma\delta$ TCR), thereby resulting in difficulties in recognising cell populations. This was tested using 2 mg/ml Collagenase D and incubating at 36°C for various timepoints up to 60 min. Expression of cell-surface markers was found to be unaffected.

Flow cytometric analyses Single cell preparations were Fc-blocked (anti-CD16/32) before being surface-stained with a multi-colour panel of monoclonal antibodies and/or permeabilised for intracellular staining. Samples were then run on a Cell Analyser and analysed with FlowJo software.

Cell culture Tissue-resident lymphocytes from the liver and naïve splenocytes were cultured in complete medium (RPMI 1640, 5% fetal calf serum, L-glutamine, 2-mercaptoethanol, penicillin-streptomycin) and stimulated with 5 µg/ml concanavalin A or 1 µg/ml anti-CD3 for a pre-determined time course. Cells were then stained with anti-CD3, CD4, CD8, CD19, PD-1 antibodies, fixed (Fixation Buffer) and run on a flow cytometer.

Results

Pattern of injury The biochemical pattern of liver injury of warm left lobe IRI showed a peak in ALT levels at 6 h. This was a similar pattern to that described by other research teams. The level of creatinine in serum also increased during the first 24 h after reperfusion and was consistent with renal injury. These findings suggested that this was not just a localised phenomenon but instead a systemic disease process.

Cellular influx after IRI After hepatic IRI, cells were mobilised from the spleen and there is a significant influx of CD45+ cells into the ischaemic lobe by 12 h; this is not seen in NILs. This may have been a non-specific mobilisation of cells from the spleen because, although the splenic cell count diminished significantly, the relative contribution of CD3+, CD11b+ and CD3-CD11b– cells (predominantly B cells in the spleen) did not alter significantly. The influx of cells into the ischemic lobe after 12 h of reperfusion was predominately due to neutrophils (CD11b+Ly6g^{hi} cells).

T-cell specificity in IRI To test whether IRI involved an epitope-specific TCR interaction, a transgenic system was employed. The OT-II mouse has ›90% of its TCR specific for chicken ovalbumin (OVA). Significant protection was not afforded by having an OVA-specific TCR. This implied that TCR activation was not required for injury, that its activation was mediated through a non-epitope specific peptide:MHC complex–TCR interaction, or that very few (if any) T cells were required in this model of IRI.

To further evaluate this finding, an antigen transgenic system was used. The AMK-35 mouse is a transgenic mouse expressing the 1–10 peptide of myelin basic protein (MBP) covalently bound to major histocompatibility complex (MHC) class II molecules. If all the MHC class-II molecules were bound covalently with MBP_{1-10}, then they would

not be free to present antigen to T cells and, theoretically, subsequent injury would be reduced in severity. This would allow the role of MHC class-II presented peptides and their interactions with the TCR in IRI to be evaluated indirectly.

Littermates not expressing the transgene were used as wild-type controls. A significant difference between wild-type and AMK-35 mice was not observed. This raised the possibility that IRI was independent of a peptide:MHC complex–TCR interaction or that the AMK-35 mice could present sufficient antigen on MBP_{1-10}-free MHC class-II molecules.

PD-1 and IRI To ascertain if there was a role for the negative regulatory signalling pathway PD-1 in hepatic IRI, PD-$1^{-/-}$mice were compared with wild-type controls. PD-$1^{-/-}$ on a C57BL/6 background showed a relatively benign phenotype and developed mild glomerulonephritis and arthritis only at the age of 6 months. Injury was significantly increased in the absence of PD-1. This finding was consistent with a report from another research team, who noted that treatment with rat anti-B7-H1 monoclonal antibody (to disrupt PD-1 signaling) exacerbated injury. Peak levels of ALT in serum in PD-$1^{-/-}$ mice were also noted at 6 h, mirroring the pattern seen in wild-type mice. Differences between PD-$1^{-/-}$ mice and wild-type mice did not merely reflect a temporal shift in the injury pattern, but increased severity.

To evaluate how quickly PD-1 was upregulated on leucocytes using flow cytometry, splenocytes and Lympholyte-M-purified hepatic lymphocytes were stimulated in culture with concanavalin A or anti-CD3 for pre-defined time points. We found that PD-1 was upregulated rapidly on CD4+, CD8+ and CD19+ cells, and this started to be visible by flow cytometry within 3–6 h of stimulation with anti-CD3 or concanavalin A.

The resting liver had a resident population of CD4+ and CD8+ cells that expressed appreciable quantities of PD-1. This may have been because the liver receives a high burden of antigen from the digestive tract *via* the portal vein and because PD-1 is important in the maintenance of peripheral tolerance in the liver.

Discussion

We noted a significant influx of neutrophils into the ischemic lobe by 12 h of reperfusion. This finding was consistent with the observations of authors who identified neutrophils as important mediators of hepatic IRI; antibody depletion of neutrophils was found to decrease the area of observed necrosis from 80% to 28%. Accumulation and activation of neutrophils at the site of injury appears to be mediated by T cells. It has been suggested that the recruitment of neutrophils may be a $\gamma\delta$ T cell- rather than $\alpha\beta$ T cell-mediated process.

Our findings suggested that if T cells are important in IRI, then it is an antigen-independent process. This finding is contrary to the work from one research team who found it to be an antigen-dependent process because they found a significant reduction in injury using OT-II mice at 4 h and 8 h of reperfusion compared with wild-type mice.

Using the PD-1 knockout mouse described above, there appeared to be a protective role for PD-1 in IRI. This was in accordance with the observations seen by one research team. They found that interruption of this signalling pathway augmented injury and that treatment with B7-H1 immunoglobulin to stimulate signalling reduced injury. PD-1 is thought to be a negative regulator of T and B cells, and this work pointed indirectly to a role for these cells in IRI.

The exact cell type through which this protective mechanism acts in IRI remains elusive. However, we demonstrated that, after stimulation, T cells can upregulate PD-1 expression within the hyperacute timeframe of IRI.

Problems encountered and steps taken to overcome them

Reliable isolation of lymphocyte populations from control and inflamed liver Collaboration was established with Professors Stephen Anderton and Sarah Howie (Autoimmunity and Chronic Inflammation Group, Edinburgh University, Edinburgh). This led to significant improvement in the yield and purity of immune cells. Introduction of a 'hepatocyte spin' to further remove unwanted debris and cells also made a significant improvement to the cleanliness of the cell preparations.

Multicolour flow panel Development of an 11-colour flow cytometry panel dramatically increased the data retrieved per experiment and enabled more thorough classification of lymphocyte subpopulations. Advice and help from Kelly Lundsten (Chicago University, Chicago, USA) was invaluable. Compensation was improved through the use of polystyrene beads rather than cells.

Collaborations established

1 Professor Stephen Anderton, University of Edinburgh, Edinburgh.
2 Professor Sarah Howie, University of Edinburgh, Edinburgh
3 Dr Chris Bellamy, University of Edinburgh/Royal Infirmary of Edinburgh, Edinburgh.
4 Professor Adrian Hayday, Kings College London, London.
5 Professor Gerry Graham, University of Glasgow, Gasgow.

Publications

1 Richards J, Wigmore SJ, Devey LR. Heme-oxygenase system in hepatic ischemia-reperfusion injury. *World J Gastroenterology* 2010; 16: 6068–6078.
2 Richards J, Wigmore S. Surgery: immunology at the cutting edge. *Immunol News* 2011; 18: 19–21.

Poster presentation

Richards J. Role of T cells in hepatic ischemia–reperfusion injury. Fifth European Network of Immunolgy Institutes/European Federation of Immunological Societies/European Journal of Immunology (ENII/EFIS/EJI) Summer School in Advanced Immunology, Sardinia, May 2010.

Higher degree

This work featured in my doctoral thesis.

Further funding

Receipt of this initial Small Research Support Grant acted as a catalyst to me securing further funding.

Peel Medical Research Trust Research Grant: £2,000.

Tenovus Scotland Small Research Grant: £10,000.

British Society for Immunology Student Fellowship: £750.

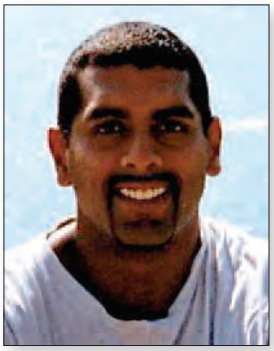
Wellcome Trust Research Training Fellowship: £249,055.

Acknowledgements

I am especially grateful for the support from the Maurice Wohl Fellowship and the RCSEd for this opportunity to pursue this important research project and help launch my career in academic surgery. I would also acknowledge the help, assistance and advice from all members of the Immunity and Chronic Inflammation research team (Profes-

sors Anderton, Howie and Schwarze) as well as Professor Wigmore's research team (Luke Devey and Stephen McNally). I greatly appreciate the input of Dr Chris Bellamy. I thank Forbes Howie (Biochemistry Department), all members of the Histopathology Unit, and the Flow Cytometry Unit at the Centre for Inflammation Research.

Derivation of photoreceptor precursors from human Müller stem cells and their application in experimental photoreceptor replacement



Hari Jayaram, Ocular Biology and Therapeutics, UCL Institute of Ophthalmology, London

Medical Research Council/RCSEd Clinical Research Training Fellowship (1 August 2008–31 July 2011)

Lay summary

The adult human retina has a population of cells named 'Müller glia' that exhibit stem cell (SC) characteristics. We investigated the feasibility of obtaining Müller stem cells (MSCs) from small samples of retinae from humans, rats, rabbits and non-human primates to provide proof-of-concept that SCs may be obtained from a human to develop a strategy of transplantation of his/her SCs. We also explored the role of human Müller SCs in providing a source of rod photoreceptor precursors that may be applied to experimental models of photoreceptor replacement.

A protocol was developed using MSCs that demonstrated increased expression of photoreceptor markers after treatment with various combinations of growth factors in the laboratory. These photoreceptor precursors showed increased signalling in response to specific chemicals and light stimulation as judged by calcium imaging and patch-clamp techniques. After subretinal transplantation into rodent models of photoreceptor degeneration, MSC-derived photoreceptor precursors migrated and integrated into the outer retina, demonstrating expression of photoreceptor markers and connectivity with the host retina. Grafting of photoreceptor-differentiated (but not undifferentiated) cells led to a significant increase in rod photoreceptor function as shown by the measurement of electrical responses of the retina to light stimulation.

These observations showed that human MSCs can differentiate into photoreceptor precursors and that these could be used in the development of therapies for human photoreceptor disease.

Grant report

Demonstration of proof of concept that MSCs may be isolated from peripheral retinal biopsies, thereby making autologous transplantation strategies a possibility. Isolation of MSCs from retinectomy specimens proved difficult. The success rate from peripheral cadaveric donor retinae improved to ≈30%. It was hypothesized that prolonged tissue storage before processing (24–72 h) could limit success. Peripheral biopsies of fresh (‹4 h) *post mortem* rabbit and primate retinae revealed a success rate of 80%. These results provided proof of concept that harvest of tissue for patient-specific therapies may be feasible (although they may be prohibited by financial restraints).

Development of an *in vitro* differentiation protocol to generate an enriched population of rod photoreceptor precursors from human MSCs. Studies of exogenous factors upon the induction of rod photoreceptor markers led to the development of an *in vitro* differentiation protocol that significantly upregulated the expression of rod photoreceptor markers over a 5-day period. Some functionality of the precursors was examined *via* intracellular calcium changes in response to cyclic guanosine monophosphate (cGMP) analogues and patch-clamp techniques. These findings indicated that cultured MSCs could constitute a useful source of rod photoreceptor precursors for subsequent transplantation.

MSC-derived photoreceptor precursors migrate and integrate within the outer nuclear layer of degenerate rodent retinae after sub-retinal transplantation *in vivo* and express markers of mature rod photoreceptors. Subretinal transplantation of enriched populations of photoreceptor precursors resulted in migration to the outer nuclear layer of degenerated rodent retinae. Transplanted cells expressed markers of mature rod photoreceptors but without generation of outer segments of the rods. These results suggested that human MSC-derived photoreceptor precursors could migrate to the appropriate retinal lamina after transplantation.

MSC-derived photoreceptor precursors restore rod photoreceptor function *in vivo* in a rodent model of human retinitis pigmentosa. Transplantation of differentiated cells shown to integrate within degenerating rodent retinae induced a significant increase in rod photoreceptor function compared with controls. Undifferentiated cells tended to span the entire retina and adopt a Müller morphology, exhibiting some rescue compared with controls, which was postulated to be a neurotrophic effect. These results suggested that subretinal transplantation of human MSC-derived photoreceptor precursors restores rod photoreceptor function in rodent models of retinitis pigmentosa.

Problems encountered and steps taken to overcome them

Difficulties in isolating cells from small biopsies of cadaveric human retinae. We sought to obtain fresh cadaveric non-human primate retinae by contacting the MRC Breeding Centre at Porton Down (Wiltshire). They contacted us when animal killing was scheduled so that I could enucleate eyes and process fresh tissue within 3–4 h *post mortem*.

Limitations with electroretinographic techniques and equipment. I attended a training course in El Paso (Texas, USA). Our research team purchased a state-of-the art apparatus, which enabled simultaneous bilateral electroretinographic measurements in animals.

Difficulties with in vitro functional analyses of differentiated cells using equipment at the UCL Institute of Ophthalmology. A collaboration was developed with Dr Zheng at Oxford University (Oxford), who had expertise in patch-clamp, multi-electrode array, and calcium imaging techniques in response to light stimulation.

Lack of access to expertise in microarray data analyses. I attended a 5-day course hosted by Imperial College London (London), which enabled me to become familiar with the software and programmes used for the analysis of microarray data.

Collaborations established

Dr Lei Zheng, Oxford Neuroscience/Nuffield Laboratory of Ophthalmology, Oxford. Dr Zheng has relocated to Shanghai (China) as a principal investigator. We hope to develop our collaboration further by promoting further research with his new research team in Shanghai.

Publications

Articles

1 Bhatia B, Jayaram H, Singhla S, Jones MF, Limb GA. Differences between the neurogenic and proliferative abilities of Müller glia with stem cell characteristics and the ciliary epithelium from the adult human eye. *Exp Eye Res* 2011; 93: 852–861.

2 Dahlmann-Noor A, Vijay S, Jayaram H, Limb A, Khaw PT. Current approaches and future prospects for stem cell rescue and regeneration of the retina and optic nerve. *Can J Ophthalmol* 2010; 45: 333–341.

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Book chapters

1 Jayaram H, Becker S, Limb GA. Stem-cell based therapies for glaucoma. In: Kubena T (ed) Glaucoma 2. Intechweb Publishing, 2011.

2 Limb GA, Jayaram H. "Regulatory and pathogenic roles of Müller glial cells in retinal neovascular processes and their potential for retinal regeneration. In: Hammes HP, Porta M (eds) Experimental approaches to diabetic retinopathy. Frontiers in diabetes, Karger, Basel, 2010, vol 20, p 98–108.

Poster presentations

1 Jayaram H, Singhal S, Bhatia B, Khaw PT, Limb GA. Derivation of photoreceptor precursors from adult human Müller stem cells. International Society for Stem Cell Research Annual Meeting, Barcelona, Spain, July 2009.

2 James LMI, Singhal S, Jayaram H, Khaw PT, Limb GA. Optimisation of cellular scaffolds for neural retinal cell replacement using human Müller stem cells. Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, USA, May 2011.

3 Jayaram H, Singhal S, Bhatia B, Khaw PT, Limb GA. Derivation of photoreceptor precursors from adult human Müller stem cells. Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, USA, May 2011.

4 Singhal S, Jayaram H, Bhatia B, Salt TE, Khaw PT, Limb GA. Retinal ganglion cell (RGC) precursors derived from adult human Müller stem cells exhibit neural function *in vitro* and partially restore RGC function *in vivo*. Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, USA, May 2011.

5 Jayaram H, Singhal S, Bhatia B, Khaw PT, Limb GA. Taurine and notch pathway inhibition promote *in vitro* differentiation of adult human Müller stem cells towards a photoreceptor fate. Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, USA, May 2011.

6 Jayaram H, Singhal S, Bhatia B, Khaw PT, Limb GA. Role of taurine and notch inhibition in promoting *in vitro* photoreceptor differentiation of adult human Müller stem cells. United Kingdom Stem Cell Network Annual Scientific Conference, Oxford, April 2009.

Oral presentations

1 Jayaram H, James LMI, Becker S, Cottrill P, Khaw PT, Limb GA. Restoration of photoreceptor function by transplantation of human Müller stem cells differentiated towards a photoreceptor phenotype in a rodent model of retinitis pigmentosa. Oxford Ophthalmological Congress, Oxford, July 2011.

2 Jayaram H, James LMI, Becker S, Cottrill P, Khaw PT, Limb GA. Restoration of retinal function by transplantation of human Müller stem cell-derived photoreceptors in P23H rhodopsin transgenic rats. Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, USA, May 2011.

3 Jayaram H, James LMI, Becker S, Cottrill P, Khaw PT, Limb GA. Müller stem cell repair of damaged photoreceptors. Royal College of Ophthalmologists Annual Congress, Birmingham, May 2011.

4 Jayaram H, James LMI, Becker S, Cottrill P, Khaw PT, Limb GA. Restoration of retinal function by transplantation of human müller stem cell derived photoreceptors in P23H rhodopsin transgenic rats. Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, USA, May 2011.

5 Jayaram H, Khaw PT, Limb GA. Regenerating the ganglion cell layer. Moorfields International Glaucoma Symposium, London, January 2010.

Prize

Association for Research in Vision and Ophthalmology/National Institute for Health Research (ARVO/NIHR) International Travel Grant Award, May 2011.

Higher degrees

Postgraduate Diploma in Research Ethics, Keele University, 2010.

PhD, University College London, November 2011.

Acknowledgements

I am very grateful to the Medical Research Council and RCSEd for providing me the opportunity of a lifetime to be trained in basic laboratory research in an institute whose main drive is the translation of basic laboratory findings into human therapies. This fellowship has enabled me to develop the motivation, skills and collaborations which will help develop my career as a clinician scientist. I also thank my supervisors (Dr G Astrid Limb and Professor Peng T Khaw) for their constant support and guidance. In particular, Dr Limb's advice and feedback enabled me to write and submit my PhD thesis before the end of my 3-year clinical research training fellowship.

Nanoscale control of cells on fabricated biomaterial surfaces

L J Spalding, Institute of Cellular Medicine, Newcastle University, Newcastle

arc/RCSEd Fellowship (April 2007–January 2011)

Lay summary

For many years, metal implants have been used to treat patients with orthopaedic injuries. However, there is a need to develop new technologies that are better able to influence the biological events at the implantation site. One strategy is to 'coat' biomaterial scaffolds with biological molecules such as signalling proteins before insertion into the skeleton.

We investigated a protein signalling molecule called AC-100. Upon surface coating with some of our engineered proteins, we were able to promote the adhesion of the cells that make bone (osteoblasts) and also adult stem cells (mesenchymal stem cells (MSCs)) that give rise to the osteoblasts. In longer-term experiments assessing the production of a mineralised matrix, we showed that the surfaces we created could stimulate the cells to make extensive bone-like nodules in a way that was comparable with a powerful regulatory molecule called bone morphogenetic protein (BMP), which is already in clinical use. We also investigated how best to harvest the cells of patients during a surgical procedure and ensure that populations of cells that are alive and healthy can be reintroduced with the developed biomaterials to provide enhanced treatments for musculoskeletal defects.

Grant report

Tissue engineering and regenerative medicine are rapidly becoming an established part of orthopaedic practice. These treatments have been based upon a few cell types and scaffolds that have been modified by the addition of chemical or biological factors. Refinements of these therapeutic strategies may ensure the regeneration of tissue that is 'fit for purpose' and exhibits significant functional longevity.

These approaches require a detailed understanding of how relevant cell types interact with scaffold materials at the biomolecular (nano) scale and how this influences their biology. This goal was addressed by using novel *in vitro* systems to investigate how controlled presentation of bioactive motifs from matrix extracellular phosphoglycoprotein (MEPE) can influence the activity of mesenchymal stem cells (MSCs) and cells from the osteoblast lineage.

MEPE is a member of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of secreted glycophosphoproteins. Several studies demonstrated that MEPE and its peptide motif, AC-100, may regulate bone mass and influence osteoblast activity. Those findings suggested its potential for inclusion in novel therapeutic strategies aimed at increasing osteogenesis. Evidence suggests that AC-100 exerts its effects (at least in part) through cell adhesion receptors. Therefore, it could be used to understand the relative roles of immobilised *versus* soluble signals in the control of cell activity.

In our work, AC-100 was cloned into a novel scaffolding protein that has a C-terminal cysteine that allows chemisorption to gold, followed by the addition of 12 amino acids that form a water-soluble coil that switches to a hydrophobic helix in the presence of alkane thiols. This approach allowed us to immobilise AC-100 on gold-coated coverslips and then ensure its controlled display by the addition of a triethyleneglycol-thiol.

Experiments demonstrated that AC-100 supported the adhesion of osteoblasts and MSCs. However, there were specific differences in that the focal complexes (points at which cells are anchored) were larger on AC-100-coated surfaces and, even though osteoblasts adhered to the osteopontin motif, MSCs did not. Cells were also cultured on these surfaces for longer periods of time to investigate their influence on bone formation. AC-100 surfaces could support osteogenesis to a greater degree than osteopontin surfaces and to a level comparable with that seen with BMP2. AC-100 is believed to not only influence cell adhesion but also activate signalling of prostaglandin E2. Experiments were also conducted to assess the relative contributions of soluble AC-100 (kindly provided by Acologix Incorporated) with immobilised AC-100. In addition, studies were undertaken to investigate how a commercial gas pressure lavage system (Carbojet™) could be used to retrieve human bone marrow cells at the time of surgery. These studies revealed that the Carbojet™ system was no more effective than simple mechanical agitation in solution at retrieving viable cells.

In summary, this work demonstrated that immobilised cell adhesive cues such as AC-100 have potent effects on regulation of the activity of musculoskeletal cells and could be utilised in the development of new scaffold materials for orthopaedic surgery.

Problems encountered and steps taken to overcome them

The overall goals of the study were retained for the duration of the project. However, there was some re-evaluation of the breadth of experimental approaches that were used. Initial experimental approaches to study cell activity on immobilised biomolecules were unsuccessful and significant work was required to develop the approach that became a central part of this study. Enforced breaks due to pregnancy-related problems and maternity leave meant that some research momentum was lost. A pre-agreed return to clinical training that could not be deferred meant that not all experimental work could be completed. Therefore, a further period of time out of training was negotiated to allow completion and submission of this work.

Collaborations established

Professor Jeremy Lakey, Professor of Structural Biochemistry, Newcastle University, Newcastle.

Poster prize

Matrix extracellular phosphoglycoprotein as an immobilised motif to control osteoblast differentiation. First prize, British Orthopaedic Research Society Meeting, Newcastle, 2009.

Acknowledgements

Arthritis Research UK and the RCSEd.

My supervisors: Dr Mark Birch and Professor Andrew McCaskie.

Professor Jeremy Lakey.

Orla Protein Technolgies.

Acologix Incorporated.

Kinamed Incorporated.

Role of sinusoidal endothelial cells in regulating hepatoblast proliferation and differentiation in developing human liver

Janet Kung, MRC Centre for Regenerative Medicine, Chancellor's Building, Edinburgh

MRC/RCSEd Clinical Research Training Fellowship (1 September 2007–31 August 2011)

Lay summary

Liver disease is the fifth leading killer in the UK. It is the only major cause of death whose prevalence has been increasing year-on-year over the past 40 years. Liver transplantation is the only cure but, unfortunately, many patients die waiting for a new liver. New treatments are urgently needed.

Liver stem cell therapy holds great promise for the treatment of liver disease. Such therapy can provide liver cells (hepatocytes) for drug discovery, bio-artificial liver support devices and cell transplantation. However, hepatocytes are unstable and have suboptimal function in the laboratory. Understanding how new hepatocytes are regenerated is key to finding ways of repairing damaged liver tissue.

We isolated stem cells that can mature into functioning hepatocytes from the developing human liver. Soluble factors secreted by neighbouring cells (including cells that line blood vessels) provide vital signals for the stimulation of maturation of liver stem cells. Using novel gene-based techniques, the network of complex biochemical signals that control the development and maturation of the human liver were unravelled. These data will be central to the development of stem-cell therapies for human liver disease.

Grant report

Human foetal liver progenitor cells (LPCs) and embryonic stem (ES) cell-derived hepatocytes have promise for liver cell therapy. However, the microenvironment and the regulatory signals governing their optimal proliferation and differentiation *in vitro* are not known. Culture of LPCs alone results in inadequate functionality and phenotypic instability. The nature of the developmental hepatocellular niche and the maturation that occurs during liver development must be understood to deliver the promise of stem-cell therapy.

We aimed to: (i) characterise the 'transcriptomic signature' of LPCs and delineate the molecular mechanisms participating in the emergence of the identity and function of LPCs; and (ii) determine the role of non-parenchymal cells (NPCs) in regulating the proliferation and differentiation of LPCs.

Methods

Cell-surface markers were used to delineate different cell populations in the developing liver. $EpCAM^{+}/CD29^{+}/CD49d^{+}/CD49e^{-}/CD45^{-}$ LPCs were isolated from second trimester (weeks 14–20) human livers. Their phenotype was confirmed through immunophenotyping and analyses of secreted liver-specific proteins. Microarray profiling of mRNA and microRNA in LPCs was undertaken and compared with adult hepatocytes. NPCs were isolated through EpCAM negative selection. LPCs, NPCs and mixed LPC/NPC cultures were grown in Williams' Medium E (WME) and NPC-conditioned medium (NPC-CM) for 13 days and their hepatocytic functions compared.

Results

EpCAM$^+$ LPCs were consistently and robustly isolated from human foetal livers from gestational weeks 14–20. Gene ontology analyses showed that differentially expressed mRNAs upregulated in LPCs were predominantly involved in cell division, chromatin modification and transcription. Genes upregulated in hepatocytes were involved in metabolism and biosynthesis. Promoter analyses suggested that LPC gene expression was driven by the proliferation-associated transcription factors E2F1 and NFYA, whereas the liver-enriched transcription factors HNF4A and HNF1A were active in hepatocytes. Fifteen microRNAs were differentially regulated between LPCs and hepatocytes. Bioinformatic integration of the microRNA and mRNA datasets revealed microRNAs upregulated in LPCs targeted genes involved in metabolic processes, implying repression of the mature hepatocyte phenotype. Conversely, microRNAs upregulated in hepatocytes targeted and inhibited genes which negatively regulated mature hepatocyte processes, thereby promoting adult liver function. MicroRNA promoter analyses suggested that LPC microRNA expression was driven by the E2F-binding protein ARID3A, whereas hepatocyte microRNA expression was driven by FOXA2 (a transcriptional activator for liver-specific genes).

Functional studies showed that LPCs had a bipotential phenotype expressing hepatocytic (AFP and albumin) and biliary (CK19) markers and could secrete liver-specific proteins *in vitro*. EpCAM$^-$ NPCs consisted of tube-forming endothelial cells and mesenchymal cells. Mixed LPC/NPC cultures had significantly greater synthetic competence than LPCs alone ($p<0.01$). This was confirmed to be mediated through soluble factor(s) by media transfer experiments. LPCs grown in NPC-CM had greater hepatocytic maturity by secreting significantly more albumin ($p<0.01$) and fibrinogen ($p<0.001$) than that grown in WME. Fibronectin was secreted by NPCs in abundance and was intimately associated with EpCAM$^+$ LPCs *in vivo*. EpCAM$^+$ LPCs expressed the fibronectin-binding integrins $\alpha4\beta1$ and $\alpha5\beta1$, suggesting fibronectin might be an important mediator of hepatocyte maturation.

Conclusions

LPCs exhibit a molecular profile consistent with a ‘stem cell signature’, cell division and certain liver-specific functions. Transcriptional control of the LPC phenotype occurs at transcriptional and post-transcriptional levels through microRNAs and involves biologically relevant transcription factors. Furthermore, NPCs, by secreting soluble factor(s), play an important part in the proliferation and differentiation of LPCs.

Problems encountered and steps taken to overcome them

The major problem was the lack of commercially available recombinant fibronectin fragments consisting of specific functional domains known to interact with different integrin heterodimers. The collaborations shown below were established to facilitate investigation of the role of fibronectin in regulating the proliferation and differentiation of foetal liver progenitor cells.

Collaborations established

1 Dr Chris van der Walle, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Strathclyde. Provided the ‘cell-binding domain’ of fibronectin (modules III_{9-10} in monomers and multimers).

2 Professor Cay Kielty, Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester. Provided (i) the fibronectin construct containing modules III_{7-15} and extra domain A (EDA), and (ii) EBNA-293 cells transfected with the construct.

Publications

Articles

1 Kung JWC, Forbes SJ. Stem cells and liver repair. *Curr Opin Biotechnol* 2009; 20: 568–574.

2 Kung JWC, Currie IS, Forbes SJ, Ross JA. Liver development, regeneration and carcinogenesis. *J Biomed Biotechnol* 2010; 2010: 984248.

Book chapters

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2 Medine CN, Kung JWC, Payne CM, et al. Human liver development as a template to generate high fidelity models. In: Hay DC (ed) Stem cells, regenerative medicine and the liver. In press.

Poster presentations

1 Kung JWC, Currie IS, Hadoke PWF, Forbes SJ, Ross JA. Non-parenchymal cells regulate proliferation and differentiation of human liver progenitor cells *via* soluble factor(s). International Liver Congress, Barcelona, April 2012.

2 Kung JWC, Currie IS, Hadoke PWF, Forbes SJ, Ross JA. Non-parenchymal cells regulate proliferation and differentiation of human liver progenitor cells *via* soluble factor(s). Lister Centenary Meeting, RCSEd, Edinburgh, February 2012.

3 Kung JWC, Gallagher IJ, Forbes SJ, Ross JA. Combined mRNA and microRNA profiling reveals microRNA regulation of liver progenitor cell phenotype. Lister Centenary Meeting, RCSEd, Edinburgh, February 2012.

Oral presentations

1 Kung JWC, Gallagher IJ, Forbes SJ, Ross JA. Combined mRNA and microRNA profiling reveals microRNA regulation of liver progenitor cell phenotype. International Liver Congress, Barcelona, April 2012.

2 Kung JWC, Gallagher IJ, Forbes SJ, Ross JA. Combined mRNA and microRNA profiling reveals microRNA regulation of liver progenitor cell phenotype. School of Surgery Day, University of Edinburgh, Edinburgh, December 2011.

3 Kung JWC, Forbes SJ, Ross JA. Non-parenchymal cells regulate proliferation and differentiation of human liver progenitor cells *via* soluble factor(s). School of Surgery Day, University of Edinburgh, Edinburgh, December 2010.

Awards

1 Chiene Medal Winner, University of Edinburgh/RCSEd, Edinburgh, 2011.

2 European Association for the Study of the Liver (EASL) Young Investigator Bursary, 2012.

Acknowledgements

Professor James A Ross.

Professor Stuart J Forbes.

Dr Patrick WF Hadoke.

Dr Ian S Currie.

Dr Iain J Gallagher.

Role of sinusoidal endothelial cells in regulating hepatoblast proliferation and differentiation in developing human liver

Dr David C Hay.

Kay Samuel.

Dr Martin Waterfall.

Professor Richard Anderson.

Dr Andrew Childs.

Anne Saunderson.

Dr Chris van der Walle.

Professor Cay Kielty.

All members of the Tissue Injury and Repair Group.

Travelling Fellowship Reports

Cutner Travelling Fellowship in Orthopaedics

Dr Mark Gaston, Department of Paediatric Orthopaedics
University Children's Hospital, Basel, Switzerland
(1 June 2009–30 June 2010)

I had the unique opportunity of undertaking this clinical fellowship working under the supervision of Professor Reinald Brunner and Professor Fritz Hefti at the University of Basel Children's Hospital in Switzerland. This was unique because even though they usually have a fellow from ASG (Austria, Switzerland, Germany), I was the first British fellow to fill the position.

I was fairly timorous when I attended on the first Monday morning in June 2009, which began with a clinical meeting with the whole department at 7 am (as every Monday morning) to discuss difficult cases from the outpatient clinics the week before and to discuss potentially interesting surgical cases in the week ahead. I rapidly learned that my 18 months of weekly lessons in high German were only a mild preparation for 'Schweizerdeutsch' (which is a very different-sounding dialect) and it was clear from day one that (quite rightly) no exceptions would be made for my lack of understanding. This allowed me to rapidly gain a good grasp of the language which now, if not fluent, is competent enough to allow me to work independently in medicine in a German-speaking country. Thankfully many of the medical teams are considerate and the secretaries were kind enough to correct my many grammatical errors in dictation.

My main role was as resident to Professor Brunner, which was my desire because his main interest lies in neurological disorders in paediatric orthopaedics. He runs a tertiary referral centre for children with complex neurological musculoskeletal deformity and gait abnormalities, and is responsible for the Gait Analysis Laboratory. He also had external clinics all over Switzerland and as far away as Rome. His team also includes an Oberarzt (essentially the level of a consultant but not the 'chief'), Dr Camathias, who helps him run the neuro-orthopaedic service but also has an independent interest in paediatric knees and has a weekly outpatient clinic dealing exclusively with this problem.

My weekly responsibilities included two specialist neuro-orthopaedic outpatients clinics with Professor Brunner and one knee clinic with Dr Camathias. I also had a general, 'poliklinic' which I did independently, seeing fractures, general paediatric orthopaedics and post-operative patients. The other 3 days were spent mainly in the operating theatre. Every Tuesday was a 12-h session with Professor Brunner doing the neurological cases (mainly multilevel lower-limb correction surgery and complex hip reconstruction for patients with cerebral palsy). This included pelvic osteotomy, long-bone osteotomy (particularly in the femur with three-plane correction), soft tissue corrections and correction of complex neurological foot deformities. Friday morning was a 6-h paediatric knee session with arthroscopy and open procedures (particularly in relation to patello-femoral instability).

Professor Hefti heads the general paediatric orthopaedic service as well as being responsible for management of musculoskeletal tumours in this area of Switzerland. I also participated in his service, which included correction of development dysplasia of the hip (which was very rarely seen late due to ultrasound screening), clubfoot and growth deformity, as well as tumour management. Extracorporeal irradiation methods

were used frequently with good outcome in children. He also has an interest in the young adult hip, and I gained experience in Ganz-type surgical dislocations of the hip and peri-acetabular osteotomy for disorders in the young adult hip.

I therefore gained excellent experience, logging nearly 500 cases in the year (including ›50 pelvic osteotomies and ›100 long-bone osteotomies). I believe I gained good understanding of how to evaluate and manage the orthopaedic problems of children with neurological disorders, including non-surgical methods such as orthotic management and interpretation of gait analysis data. The time spent with Professor Brunner was invaluable because his depth of understanding in neuro-orthopaedics in Europe is unmatched. I also gained good experience in managing paediatric knee disorders, including meniscal repair, medial patello-femoral ligament reconstruction and trochleaplasty. I was also exposed to, and involved in, many of the cases in one of the most respected centres for paediatric orthopaedics. I have a log of radiographs of ›50 interesting cases. I was also on the call rota for paediatric trauma 1 in 4, which helped to maintain my trauma experience.

I also undertook a research project in the Gait Analysis Laboratory to further my understanding of this difficult area. Professor Brunner, along with Jacqueline Romkes (a full-time researcher in the Gait Analysis Laboratory) have published extensively in kinetics, kinematics and electromyography in children with neuromuscular disorders as well as able-bodied children. My aim is to continue a research interest in this area in the UK and to collaborate with the colleagues I worked with in Basel. In addition I was involved in several other article during this year (see below).

This was an incredibly busy fellowship consisting of hard work with many challenges, not least communication (certainly in the first period). It was equally as rewarding in every aspect (clinical, surgical and academic) and I believe the experience I have gained in paediatric orthopaedics is second to none. It also presented a prolonged insight into the working of a very different medical system and culture, where the chief holds all the influence clinically and administratively, presenting a striking contrast to the current NHS. It is a insurance company-funded system which, while providing tangible benefits such as short waiting times and access to top-quality instrumentation, also has its flaws such as time spent in securing funding for patients whereas companies try to find ‘ways out of paying’.

Time was found to travel with my family to the Alps, southern France, Austria, Italy and most of Switzerland. My 5-year-old son developed what is sure to be a long-lasting love for skiing! I will bring the experience and knowledge of this fellowship back to the UK and put it to good use as a consultant in a respected paediatric orthopaedic centre.

Publications

1 Camathias C, Rutz E, Gaston MS. Massive osteochondritis of the lateral femoral condyle associated with discoid meniscus: management with meniscoplasty, rim stabilization and bioabsorbable screw fixation. *J Pediatr Orthop B* 2012; 21: 421–424.

2 Camathias C, Festring JD, Gaston MS. Bioabsorbable lag screw fixation of knee osteochondritis dissecans in the skeletally immature. *J Pediatr Orthop B* 2011; 20: 74–80.

3 Bachmann M, Gaston MS, Hefti F. Supracondylar stress fracture of the femur in a child. *J Pediatr Orthop B* 2011; 20: 70–73.

4 Krieg AH, Lenze U, Gaston MS, Hefti F. Pelvic reconstruction with non-vascularised fibular grafts after resection of bone tumours. *J Bone Joint Surg Br* 2010; 92: 1568–1573.

Cutner Travelling Fellowship in Orthopaedics

Major Raymond Anakwe, RAMC, Landstuhl Regional Military Medical Center, Landstuhl, Germany
(2010)

Grant report

This travelling fellowship gave me a tremendous opportunity to visit this centre for military trauma care. It receives injured military personnel from the USA and Canada fighting in Afghanistan and Iraq. Initial surgery was undertaken in field hospitals deployed in these countries. Casualties were evacuated by a dedicated MEDEVAC/Critical Care Team, often within 24 h of injury. I was able to 'shadow' and operate with several trauma and orthopaedic surgeons serving as military personnel and volunteers.

I visited the unit for 2 weeks and in this time I was attached to the on-call/receiving orthopaedic/trauma team. We received regular MEDEVAC flights, reviewed all casualties, undertook early revision surgery (including external fixation, wound debridements, fracture fixation and limb fasciotomies). I was able to sit in on clinical conferences held between teams in the USA, Afghanistan and Iraq in which cases and progress were discussed. As a British military surgeon, this fellowship allowed me to see how colleagues from other nations manage similar problems, structure their services, and to form new professional contacts and friendships.

Problems encountered and steps taken to overcome them

Security clearances to visit were required: my hosts arranged these for me. This visit was planned ≥1 year in advance with the Chief Surgeon at Landstuhl Regional Military Medical Center (who had been posted by the time I visited).

Collaborations established

Planned future visits.

Acknowledgements

Colonel S Flaherty, US Army Surgeon.

Lieutenant Colonel D Gajewski, US Army Surgeon.

Cutner Travelling Fellowship in Orthopaedics

Mr Lukman Khan, SpR Trauma and Orthopaedic Surgery, Royal Infirmary of Edinburgh

Clinical and Research Fellowship in Shoulder and Upper Limb Surgery, Australia (August 2010–July 2011)

Lay summary

The funding from the Cutner Travelling Fellowship allowed me to undertake a clinical and research fellowship in Geelong, Australia. The fellowship was under the supervision of Associate Professor Richard S Page. It provided me with experience in the assessment and management of a wide range of shoulder and upper-limb problems. It also provided me with hands-on training using the latest surgical methods in reconstructive arthroscopic (keyhole) and arthroplasty (joint replacement) procedures as well as in the treatment of traumatic injuries.

The research component allowed me to analyse data from the Australian National Joint Registry to identify factors which may lead to early failure after shoulder replacement surgery. Results from this study may allow surgeons to alter their practice, leading to a better and more durable outcome for patients undergoing shoulder replacement surgery. In addition, I had access to a biomechanical laboratory. I undertook biomechanical testing of materials used in the treatment of shoulder injuries to identify which materials and methods provide the best outcome. I also held a conjoint appointment as Clinical Lecturer with Deakin University Medical School in Geelong, and was involved in undergraduate and postgraduate teaching.

Grant report

The clinical component allowed me to practice advanced techniques in arthroscopic, open and arthroplasty surgery of the shoulder and upper limb for use in reconstructive elective and trauma surgery. This included: arthroscopic shoulder stabilization; arthroscopic rotator cuff repair; diagnostic and therapeutic elbow and wrist arthroscopy; total and reverse shoulder arthroplasty; total elbow replacement; and the use of internal fixation and arthroplasty for complex upper-limb trauma.

The research component allowed: a study of factors which may lead to early failure and early revision after shoulder arthroplasty; biomechanical testing of different methods of internal fixation of clavicle fractures; biomechanical testing of different suture materials and anchors commonly used in shoulder surgery; and testing of functional outcome of patients who had been treated for bilateral posterior fracture dislocation of the shoulder.

Problems encountered and steps taken to overcome them

To familiarize myself with new equipment and techniques, I used an arthroscopic shoulder model. This allowed me to practice arthroscopic techniques and become accustomed to the new equipment in a controlled environment.

Collaborations established

1. Clinical staff at Barwon Health and Bayview Orthopaedics.
2. Research staff at the Department of Material Testing, Deakin University.
3. Industry partners at Depuy and Acumed.

Oral presentations

1 Factors associated with early revision after shoulder arthroplasty: 7113 shoulder arthroplasties from the Australian Orthopaedic Association National Joint Registry. British Orthopaedic Association/Irish Orthopaedic Association (BOA/IOA) Joint Annual Meeting, Dublin 2011.

2 A biomechanical study to compare locking and standard pre-contoured plate fixation for mid-shaft clavicle fractures. British Orthopaedic Association/Irish Orthopaedic Association (BOA/IOA) Joint Annual Meeting, Dublin 2011.

3 Bending and abrasion fatigue of common materials used in shoulder surgery. New Zealand Orthopaedic Association/Australian Orthopaedic Association (NZOA/AOA) Joint Shoulder and Elbow meeting, Tahiti, 2011.

Prize

HMO Research Prize (third place), Barwon Health Scientific Meeting, Melbourne, Australia, 2010.

Acknowledgements

I acknowledge the Cutner Travelling Fellowship and RCSEd for their generous support. I thank Professor Page and his colleagues in Geelong for all their hard work to ensure this fellowship was a success.

John Steyn Travelling Fellowship in Urology

Hugh O'Kane, Department of Urology, Royal Adelaide Hospital, South Australia

Urology Fellowship in laparoscopic and robotic urology (3 February 2010–1 February 2011)

Lay summary

Urologists have been at the forefront in the development of robotic-assisted surgery (specifically robotic-assisted laparoscopic radical prostatectomy) for the management of localised prostate cancer. This surgical technique has become increasingly popular worldwide over the past several years, with very encouraging results. The operating robot uses three-dimensional (3D) vision and robotic surgical arms attached to the patient controlled by the operating surgeon *via* a separate operating console to carry out the procedure to remove the prostate gland. The benefits of this minimally invasive type of surgery to the patient include shorter recovery time and length of hospital stay, reduced blood loss and earlier return to normal activities.

This technique has been introduced in only a few centres within the UK and, as a result, training opportunities are limited. The Robotic Surgical Unit at the Royal Adelaide Hospital was set up in 2004. Since then, ≈1,000 robotic prostatectomies have been carried out. Upon returning to the UK, I hope I will be able to develop and utilise the skills that I obtained during my fellowship for the benefit of patients in the NHS.

Grant report

In February 2010, I started a Urological Fellowship at the Royal Adelaide Hospital in South Australia. This fellowship was kindly supported by the John Steyn Travelling Fellowship grant. The reason to travel to Adelaide was to gain experience in the novel surgical technique of robotic-assisted laparoscopic prostatectomy used in the management of localised prostate cancer.

The Royal Adelaide Hospital is the largest teaching hospital in South Australia with ›900 inpatient beds and 14 operating theatres. It is the regional trauma centre for the state of South Australia and caters for 2 million people. It includes an area stretching 3,000 km to the north of Australia, covering Darwin in the Northern territories. The Urology Department has 20 inpatient beds and is staffed by 7 consultant urologists and 2 middle grades (which included myself and a junior registrar along with 2 junior doctors and a specialist nurse). All subspecialisations in urology were catered for at the Royal Adelaide Hospital (including oncology, endourology, stone disease, female urology, andrology and reconstruction).

My week was concentrated mainly on surgery, with 4 full days per week spent in the operating theatre and one half-day outpatient clinic. One full day and often a second half-day was spent in the Robotic Operating Theatre. Management of ward patients was carried out mainly by the registrar, and included a daily 7.30 am ward round followed by a further evening round after the surgical list was finished. For the first 4 months I participated in a 1:2 on-call rota changing to 1:3 on the arrival of a second fellow to the unit. The concentration of such a large volume of surgical experience in a short period of time gave me appreciable exposure with is I feel is not possible in any UK-based training position.

Over the 5 years before commencing the fellowship I had undergone urological training in the Northern Ireland Urology training programme. During this training, I gained experience in open radical prostatectomy (the most commonly used surgical technique used in the UK for the management of localised prostate cancer).

Most robotic-assisted surgery was carried out by Mr Peter Sutherland (who founded the unit and who provided me with most of the training during the fellowship year). Further training was given by second consultant, Mr Richard Wells, who had been carrying out the procedure for 3 years and who had experience in >150 robotic-assisted radical prostatectomies. During the fellowship I was involved in ≈100 robotic prostatectomies. To put this in perspective, over the previous 5 years of my training in the UK I had been involved in <60 standard prostate procedures.

My training initially involved becoming familiar with the robotic control console and practicing with the robot using various training aids. This included tissue handling, suturing as well as carrying out over 20 vesicourethral anastamoses on a plastic training module in a timed fashion. I spent the first month watching DVD recordings of the procedure carried out by Mr Sutherland and other experts. I assisted Peter Sutherland in ≈20 cases to become familiar with the steps of the procedure.

Robotic-assisted radical prostatectomy can be divided into steps. I learned each step and, under supervision, became competent in all steps of the procedure. One of the main advantages of robotic surgery is that the trainer can observe the trainee carrying out the procedure on a TV monitor and guide the trainee safely. Over a 12-month period I was involved in ≈100 procedures and was on the operative console for 50 of these cases. During the final 3 months of the fellowship I was completing procedures without assistance in <3 h.

I was involved in the development of the robotic surgery programme at the Royal Adelaide Hospital with the introduction of robotic-assisted renal surgery. The first cases of partial nephrectomy and pyeloplasty were completed during my fellowship year, and I participated as the first assistant in all of these cases. This development can shorten post-operative recovery, allowing for a earlier return to normal activities in a similar way to robotic-assisted prostate surgery. Four cases were carried out without conversion to open surgery. I had the opportunity to present these data at the South Australia State urology meeting in Adelaide in 2010.

In addition to the training that I received in robotic surgery I have also been carrying out a high volume of surgical training in conventional laparoscopic surgery and other surgical procedures. My interest is in oncology and, in addition to radical prostate surgery, I gained significant experience in open radical pelvic surgery (including radical cystectomy, open radical prostatectomy and complex reconstructive surgery). Adding to my previous UK-based experience, the opportunity to operate with a junior registrar meant that I was the primary surgeon with minimal supervision from a consultant. This increased my experience and confidence to carry out these procedures independently. This honing of skills I feel is invaluable before taking the next step to independent consultant practice in the UK.

Before commencing the fellowship I received excellent training in Belfast in laparoscopic renal surgery. During the fellowship, this previous training allowed me to complement these skills and meant I could carry out several laparoscopic nephrectomies independently and further my competence with this procedure.

Alongside the uro-oncology practice, a large volume of core endo-urology and complex stone work was carried out at the Royal Adelaide Hospital. As the fellow, independent

surgical activity in these procedures was expected, and opportunities to gain experience with transurethral resection of prostate gland, rigid and flexible ureteroscopy, and percutaneous renal stone surgery was available. The Royal Adelaide Hospital is one of the few urology institutions worldwide that practices supine percutaneous renal stone surgery (which holds many potential advantages for patients undergoing this form of treatment for stone surgery). Mr Denby Steele has appreciable experience in this type of surgery and provided me with excellent training in >20 cases.

I travelled to Melbourne in August 2010 for the Eleventh National Prostate Cancer Symposium. This excellent meeting (organised by Professor Tony Costello and Declan Murphy) focuses on all aspects of prostate cancer had world-expert speakers such as Peter Scardino, Peter Carroll and Eric Klein presenting data on prostate cancer survival, treatment of high-grade non-metastatic disease, and basic scientific research on the role of infection in the aetiology of prostate cancer. The opportunity to hear such luminary figures deliver state-of-the-art lectures was a highlight.

Acknowledgements

Having returned to the UK my focus is to try to develop and utilise the skills that I obtained during my fellowship for the benefit of patients in the NHS. I have been very fortunate to be able to travel to Adelaide to do this fellowship, and I am extremely grateful for the support from the RCSEd.

Ophthalmology Reports

Developing gene therapy for cone neuroprotection in rod cone dystrophies

Professor Robert E MacLaren DPhil FRCSEd, Nuffield Laboratory of Ophthalmology, University of Oxford, John Radcliffe Hospital, Oxford

Ophthalmology Major Project Grant (1 October 2008–31 September 2009)

Lay summary

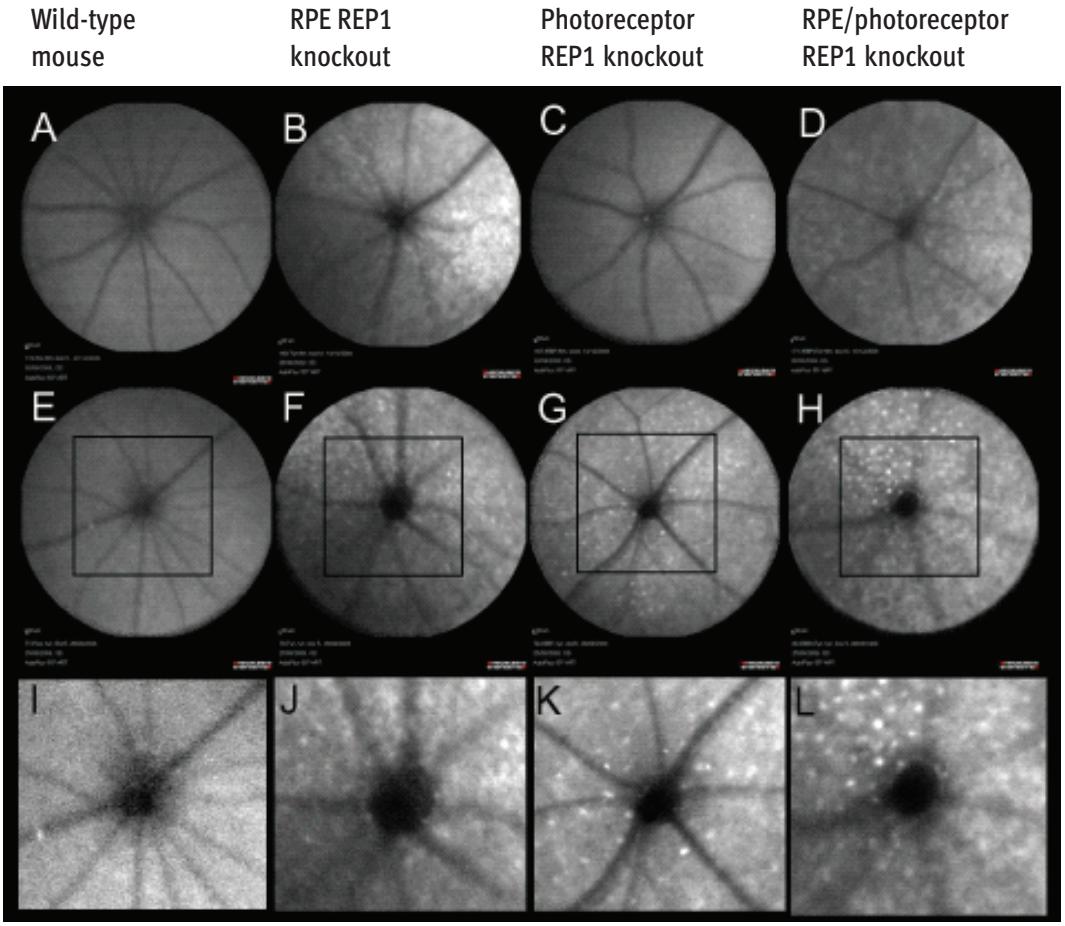
Cone photoreceptors are the light-sensitive cells in the retina that are most important for vision in humans. It is the loss of these cells that leads to blindness in retinal diseases such as age-related macular degeneration (AMD), retinitis pigmentosa (RP) and diabetic maculopathy. Most laboratory models, however, investigate models of retinal disease in the rodent retina, which comprises 97% rods (light-sensitive cells used mainly at night).

The purpose of this project was to apply advances in genetic biotechnology to develop a mouse with fluorescent green cones and retinal degeneration. This was to ensure that the small population of cones can be seen as 'fluorescent dots' against a background of mainly rods. Using a scanning laser ophthalmoscope, the number of individual cones at various stages of retinal degeneration in the living mouse could be counted and therefore a means of delaying cone loss assessed. The project was highly successful at establishing a unique new model and is currently being used to test several proteins that might be of benefit in AMD or RP.

Grant report

In setting up this imaging project we established a method for viewing the mouse retina using the scanning laser ophthalmoscope to detect autofluorescence. At the time this was the first such application. In addition to this project, we also applied it to image a mouse model of choroideraemia to detect degeneration in layer-specific knockout of the choroideraemia (REP1) gene. By observing the changes in fluorescence we determined that the choroideraemia gene was necessary for normal photoreceptor function. This information formed a critical part of a successful application to the Gene Therapy Advisory Committee (GTAC) to set up a clinical trial for choroideraemia. Hence, this project funded by the RCSEd has led directly to the setting up of a new clinical trial.

Fluorescence imaging in the choroideraemia knockout mouse. A–D show retinal changes at 6 months whereas E–F show changes at 12 months (I–J are enlargements of disc areas). The white dots represent macrophages which accumulate secondary to retinal cell death. In this model, the choroideraemia gene (REP1) is knocked out in the retinal pigment epithelium (RPE; B, F, J), the photoreceptors (C, G, K) or both (D, H, L). The appearance of the fluorescent dots in K is evidence that the REP1 gene (deficient in choroideraemia) is necessary for photoreceptor function. This supports clinical observations and confirmed that gene therapy would need to target the neurosensory retina and RPE.



Problems encountered and steps taken to overcome them

We screened several agents that might be neuroprotective in this model. We did not find neuroprotective effects with lamotrigine or glial cell-derived neurotrophic factor (GDNF). We screened other proteins for potential delivery by gene therapy. We found recently that XIAP is neuroprotective when delivered by gene therapy on cells *in vitro* and we are assessing it in this model.

Collaborations established

We set up a collaboration with Professor Miguel Seabra's group at Imperial College London (London) for investigating retinal neuroprotection using AAV.REP1 in a mouse model of choroideraemia.

Publications

1 Lee E, MacLaren RE. Sources of RPE for replacement therapy. *Br J Ophthalmol* 2010; 95: 445–459.

2 Tolmachova T, Wavre-Shapton ST, Barnard AR, MacLaren RE, Futter CE, Seabra MC Retinal pigment epithelium defects accelerate photoreceptor degeneration in cell type-specific knockout mouse models of choroideremia. *Invest Ophthalmol Vis Sci* 2010; 51: 4913–4920.

3 Pearson RA, Barber AC, West EL, et al. Targeted disruption of outer limiting membrane junctional proteins (Crb1 and ZO-1) increases integration of transplanted photoreceptor precursors into the adult wild-type and degenerating retina. *Cell Transplant* 2010; 19: 487–503.

4 Comyn O, Lee E, MacLaren RE. Induced pluripotent stem cell therapies for retinal disease. *Curr Opin Neurol* 2010; 23: 4–9.

5 MacLaren RE. An analysis of retinal gene therapy clinical trials. *Curr Opin Mol Ther* 2009; 11: 540–546.

6 West EL, Pearson RA, MacLaren RE, Sowden JC, Ali RR. Cell transplantation strategies for retinal repair. *Prog Brain Res* 2009; 175: 3–21.

7 Luhmann UF, Robbie S, Munro PM, et al. The drusenlike phenotype in aging Ccl2-knockout mice is caused by an accelerated accumulation of swollen autofluorescent subretinal macrophages. *Invest Ophthalmol Vis Sci* 2009; 50: 5934–5943.

8 West EL, Pearson RA, Tschernutter M, Sowden JC, MacLaren RE, Ali RR. Pharmacological disruption of the outer limiting membrane leads to increased retinal integration of transplanted photoreceptor precursors. *Exp Eye Res* 2008; 86: 601–611.

9 Kokkinopoulos I, Pearson RA, MacNeil A, et al. Isolation and characterisation of neural progenitor cells from the adult Chx10(orJ/orJ) central neural retina. *Mol Cell Neurosci* 2008; 38: 359–373.

Further funding

As a direct result of the research supported by the RCSEd with this grant, we have used the the preliminary data to gain further funding from Fight for Sight (Dan Lipinski, PhD studentship) and the Wellcome Trust (Mr Ed Lee, Clinical Research Fellow). The total amount accrued is ≈£300,000.

Acknowledgements

I remain extremely grateful to the RCSEd, and Royal Blind and Scottish War Blinded to have this support. This grant was the only one I held when I transferred my research to take up a new chair at the University of Oxford. It enabled me to employ a post-doctoral assistant and continue this work straight away. I now have three post-doctoral assistants with four clinical PhD students. The work from this project has supported other successful grant applications which have led to a large grant to support a new gene therapy clinical trial in patients with an inherited retinal disease.

Testing clinical treatments that might preserve cones in retinitis pigmentosa

Professor Robert E MacLaren, Nuffield Laboratory of Ophthalmology, University of Oxford, Oxford
Ophthalmology Major Project Grant (October 2009–September 2010)

Lay summary

We bred a genetically modified mouse which has retinitis pigmentosa (RP). In RP, the light-sensing cells (photoreceptors) degenerate over a period of several years. The photoreceptors for night vision (rods) are the first to go, but blindness follows if the photoreceptors (cones) also degenerate. Cones are used for daytime vision and for most of the tasks we carry out. It is not known why the cones degenerate because they do so even if the genetic problem is present only in the rods. Trying to understand ways of preventing this cone loss was the aim of this project.

To examine cone loss in the living mouse we crossbred a mouse with RP onto another mouse in which the cone photoreceptors were fluorescent green. This allowed us to see the cones at the back of the eye of the mouse with a scanning laser ophthalmoscope and observe the degeneration in real time. This provides an ideal model in which to investigate ways of preventing cone loss. We are testing several other compounds (including simple products) that could be taken by mouth or injected into the eye.

Grant report

To understand the mechanism of cone loss in RP would have a significant impact because it could provide a treatment that might be broadly applied to anyone with the disease. The loss of cones is not dependent on a particular genetic mutation present in the rods. We have identified CNTF as a major neuroprotectant in our experimental setup, and are also looking at other molecules such as GDNF as well as antioxidants and neuronal stabilising agents.

We have developed a viral vector which expresses a protein in the subretinal space and are monitoring the progression of degeneration. The model allows very reliable and precise measurement of cone loss in the mouse, which will hopefully provide us with potential therapeutic option for patients with RP.

Problems encountered and steps taken to overcome them

The imaging tests led to the development of cataract due to drying of the cornea. We modified the technique using a contact lens, which improved the clarity of the images.

Collaborations established

As a result of successful work on this project in part, Dan Lipinski was awarded a Fulbright Scholarship to enable him now to continue some parts of the project in Florida (USA) with the gene therapy group led by Professor William Hauswirth. Peter Charbel Issa has recently been appointed to a Professorship in Ophthalmology in Bonn, and has optimised much of the imaging work on this project. We will remain close collaborators from now on.

Several students have worked on this project under the guidance of the post-doctoral assistant who was supported by the award: Dan Lipinski.

Publications

1 West EL, Pearson RA, Duran Y, et al. Manipulation of the recipient retinal environment by ectopic expression of neurotrophic growth factors can improve transplanted photoreceptor integration and survival. *Cell Transplant* 2012; 21: 871–887.

2 You Q, Brown LA, McClements M, Hankins MW, MacLaren RE. Tetradecanoylphorbol-13-acetate (TPA) significantly increases AAV2/5 transduction of human neuronal cells *in vitro*. *Exp Eye Res* 2012; 97: 148–153.

3 Charbel Issa P, Singh MS, Lipinski DM, et al. Optimization of *in vivo* confocal autofluorescence imaging of the ocular fundus in mice and its application to models of human retinal degeneration. *Invest Ophthalmol Vis Sci* 2012; 53: 1066–1075.

4 West EL, Pearson RA, Duran Y, et al. Manipulation of the recipient retinal environment by ectopic expression of neurotrophic growth factors can improve transplanted photoreceptor integration and survival. *Cell Transplant* 2012; 21: 871-887.

5 Charbel Issa P, MacLaren RE. Non-viral retinal gene therapy. *Clin Experiment Ophthalmol* 2012; 40: 39–47.

6 Barnard AR, Issa PC, Perganta G, et al. Specific deficits in visual electrophysiology in a mouse model of dominant optic atrophy. *Exp Eye Res* 2011; 93: 771–777.

7 Shan H, Ji D, Barnard AR, Lipinski DM, et al. AAV-mediated gene transfer of human X-linked inhibitor of apoptosis protects against oxidative cell death in human RPE cells. *Invest Ophthalmol Vis Sci* 2011; 52: 9591–9597.

8 Lipinski DM, Singh MS, MacLaren RE. Assessment of cone survival in response to CNTF, GDNF and VEGF165b in a novel *ex vivo* model of end-stage retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 2011; 52: 7340–7346.

9 Singh M, MacLaren RE. Stem cells as a therapeutic tool for the blind: biology and future prospects. *Proc Biol Sci* 2011; 278: 3009–3016.

10 Lipinski DM, Yusuf M, Barnard AR, et al. Characterization of a dominant cone degeneration in a GFP-reporter mouse with disruption of loci associated with human dominant retinal dystrophy. *Invest Ophthalmol Vis Sci* 2011; 52: 6617–6623.

11 West EL, Pearson RA, Barker SE, et al. Long-term survival of photoreceptors transplanted into the adult murine neural retina requires immune modulation. *Stem Cells* 2010; 28: 1997–2007.

12 Tolmachova T, Wavre-Shapton ST, Barnard AR, et al. Retinal pigment epithelium defects accelerate photoreceptor degeneration in cell type-specific knockout mouse models of choroideremia. *Invest Ophthalmol Vis Sci* 2010; 51: 4913–4920.

Acknowledgements

Royal Blind, Scottish War Blinded and the RCSEd.

An analysis of retinal digital image abnormalities seen in acute retinopathy of prematurity – is reduced oxygen therapy protective? The Benefits Of Oxygen Saturation Targeting Trial II UK Retinal Image Digital Analysis (BOOST-II UK RIDA) Study.

Professor Brian W Fleck, Princess Alexandra Eye Pavilion, Edinburgh

Ophthalmology Major Project Grant (1 April 2010–31 March 2011)

Lay summary

This was a three-year project, and this report covers progress made during the second period of the study: April 2010 to March 2011. Substantial progress with the study was made during this period.

The study was part of a large clinical trial of oxygen therapy in premature babies: BOOST II UK. The aim of the trial was to determine the optimal amount of oxygen to use when treating such babies. One of the effects of prematurity is blindness due to retinopathy of prematurity (ROP).

Many of the study centres in the UK use specialised retinal photographs to examine babies' eyes for ROP. The Retinal Image Digital Analysis (RIDA) study will collect all the photographs taken of babies in the BOOST II UK trial. The photographs will be analysed as part of the BOOST II UK trial. The photographs will be analysed with new software that can measure retinal blood vessels automatically. This will allow us to develop software to measure the severity of ROP when babies' eyes are examined. This measure of severity will allow ophthalmologists to decide which babies need treatment for ROP, and when they need treatment, in a more reliable way than is done at present.

Grant report

The BOOST-II UK Trial is a double-blind randomized controlled trial (RCT) to compare the effects of two levels of oxygen therapy on premature infants. The maximum stage of acute ROP and the need for retinal treatment are secondary outcomes during the short-term phase of the trial. Visual disability at age 2 years is a primary outcome of the trial.

A significant proportion of participating ophthalmologists use digital imaging for retinal screening: this is a unique feature of the trial. We developed processes to collect these images onto a secure study server, and organised them into a format accessible for analysis by expert readers and by quantification software. Several hypotheses were tested: (i) infants managed with lower oxygen saturation will have more mature retinal vascularisation, and will develop less severe ROP; (ii) retinal structural and functional outcomes are influenced by ROP severity at the time of treatment, and the area of avascular retina covered by laser treatment burns; (iii) digital retinal photographs may be used for remote 'telemedicine' reading and diagnostic decisions; (iv) ROP severity plus disease may be quantified by computerised image analysis, and this approach may allow more consistent diagnostic treatment decisions to be made than at present.

Progress made during the second year of the study included recruitment to the BOOST II UK Trial. The trial was designed to recruit 1,200 infants. An interim international meta-analysis of mortality in the two arms of the trial was conducted in autumn 2010. This showed a marginal (but statistically significant) reduced mortality in the higher oxygen

group. The Trial Steering Committee met and discontinued the trial on 24 December 2010. A total of 973 babies were enrolled in the study when it closed.

Several tasks for the RIDA study were also completed. A research ophthalmologist, Dr Laude, visited six sites in the UK on at least one occasion. At each visit he transferred images to a secure laptop, and later selected suitable sets of images from each screening examination. These images were anonymised and uploaded onto the secure trial server using software developed by Mr Ken Cocker. Each site visit took 1–2 days. Off-site selection, processing and upload of images took 3-4 days. On 31 October 2010, 1,536 images from 345 examinations of 85 infants had been loaded onto the trial server. Dr Laude relocated to Singapore in October 2010. A new researcher, Catriona McIntyre-Beon, was recruited. Further uploading did not occur until Catriona McIntyre-Beon started visiting centres in May 2011. Hence, infants were not added to the database between 31 October 2010 and 31 March 2011. Throughout the study, the payment of Dr Laude and Catriona McIntyre-Beon has been on an *ad hoc* basis, for the hours that they worked.

Mr Kenneth Cocker built the server-based image database. Mr Cocker was paid for the hours he worked each month. He developed software during periods when new images were not uploaded. He has written updated versions of RetView, and has developed processes to allow remote reading experiments to be conducted. He has also worked on the further development of image analysis software (with additional funding for this from the image analysis group lead by Professor Fielder), which will form a vital part of the RIDA analysis.

Mr Andrew King from the National Perinatal Epidemiology Unit in Oxford provided secure linkages between information held in the BOOST-II UK trial database and the work undertaken by Dr Laude, Catriona McIntyre-Beon, and Mr Cocker.

Problems encountered and steps taken to overcome them

Dr Laude was a Research Ophthalmologist based in Edinburgh until he relocated to Singapore in October 2010. Catriona McIntyre-Beon (Specialist Neonatal Nurse and lead BOOST II UK Nurse for Scotland) was recruited to continue this project on a part-time basis from January 2011.

Several information-technology (IT) issues arose while using NHS Lothian IT systems for the study. These were dealt with by Professor Fleck, Mr Cocker and Dr Laude.

Collaborations established

1. Close links with the National Perinatal Epidemiology Unit (NPEU) in Oxford were maintained and developed further. We are planning a RCT of laser *versus* anti-VEGF treatment for ROP.
2. Closer links with the Wellcome Trust Clinical Research Facility Image Analysis Core Facility in Edinburgh were developed (Dr Tom MacGillivary).
3. Links with the Department of Public Health in Edinburgh (Professor Harry Campbell) were developed.

Publications

The BOOST Trial Steering Committee controls all publications from the trial. They will not sanction communication of any outcomes of the RIDA trial in advance of publication of the main BOOST trial.

Poster presentations

1. World Congress on ROP, New Delhi, India, November 2009.
2. British and Ireland Paediatric Ophthalmology Association meeting, Glasgow, Sept 2009.

A national study of rhegmatogenous retinal detachment in Scotland: clinical epidemiology and genetic aetiology

Mr David Charteris, Princess Alexandra Eye Pavilion, Edinburgh, Department of Public Health Sciences, University of Edinburgh and Moorfields Eye Hospital, City Road, London.

Ophthalmology Major Project Grant (1 August 2007–31 December 2010)

Lay summary

This was the first study in the UK to establish the annual incidence of primary rhegmatogenous retinal detachment (RRD). Over 1,200 incident cases were identified during the study period, making it one of the largest reports in the literature. It established contemporary clinical and demographic associations of RRD which created an important clinical distinction and which may have important implications in the surgical management of RRD. In addition, we examined the familial tendency of RRD and, with our collaborators, established a genetic database of ≈2,000 individuals with RRD. As part of our ongoing work we will undertake a genetic association study to determine the potential role of genes in causing RRD. Finally, our database will allow us to audit the outcomes of all patients undergoing surgery for RRD in Scotland to determine if national standards of care are being met.

Grant report

The primary objectives of the study were to:

1. establish a complete case register of new cases of primary RRD in the Scottish population during a 2-year period,
2. calculate the incidence of retinal detachment,
3. determine the distribution of known associations of retinal detachment within the study population,
4. determine the incidence of familial retinal detachment in Scotland, and the distribution of known associations within this population,
5. determine the incidence of familial retinal detachment associated with pathological myopia, and undertake genetic linkage studies in these families,
4. obtain a DNA database from a well-characterised population of subjects with retinal detachment to screen for genes for this condition.

The secondary objectives of the study were to:

1. provide data to inform the planning of vitreoretinal surgical services in Scotland,
2. define abnormalities present in the second eye of patients presenting with RRD,
3. establish a cohort of patients for potential future longitudinal studies of the natural history of the second eye in retinal detachment cases.

Principal findings

The Scottish Rhegmatogenous Retinal Detachment study was a cross-sectional, prospectively recruited study that sought to recruit all incident cases of primary RRD presenting to each vitreoretinal surgical site in Scotland over a 2-year period. This study was conducted between November 2007 and November 2009. We recruited 1,202 incident cases of primary RRD from six centres across Scotland, representing ›95% of all operated cases in Scotland during this time period.

After informed consent, clinical and demographic details were taken from each participant and a blood sample obtained. Based on the mid-year population estimates for 2008, the annual incidence of RRD was 12.05 per 100,000 (95% confidence interval (CI):11.35–12.70), meaning ≈7,500 people are affected annually in the UK.

Deographic associations

The age-specific incidence increased to a peak in both sexes in the 60–69 year age group. RRD was significantly more frequent in males than in females (14.70 *versus* 8.75 per 100,000). A total of 53.2% of cases were myopic with a spherical equivalent refractive error ›-1 dioptres. A total of 23.4% of cases had previous cataract surgery and 10.4% were traumatic. A strong association was found between RRD incidence and affluence, with a highly significant rising trend across quintiles of deprivation. The age-standardised incidence of RRD rose from 9.15 to 13.5 per 100,000 between the most deprived and the least deprived quintile, with a strong association across quintiles of increasing affluence. In summary, RRD incidence increased with age, was more common in men and right eyes, and was strongly associated with affluence.

Clinical associations

Detailed clinical information was available on 1,130 (94%) of cases. By causative break, the proportions of RRD were: horse-shoe tear (HST) associated with posterior vitreous detachment (PVD) in 86.2%, giant retinal tear (GRT) and PVD in 1.3%, non-PVD round hole (RH) in 4.9%, retinal dialysis in 5.9%, and retinoschisis RRD in 1.6%. RH RRD presented more frequently with multiple retinal breaks compared with HST RRD (67.8% *versus* 48.7%). In PVD-associated RRD, 56.1% (95% CI= 53.8–58.3%) of breaks were identified in the supero-temporal retina. In non-PVD RRD, 54.6% (95% CI = 47.9–61.1%) of breaks were infero-temporal followed by supero-temporal in 34.9% (95% CI = 28.7–41.5%). Lattice degeneration was present in 18.7% of affected eyes and was more common in RH RRD (35.7%) than in PVD-associated RRD (18.9%). Seven percent reported an affected first-degree relative, and these cases were significantly more myopic than non-familial cases.

In summary, ›85% of RRD cases were associated with PVD and related tractional tears. Non-PVD RH RRD occurred in younger, more myopic, individuals. Most cases were caused by more than one retinal break, and the macula was affected in ›50% at presentation. Ocular trauma, previous cataract surgery, family history and lattice degeneration were important predisposing features for RRD development.

Fellow eye disease

Full-thickness retinal breaks were found in 8.4% (95/1,130) of fellow eyes on presentation. Lattice degeneration was present in 14.5% of fellow eyes. Thirteen percent of affected fellow eyes had a best corrected visual acuity of 6/18 or worse with previous RRD (the second most common cause of poor vision). Overall 7.3% of cases had RRD in both eyes. Sixty percent of cases with consecutive bilateral RRD presented before the macula was affected.

In summary, rhegmatogenous disease in the fellow eye represented an important threat to vision. Fellow eye detachments were more common in pseudophakic individuals and in those with a more myopic refractive error. Fellow eye RRD had a greater likelihood of prompt presentation.

Familial aggregation of RRD

Self-addressed questionnaires were sent to all study participants. Sixty-five percent of probands returned completed questionnaires. Of these 602 families (parents, sib-

lings, offspring), 7.8% (47) had one affected member and 0.5% (3) had two affected members. A total of 501 sibships were included in the regression analysis. The odds ratio (OR) of a sibling being affected given another affected sibling was 1.91 (95% CI = 1.18–3.05). Adjusting for age and sex, the OR of a sibling being affected increased by 9.8% for each additional dioptre of spherical equivalent refractive error towards myopia in the proband. The λ_s and the parent–offspring recurrence risk ratio of RRD was 2.1 (95% CI = 1.3–3.2) and 2.9 (95% CI = 1.9–4.2), respectively. Thus, genetic factors were likely to be important in the aetiology of myopic and non-myopic RRD. The risk of having an affected sibling with RRD increased twofold given that a sibling had the condition. The sibling risk increased with the level of spherical equivalent myopia in the proband.

Genome-wide association

DNA was extracted from 1,100 participants. With our established collaborations (see below), we are conducting a genome-wide association study. Stage 1 of the study has been completed, and we have genotyped 912 Scottish cases with primary RRD using 385,000 single nucleotide polymorphisms (SNPs) and undertaken a case–control analysis with age- and region-matched controls. The top 5,500 SNPs have been selected from this case–control analysis and are being genotyped on a further 1,000 cases of primary RRD derived from our collaborations.

Problems encountered and steps taken to overcome them

Ensuring complete participation and co-operation of all vitreoretinal surgical sites in Scotland. To achieve this, the lead investigator visited all sites at the outset and at regular intervals. A didactic lecture was given in all vitreoretinal sites. Local investigators were contacted regularly to ensure complete and accurate case capture.

Achievement of sample size for the genome-wide association study. The sample size needed to identify common genetic variants involved in RRD pathogenesis was not known. However, estimates from other complex disorders have indicated a minimum sample size of 2,000 cases and controls to allow identification of a variant that is likely to be of clinical and public-health importance. To achieve this, we established case–control collection in conjunction with several collaborators in the UK, Europe and USA.

Collaborations established

We succeeded in establishing several collaborations that allowed us to develop one of the largest genetic databases of primary RRD. We established a collaboration and ongoing case-control collection with:

1. Professors Carel Hoyng and Anneke den Hollander at Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands.
2. Mr Martin Snead at Addenbrookes NHS University Hospital, Cambridge.
3. Professor Dwight Stambolian at Penn Presbyterian Medical Centre, Scheie Eye Institute, Philadelphia, USA.

Publications

1. Mitry D, Singh J, Yorston D, et al. The fellow eye in retinal detachment – findings from the Scottish Retinal Detachment Study. *Br J Ophthalmol* 2012; 96: 110–113.
2. Mitry D, Chalmers J, Anderson K, et al. Temporal trends in retinal detachment incidence in Scotland between 1987 and 2006. *Br J Ophthalmol* 2011; 95: 365–369.
3. Mitry D, Campbell H, Charteris DG, et al. SNP mistyping in genotyping arrays – an important cause of spurious association in case-control studies. *Genet Epidemiol* 2011; 35: 423–426.

4 Mitry D, Singh J, Yorston D, et al. The predisposing pathology and clinical characteristics in the Scottish Retinal Detachment Study Ophthalmology. *Ophthalmology* 2011; 118: 1429–1434.

5 Mitry D, Williams L, Charteris DG, et al. Population-based estimate of the sibling recurrence risk ratio for rhegmatogenous retinal detachment. *Invest Ophthalmol Vis Sci* 2011 20; 52: 2551–2555.

6 Mitry D, Tuft S, McLeod D, Charteris DG. Laterality and gender imbalances in retinal detachment. *Graefes Arch Clin Exp Ophthalmol* 2011; 249: 1109–1110.

7 Mitry D, Charteris DG, Fleck BW, Campbell H, Singh J. The epidemiology of rhegmatogenous retinal detachment - geographic variation and clinical associations. *Br J Ophthalmo*l 2010; 94: 678–684.

8 Mitry D, Fleck BW, Wright AF, Campbell H, Charteris DG. Pathogenesis of rhegmatogenous retinal detachment: predisposing anatomy and cell biology *Retina* 2010; 30: 1561–1572.

9 Mitry D, Charteris DG, Yorston D, et al. The clinical epidemiology and socio-economic associations of primary retinal detachment in Scotland: a two-year prospective population-based study. *Invest Ophthalmol Vis Sci* 2010; 51: 4963–4968.

10 Saidkasimova S, Mitry D, Singh J, Yorston D, Charteris DG. Retinal detachment in Scotland is associated with affluence. *Br J Ophthalmol* 2009; 93: 1591–1594.

11 Mitry D, Charteris DG, Yorston D, Fleck BW, Wright A, Campbell H, Singh J. Rhegmatogenous retinal detachment in Scotland: research design and methodology. *BMC Ophthalmol* 2009; 9: 2.

Poster presentations

1 Mitry D, Charteris D, Siddiqui R, et al. The epidemiology of retinal detachment in Scotland. Royal College of Ophthalmology Annual Congress, Liverpool, May 2010.

2 Mitry D, Yorston D, Charteris D, Campbell H, Singh J. The epidemiology of rhegmatogenous retinal detachment in Scotland. Royal Society of Medicine, London, May 2009.

3 Mitry D, Yorston D, Charteris D, Campbell H, Singh J. The incidence and epidemiological characteristics of rhegmatogenous retinal detachment in Scotland. ARVO, Fort Lauderdale, USA, May 2009.

4 Mitry D, Yorston D, Charteris D, Campbell H, Singh J. Rhegmatogenous retinal detachment – a national study. Allergan Achievements Award, Liverpool, May 2008

Oral presentations

1 Mitry D, Williams L, Charteris DG, Fleck BW, Wright AF, Campbell H. Population-based estimate of the sibling recurrence risk ratio for rhegmatogenous retinal detachment. Royal College of Ophthalmology Annual Congress, Birmingham May 2011.

2 Mitry D, Charteris D, Siddiqui R, et al. The epidemiology of retinal detachment in Scotland. Royal College of Ophthalmology Annual Congress. Liverpool, May 2010.

3 Mitry D, Charteris D, Siddiqui R, et al. Rhegmatogenous retinal detachment in Scotland: Two years of prospective recruitment. ARVO, ARVO, Fort Lauderdale, USA, May 2010.

4 Mitry D, Charteris D, Siddiqui R, et al. The epidemiology of retinal detachment in Scotland

5 New England Ophthalmological Society and Irish College of Ophthalmology Annual Conference, Dublin, April 2010.

6 Mitry D, Charteris D, Siddiqui R, et al. Retinal detachment in Scotland: 2 years of prospective recruitment. British and Eire Association of Vitreoretinal Specialists (BEAVRS), Amsterdam, November 2009.

7 Mitry D. Epidemiology and molecular genetics of retinal detachment. Moorfields Eye Hospital, London, May 2009.

8 Mitry D. Genetics of rhegmatogenous retinal detachment and related disorders. Clinical Genetic SpR Training Day, Western General Hospital, Edinburgh, March 2009.

9 Mitry D, Saidkasimova S, Yorston D, et al. Epidemiology and clinical characteristics of the first 500 cases. BEAVRS, Reading, November 2008.

10 Saidkasimova S, Mitry D, Singh J. Is retinal detachment associated with affluence? BEAVRS, Reading, November 2008.

11 Mitry D. Scottish RRD Study: interim report. Scottish Association of Vitreretinal Specialists (SCIVRS), Perth, June 2008.

12 Mitry D. Rhegmatogenous retinal detachment – a national study (introductory presentation). BEAVRS, Saint Andrews, November 2007.

Prizes

1 Barbara Knox Medal for best overall research presentation, Irish College of Ophthalmology Annual Conference 2010, Dublin.

2 ARVO International Travel Award, Fort Lauderdale, USA.

Further funding

1 WH Ross Foundation for the Prevention of Blindness: £13,647 for 1 year.

2 Chief Scientist Office Major Grant (CZB/4/705): £285,300 for 15 months.

3 Moorfields Eye Hospital Special Trust: £40,000 for 1 year.

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Limbal stem cell (LSC) isolation and characterisation for *ex vivo* transplantation

Harminder Dua, Division of Ophthalmology and Visual sciences, University of Nottingham, Nottingham
Ophthalmology Major Project Grant (August 2010–August 2011)

Lay summary

The surface of the eye is covered by a thin and transparent layer known as the corneal epithelium. It undergoes constant wear and tear but is continually regenerated by dividing epithelial cells at the limbus, which is a 1-mm zone between the cornea and conjunctiva. Clinical and scientific evidence suggests that damage and depletion of these stem cells (SCs) leads to limbal stem cell deficiency (LSCD), and ultimately causes blindness. The treatment for this condition is transplantation of donor epithelial cells grown in the laboratory, on the human amniotic membrane (AM). We optimised the techniques for efficient growth of limbal stem cells (LSCs) using *in vitro* conditions for transplantation. The efficiency of separation of stem/progenitor cell populations by antibodies to various SCs was determined by measuring the growth efficiency of the separated cells. Also, the culture media and various substrates were tested to determine their efficiency to sustain SCs over long periods of time. This would standardise the protocol for *ex vivo* expansion of limbal epithelial SCs. It would improve the surgical and visual outcome of LSCD patients treated with transplantation of *ex vivo* expanded LSCs on the AM.

Grant report

The aim of this project was to improvise the methodology for efficient isolation, expansion and sustenance of LSCs under *in vitro* conditions.

Optimisation of culture conditions

We have shown that LSCs are known to adhere strongly to the basement membrane deep within limbal epithelial crypts. To miximise isolation of the limbal epithelium from corneal tissue, several enzymatic recovery techniques were investigated. These were, collagenase type-II (which releases cells by the digestion of the extracellular matrix (ECM)) and the neutral serine proteases dispase II and thermolysin (which cleave the BM at the hemidesmosomes to release cells). Dispase digestion is used widely for the isolation of limbal epithelial sheets. We previously investigated dispase and thermolysin for the de-epithelialisation of the AM and showed that dispase is the most effective agent for releasing the epithelium.

Enzymes

Using phenotypic characterisation we showed that, due to digestion of the ECM, keratocyte contamination prevented epithelial expansion. Infection of the culture was a recurring issue with collagenase extractions. Due to the milder nature of thermolysin, it was ineffective at releasing limbal epithelium, producing low cell yields. Initial dispase-related cellular isolations were found to contain few cells possessing poor progenitor activity. This suggested that the high progenitor cells were not being released. The dispase-II digestion protocol was therefore optimised to yield more epithelial cells using 10 mg/ml enzyme and a divalent metal ion activator (2 mM Ca^{2+} as $CaCl_2.2H_2O$). The cell count was 45% higher with 2 mM Ca^{2+} used as an activator in dispase-II digestion. We also discovered that extracting the cells using a corneal progenitor medium

CnT20 (CellnTec) improved retention of progenitor marker expression. Digestion with dispase did not result in infection.

Substrates

Substrate optimisation was undertaken using whole limbal dispase isolations compared with plastic. We determined that plates coated with 4.71 µg/ml fibronectin and 0.1% gelatin resulted in the best progenitor phenotypes. Cells were grown on different substrates and fully characterised at the gene expression level for a range of corneal differentiation (K3, K12, K24, DSG3) and standard progenitor (ABCG2, DeltaNP63) markers. Our previous transcriptome study of limbal epithelial crypts identified several potential novel SC markers, including K19, HES1, FRZB1, SOD2, DTC, and CDH1 (ECaderin), which we also profiled. We discovered that, compared with plastic, dispase-isolated limbal cells cultured in fibronectin and gelatin had significantly lower (almost undetectable) expression of differentiation markers (K3/12, DSG, Cx43), but significantly (all p=0.001) greater expression of the progenitor markers (K19 ‹20 fold, ABCG2 ‹20 fold, HES1 ‹20 fold, and FRZB1 ‹30 fold). SOD2 expression was marginally greater. We also established that the increased progenitor marker expression was significantly higher on fibroenction than on gelatin for all markers, suggesting fibronectin to be the choice for preserving progenitor activity.

Expand *versus* dispase isolation

There are many potential advantages and disadvantages between culturing explants (limbal tissue seeded directly onto a substrate) or culturing single cell suspensions of isolated cells. We found that, from a single limbus, 10–12 explant cultures could be established and reach confluence within 21 days. The explant could then be re-seeded to grow again. Depending on the age and quality of the donor tissue, we could obtain 3–4 repeat cultures from explants before expiration. Although many cells can be expanded from explants, the drawback is that control of cell differentiation/progenitor activity is not possible. It has been reported that the SCs remain within the explant and are not transplanted to the ocular surface. The resulting population would therefore be highly heterogeneous, reducing their long-term survival. Dispase isolation maximised potential recovery of SCs. However, we found that cells isolated from an entire limbus were difficult to passage, producing a fraction of the cells obtained from explants.

Comparing the differentiation cell sheets resulting from both techniques on fibronectin, we showed that expression of K3 (indicative of terminal differentiation) was significantly less (p=0.026) and the progenitor marker (ABCG2) significantly higher (p=0.003) in dispase-isolated cells compared with explant cultures. This suggested that explant cultures failed to maintain progenitor capacity as cells moved away from the SC *niche*. One of the causes for failure of ex vivo transplantation is transfer of insufficient numbers of progenitor/SC-like cells with small populations of differentiated cells on ocular surface. We also assessed the expression of markers at different proximities to the explant. We discovered that, in the initial zone immediately surrounding the explant, expression of differentiation markers (K3, K12, K24, DSG3, Connexin 43) was low and expression of undifferentiated markers (ABCG2, CDH1, K19, HES1 and FRZB1) was high. Further away from the explant (and SC-containing *niches*), cells became more differentiated and less progenitor-like. We found that this decrease in progenitor activity also occurred during culture on the intact AM, which has been reported to preserve SC characteristics. This finding therefore called for modifications in the existing technique to enable success in *ex vivo* expansion.

Isolation of progenitor cells

The work described above demonstrated fibronectin to be the optimum substrate for maintaining progenitor properties. As well as the standard progenitor markers (ABCG2, p63), we also confirmed K19, HES1, FRZB1, CDH1, and DTC as progenitor markers to be potentially more specific to LSCs. Focusing on cell isolates from fresh donor eyes, we isolated single cells that expressed hairy and enhancer of split1 transcription factor (HES1), lymphoid enhancer-binding factor 1 (LEF1) or DeltaNp63. However, these markers are intracellular proteins, and require double permeabilization (i.e plasma membrane and nuclear envelope), rendering cells non-viable and unusable for further expansion to assess colony-forming units as stated in the grant application.

Novel discovery and additional work in this study

We have made a major discovery that may result in a paradigm shift in ocular surface engineering. We were aware that even after the development of the most refined LSC isolation and expansion techniques, the number of SCs isolated from an entire limbus would be low, making expansion of these cells from a 1-mm biopsy for transplantation a huge challenge. Therefore, in parallel projects established to investigate alternative SC sources for corneal engineering, we discovered and comprehensively characterised (using FACS, gene analyses, and multipotency analyses) a population of corneal stromal MSCs (cMSCs) based on the widely accepted standards of the International Society for Cellular Therapy's (ISCT) minimal criteria. Our groundbreaking discovery is that cMPC readily undergoes mesenchymal corneal epithelial transition (McET). MSCs can undergo MSC–epithelial transdifferentiation (MET) and have been linked to CEC regeneration in animals. Our preliminary phenotypic characterisation suggested that the resulting corneal epithelium contained isolatable progenitor (K19, ABCG2) and differentiated (K3, Cx34) populations.

Interconnecting with this study, we considered the possibility that our generated corneal SCs could be substitutes for the LSCs this project aimed to isolate and expand. We could potentially expand sufficient cells to treat many patients. As part of this study, we isolated McET corneal populations and characterised the expression of progenitor and differentiated markers (as described previously). We discovered that the progenitor/differentiated population split was 40:60 and that, when grown on fibronectin, the progenitor population highly expressed all the corneal progenitor markers (ABCG2, p63, K19, SOD2, DTC, HES1, FRZB1), but not differentiated (K3/12, Dsg 3, Cx43) markers. We found that expression of the progenitor markers was significantly higher than with dispase-isolated LSCs. Furthermore, these calls could be passaged (currently beyond 3–4 passages) and appear to posseses considerable self-renewal capacity while remaining non-immunogenic. Therefore, cMPCs have great therapeutic potential as alternatives for LSC cultures and for corneal engineering for novel regenerative therapies.

Problems encountered and steps taken to overcome them

Marker selection for SC isolation The obstacle of determining an external cell marker for isolation was overcome by selecting set of cell-surface protein markers for isolation and culture. Markers like K3, K24, and DSG3 were used to separate and isolate differentiated cells from the population. The remaining cells were labelled and isolated with our novel progenitor markers K19, Cdh1, ABCG2, and DCT, and then fully characterised at the gene level.

Tissue supply Due to the nature of our research, we obtained donor material from several sources throughout the UK to meet the demands of research. We needed entire eyes for a single experiment to be able to isolate cells using flow cytometry. We used

the courier DHL for overnight tissue transport. Unfortunately due to internal university issues outside of our control, this contract was suspended for 5 months. It was then reactivated, but to provide additional backup we also established additional contracts with other couriers.

Standardisation of quantitative realtime gene analyses Different cells grown in different environments can suppress or activate expression of genes, including some housekeeping genes (e.g., GUSB, RPLP0, PPIA, GADPH, PGK1, B2M, HPRT, and 18S rRNA). In parallel projects, we encountered issues regarding effective quantitative gene analyses. To overcome this problem we established research techniques to normalise these quantitative differences ensuring the robustness of our data.

Collaborations established

We established a partnership with CellnTec Advanced Systems to develop clinical-grade media (€14,000 pilot study) and to manipulate the McET process for therapy, which supports the future progression of this project.

Publications

1 Branch M J, Hashmani K, Dhillon P, Jones DRE, Dua HS, Hopkinson A. Mesenchymal stem cells in the human corneal stroma. *Invest Ophthalmol Vis Sci* 2012; 53: 5109–5116.

2 Kulkarni BB, Mohammed I, Hopkinson A, Dua HS. Validation of endogenous control genes for gene expression studies on human ocular surface. *PLoS ONE* 2011; 6: e22301.

3 Kulkarni BB, Tighe PJ, Mohammed I, et al. Comparative transcriptional profiling of the limbal epithelial crypt demonstrates its putative stem cell niche characteristics. *BMC Genomics* 2010; 11: 526.

4 Yeung AM, Tint NL, Kulkarni BB, et al. Infant limbus: an immunohistological study. *Exp Eye Res* 88: 1161–1164. 2009.

Oral presentations that resulted in awards

1 Hashmani A, Branch M, Dua HS, Hopkinson A. Mesenchymal stem cells of the corneal stroma. Limbal Stem Cell meeting, 2011. First prize for an oral presentation.

2 Hashmani K, Branch M, Dua HS, Hopkinson A. Mesenchymal stem cells of the corneal stroma. European Society of Cornea & Ocular Surface Disease Specialists (EuCornea) Congress ESCRS, Vienna, Austria, 2011. First prize for oral presentation.

3 Dhillon P, Hashmani A, Branch M, Dua HS, Hopkinson A. Characterisation of human corneal stromal cells as a novel source of mesenchymal stem cells. Fifteenth Nottingham Eye Symposium, 2011. David Mayer Research Prize.

4 Dhillon P, Hashmani A, Branch M, Dua HS, Hopkinson A. Characterisation of human corneal stromal cells as a novel source of mesenchymal stem cells? United Kingdom Medical Students Association (UKMSA), 2011. British Journal of Hospital Medicine Scientific Research Prize.

Oral presentations

1 Dua HS, Branch M, Hashmani K, Hopkinson A. Mesenchymal–epithelial transition at the ocular surface. German Ophthalmological Society, Berlin, September 2011.

2 Dua HS, Branch M, Hashmani K, Hopkinson A. Mesenchymal–epithelial transition at the ocular surface. Japan Society for the Promotion of Science (JSPS) Research Symposium, Cardiff, September 2011.

3 Dua HS, Hashmani K, Branch M, Hopkinson A. MET at the ocular surface. Ocular Information Focus Group Barcelona, Spain, 2011. September 2011.

4 Branch MJ, Hashmani K, Dua HS, Hopkinson A. Ocular mesenchymal stem cells? Third Limbal Stem Cell meeting, Reading, September 2010.

5 Branch MJ, Al Echrish NHJ, Jones DRE, Dua HS, Hopkinson A. Transdifferentiation of hMSCs into corneal epithelial cells. Fourteenth Nottingham Eye Symposium, Nottingham, 2010.

Further funding

1 Rose FR, Hopkinson A. A novel tissue-engineered construct of mesenchymal stem cell (hMSC)-derived corneal epithelium for ocular surface reconstruction. EPSRC Doctoral Training Centre (DTC) in Regenerative Medicine. Studentship. 2011 for 36 months: £90,000.

2 Hopkinson A. Development of dedicated media for MET for commercialisation. CellnTec industrial partner, Switzerland. Student sponsorship. 2011: £7.500.

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King James IV Professorship Lectures

Periodontitis: discovering new treatments through understanding the disease

Professor Philip Preshaw, Professor of Periodontology and Consultant in Restorative Dentistry, School of Dental Sciences and Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne

Periodontitis is a chronic inflammatory disease in which plaque bacteria on the tooth surface initiates inflammatory responses in the underlying gingival and periodontal tissues. This localised inflammation is characterised by redness and swelling of the gingiva in the early stages (referred to as 'gingivitis'). If the plaque challenge persists, inflammation spreads to involve the deeper tissues of the periodontium, including the periodontal ligament and alveolar bone. The long-term consequence is loss of support for the teeth, tooth mobility and ultimately, tooth loss.

There are several variants of periodontal disease, the most common of which is chronic periodontitis, which tends to affect patients in their forties and fifties. More serious forms of periodontitis include aggressive periodontitis, which affects younger individuals in their late teens and early twenties. This can be a very distressing condition for young adults who, despite maintaining good oral hygiene, have an increased susceptibility to the disease, resulting in early tooth loss.

Periodontitis has multiple negative impacts on quality of life, including discomfort, disability, functional limitation, handicap, stigma and retrospective regret [1]. Examples include embarrassment relating to appearance, loose teeth or bad breath, social disability (such as choosing not to eat out in restaurants), and physical disability in terms of inability to eat certain types of food. Patients often feel that others will perceive that 'it is their fault for not maintaining a good level of oral hygiene', and also express a strong sense of regret (e.g., relating to their own health behaviours when they were younger) or directed at dentists who perhaps should have identified the disease earlier. This research presents a complex picture of the impact of periodontitis on the day-to-day lives of patients.

An important consideration is the link between periodontal disease and systemic health. This is not a new subject. In the late 1800s, Willoughby Miller published an article in *Dental Cosmos* which described the human mouth as a focus of infection which was responsible for causing various diseases throughout the rest of the body, including gangrene, noma, tuberculosis, meningitis, syphilis, diphtheria, pneumonia and septicaemia [2]. Indeed, dental clearance (extraction of all the teeth) was considered a viable option for the management of rheumatoid arthritis [3]. However, by 1952, an editorial in *JAMA* indicated that removal of teeth for the management of systemic diseases was no longer a valid strategy [4].

In the 1970s, we had a very poor understanding of the pathogenesis of periodontal disease, and plaque was considered the sole aetiological agent. In the 1980s, researchers began to focus on the inflammatory host response in the disease process. It became clear that certain patients had an upregulated inflammatory response to the presence of subgingival bacteria, and this was responsible for most of the tissue breakdown in periodontal tissues. For example, patients with periodontitis secreted excessive levels of inflammatory mediators such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α, prostaglandin (PG)E2, and matrix metalloproteinases (particularly

MMP8 and MMP9). Simultaneously, advances in microbiological research led to the development of the concept of plaque as a biofilm. This can be broadly described as a complex community of bacterial species living in a matrix attached to the tooth surface and other non-shedding surfaces in the oral cavity.

We now understand that bacterial challenge initiates the inflammatory response (which is essentially protective by intent) to prevent ingress of bacteria into the periodontal tissues. For most patients, the inflammatory response controls the bacterial challenge without resulting in significant breakdown of tissue. However, in patients who are more susceptible to disease, the inflammatory response is dysregulated or excessive, resulting in high levels locally of destructive mediators and enzymes (including various cytokines, prostanoids and MMPs). Excessive production of these inflammatory mediators is responsible for the breakdown in connective tissue (resorption of alveolar bone and destruction of periodontal ligaments) which results in the signs of disease (alveolar bone loss and loss of attachment between the tooth and its supporting structures).

Certain patients appear to be genetically susceptible to periodontitis, and specific periodontal conditions run in families (particularly aggressive periodontitis). It is believed that these patients mount an excessive inflammatory response to the bacterial challenge, resulting in advanced periodontitis at an early age. These conditions are also associated with specific pathogens in the subgingival biofilm, notably *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. These species are probably present in most patients (even healthy individuals) in low quantities. However, in patients with aggressive periodontitis, the levels of these organisms are particularly increased. They possess many virulence factors which perpetuate the inflammatory response, resulting in enhanced breakdown of tissue.

Smoking is also a major risk factor for periodontitis because it alters aspects of the immune response (particularly neutrophil function), resulting in increased susceptibility to disease. Indeed, smoking cessation should now be considered a routine part of periodontal therapy [5]. In the only longitudinal study of its type, we showed that smokers with periodontal disease who ceased smoking demonstratedd a better response to treatment compared with patients with periodontitis who continue to smoke [6].

The other major risk factor for periodontitis is diabetes. The precise mechanistic links between diabetes and periodontal disease are not entirely clear, but are likely to involve multiple complex molecular and cellular pathways. For example, in conditions of hyperglycaemia, neutrophil function is altered with delayed neutrophil apoptosis, resulting in prolonged retention of neutrophils in inflamed tissues, leading to more tissue breakdown. Importantly, there is emerging evidence that treating periodontitis in patients with diabetes could potentially improve glycaemic control, underscoring the importance of the two-way relationship between these common conditions.

As we improve our understanding of the pathogenesis of periodontal disease through careful clinical and laboratory-based studies, this provides the opportunity to develop novel therapeutics. Modulation of host response is starting to emerge as a viable method for managing periodontal disease. In broad terms, this treatment aims to modify the inflammatory host response so that the progression of disease is reduced and tissue breakdown halted. One such treatment is doxycycline (20 mg doxycycline twice daily for 3–9 months). We have shown that this treatment, if used as an adjunct to conventional periodontal therapy, improves treatment responses by ≈30%, representing a significant benefit for patients [7]. The drug is well tolerated at this dose, with no impact on the subgingival, gastrointestinal or genitourinary microflora. The mode of action is the inhibition of MMPs, a property shared by all tetracyclines. At 20 mg twice

daily, the drug does not exert an antibiotic effect and its therapeutic efficacy relates to the inhibition of MMPs locally in the periodontal tissues and elsewhere in the body.

We are entering a new phase of periodontal therapy in which we focus on managing inflammation rather than focussing purely on the removal of the bacterial biofilm. In the future, dentists will work much more closely with medical colleagues for joint management of patients and will use molecular diagnostic techniques for identifying diseases at a much earlier stage. This will probably involve salivary diagnostics for identifying the earliest signs of periodontal breakdown so that the condition can be intercepted and prevented before it becomes too advanced. Future treatment strategies will probably look very different from those in use today.

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Science, surgery, and surveillance: 'stopping the clock' in oral cancer

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Introduction

Oral squamous cell carcinoma (OSCC) remains a lethal and deforming disease despite advances in management. Its rising incidence in young female patients is concerning. Approximately half of OSCC patients die from their disease, whereas the morbidity after tumour ablation and reconstructive surgery remains high. Only by better understanding of oral carcinogenesis and early, effective therapeutic intervention will treatment outcomes improve.

OSCC may be preceded by clinically distinct potentially malignant lesions (OPLs) such as leukoplakia and erythroplakia, offering opportunities for interventional therapy. They display epithelial dysplasia, a variable histopathological presence of tissue disorganisation and dysmaturation preceding the development of invasive carcinoma. Dysplasia is subjectively graded into 'mild', 'moderate' and 'severe' categories or carcinoma *in situ*. No tools help to predict the behaviour of individual lesions or patients, it is assumed that more severe dysplasias are at the greatest risk of malignant transformation [1].

There are no agreed treatment protocols for OPL management, which remains polarised between interventional surgical excision and conservative or medical therapies. Quoted malignant transformation rates vary from 1% to 40%, making management and prognosis difficult [2].

This lecture summarises 10 years of translational research into the scientific basis of oral carcinogenesis and the development and assessment of interventional surgery protocols for the management of oral precancer.

Scientific basis of oral carcinogenesis

The transformation of the normal oral mucosa into premalignant and subsequently malignant tissue remains poorly understood, although it is recognised that increased cell proliferation is important in carcinogenesis. Identifying reliable markers of proliferative activity remains an important goal in clinical oncology.

In Newcastle we have utilised the biology of epithelial cells, cell-proliferation analyses and characterization of oral carcinogenesis to develop interventional management strategies to prevent patients developing OSCC.

Initial work in animal models revealed increased oral epithelial cell proliferative labelling indices (LIs) during experimental carcinogenic influence [3]. We then mapped proliferative activity in the human oral cavity. This demonstrated significantly increased cell proliferation in the floor of the mouth and ventrolateral tongue (LIs, 10–12%), sites most prone to cancer and precancer development. This finding suggested an inherent, underlying anatomical predisposition to carcinogenic influence [4, 5].

Such proliferative variation in the normal mucosa prompted us to measure LIs in OPLs: we found consistently increased proliferative activity. From mean values of 12% in mild dysplasia, we identified 2% increases with each worsening dysplasia grade. These reproducible measurements had a predictive role in identifying lesions at risk

of malignant transformation, offering objective and independent assessments of adverse lesion behaviour [6, 7].

We also found distorted LIs in clinically normal tissue harvested from patients with a history of oral cancer. We subsequently confirmed widespread changes in the oral mucosa from oral cancer and precancer patients [5, 8]. Thus, 'field change' carcinogenesis was seen in 60% of patients, with clinically normal mucosa exhibiting significant histopathological features of dysplasia and abnormally high LIs >14% [8, 9].

We hypothesised that the whole mucosal field in OPL patients was 'at risk'. We demonstrated this in a series of cohort studies, lending support to the concept that oral precancer is a widespread 'potentially malignant disorder' [8, 9]. This is now the accepted definition by the World Health Organisation, thereby recognising risk to the entire upper aerodigestive tract.

To investigate changes in the oral mucosa in detail during carcinogenesis, we assessed LIs in a range of OPLs. We consistently demonstrated increased cell activity in increasingly dysplastic and neoplastic tissue [5, 7] together with significant structural changes during epithelial dysregulation [7, 12].

We subsequently investigated the use of cell proliferation analyses measured in OPL excision specimens and compared individual lesion LI with outcome. We demonstrated a role for it as a predictive tool and as an adjunct to clinical management [1, 2].

The cell-cycle markers cyclin A and B1 and the proliferative marker Ki67 showed definite predictive value in identifying lesions at high risk, with quantitative LIs >12% for cyclins and 22% for Ki67 equating with recurrent disease [1].

Surgical management of oral precancer

In the absence of evidence-based treatment protocols for oral precancer, we hypothesised that surgical excision of dysplastic lesions should reduce the risk of malignant transformation, and confirmed this finding in clinical studies [10, 11]. CO2 laser surgery offers precise OPL excision, full histopathological assessment, minimal post-operative morbidity and facilitates coordinated follow-up.

Five-year clinical outcome data in cohort studies of OPL patients undergoing laser excision demonstrate that 70% of patients remain disease-free, whereas 28% exhibit recurrent (same site) or further disease (new site) during follow-up, with <2% developing OSCC but not at the site of the treated precancer [11].

Laser-excision specimens allowed definitive histopathological diagnosis compared with initial, diagnostic incisional biopsies, which required 'upgrading' in 25% of cases due to increased severity of dysplasia seen at excision [10].

In 9% of cases, pre-existing 'unexpected' carcinomas were identified in excision specimens despite incisional-biopsy evidence of dysplasia only; this facilitated early diagnosis and concomitant treatment of invasive carcinoma [12].

Developing reliable and reproducible CO_2 laser surgery has helped us develop 'organ-sparing' ablative procedures for early-stage OSCC [13].

Surveillance

Interventional surgical protocols and resultant structured patient reviews in dedicated dysplasia clinics have facilitated coordinated and effective surveillance strategies. These allow direct patient education with regard to risk factors and behaviour modification for smoking and alcohol habits [14,15].

We have shown that specialist non-smoking programmes are an integral part of secondary-care dysplasia services if long-term smoking cessation is to be effective [15].

We discovered that, even though patients who continue to smoke and drink alcohol are at a high risk of recurrent or further disease, the highest risk for the development of new lesions occurs in lifetime non-smokers and non-drinkers. This finding emphasised the significance of pre-existing and widespread mucosal genetic abnormalities in certain patient subgroups [11].

We also identified a low dietary intake of fruit and vegetables in OPL patients as an independent risk factor for pan-oral precancer [16].

We have highlighted significant clinicopathological differences between single and multiple precancer. Single OPLs are most commonly seen on the floor of mouth and ventro-lateral tongue, whereas multiple lesions are concentrated on the buccal mucosa and palate. The latter tend to exhibit less severe dysplastic features than single lesions, and lower proliferative LIs. However, the overall risk of malignant transformation is higher in multiple lesion disease, probably due to wider areas of inherently unstable mucosa at risk of carcinogenesis [17].

The recognition of such field-change carcinogenesis and the propensity for multiple precancer disease (which affects ≈19% of OPL patients) presents a challenging management situation due to the diffuse nature of the disease and the increased risk of OSCC development [18].

By adapting our proliferative mapping technique, we devised a new strategy of 'field mapping' dysplasia in patients with multifocal disease. Examination under anaesthesia was followed by multiple field-mapping biopsies to identify the most significant pathological sites [8, 9]. This allowed, for the first time in clinical practice, objective targeting of the most significant areas of disease, which were excised by laser.

Conclusions

Combining scientific cell biology analyses, surgical intervention and targeted patient surveillance has enabled a coordinated and methodical clinical management protocol for oral precancer. This has allowed us to effectively 'stop the clock' to prevent OSCC development in high-risk precancer patients.

Future research work involves identification of biochemical markers indicative of dysplastic change, genetic profiling of the abnormal mucosa, and quantification of risk with regard to malignant transformation.

Randomised, prospective multicentre clinical trials are needed to further investigate the role of interventional management strategies in oral precancer.

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Pain and power

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James IV possessed the virtues which marked the Stuart line: courage, firmness of purpose, and high cultivation. However, James IV also shared the misfortune that touched all of that line and for whom it might be that Dante predicted the outcome:

"Li si vedrà la superbia ch'asseta,
Che fa lo Scotto e l'Inghilese folle
Si che non può soffrir dentro a sua meta."
Paradiso XIX 121–123

Neuropathic pain arising from a focal injury to a nerve or nerves inhibits the power: and function within a limb; to work or remain independent; to engage in rehabilitation. Such neuropathic pain is usually resolved by restoration of power within the part by the reinnervation of muscle and by engagement in rehabilitation [8].

From the days of Horsley [23] and Sherren [35], alert clinicians have recognised that the sooner a great nerve is repaired the better. Understanding the affect of transection of a main nerve upon the central nervous system (CNS) came later. Neurones in the dorsal root ganglion die after peripheral axonotomy; the ventral horn cells perish after avulsion of the ventral root so that ≥80% may be lost as early as 2 weeks after injury [9]. Extensive studies of snap-frozen neurones and, more recently, of the cell bodies cultured from avulsed dorsal root ganglia, reveal dramatic upregulation and downregulation of the genes controlling nerve function [2, 3, 30] (Fig. 2).

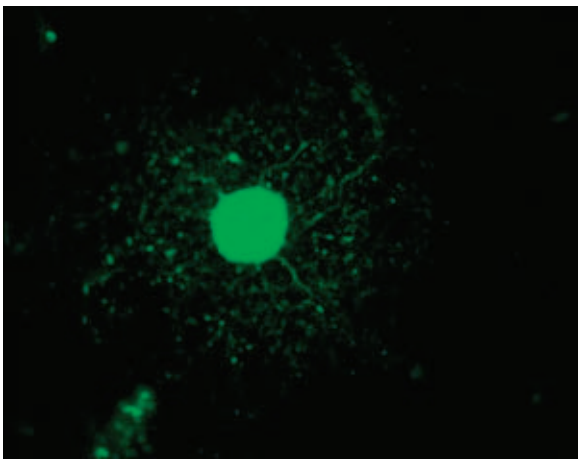


Fig 2 Cultured human DRG neurones immunostained for Gap 43 (green) and for the vanilloid receptor TRPV1 (red), the nuclei of satellite cells are stained blue (DAPI). Bar = 25 µm. Courtesy of Dr Uma Anand.

These events represent a biological imperative: regeneration diminishes with every day of delay before a main nerve is repaired; successful unimpaired regeneration is the most secure method of preventing or alleviating neuropathic pain.

Our observations are drawn from the Nerve Injury Clinics at St. Mary's and the Royal National Orthopaedic Hospitals and, over the last 6 years, from the War Nerve Injury Clinic at Headley Court. Four syndromes are described: the pain of avulsion (preganglionic) lesions; causalgia; neurostenalgia, and post-traumatic neuralgia. These are recognised from history-taking and clinical examination.

Brachial plexus

In the preganglionic injury the lesion lies between the first- and the second-order afferent neurones; it may involve the latter. The first clear description came from a patient described by Frazier and Skillern [20]. This subject experienced constant, crushing, burning pains in the hand with superimposed shooting pains, up to 30–40 times every hour, which were like "lightning zigzagging through the sky". Bonney [12, 13], in a series of meticulous clinicopathological studies, defined the lesion, established the methods of diagnosis and described the natural history of the untreated lesion. Pain persisted in patients who did not recover; recovery after rupture or through 'lesions in continuity' was poor and generally complicated by co-contraction. Bonney introduced, at St Mary's Hospital in 1956, a policy of urgent repair of the main nerves and of the main artery at emergency surgery. There, Taggart's prospective study of ›300 patients over 15 years stimulated further investigations [5, 6, 10, 24, 37]. The seven main findings are summarized below.

1 There are two patterns of pain. The first is constant, in the anaesthetised hand and forearm and usually described as 'crushing', 'bursting' or 'burning'. The second is lightning-like, shooting down the injured limb.

2 Most patients who were conscious throughout, or who suffered only a transient loss of consciousness, experienced constant pain on the day of injury. More than 50% of subjects described shooting pain within 24 h of injury (Table 1).

Table 1 Onset and nature of pain in 198 patients (2000–2004)

Interval (days)	Constant	Shooting	Total
0–7	147	112	259
8–14	12	16	28
15–28	18	16	34
›28	32	48	80
Total	209	192	401

15 patients in coma after the accident were excluded. They all reported pain upon awakening. Some patients described new types of pain during recovery.

3 The radiation of convulsive pain into the distribution of a spinal nerve which is not working strongly suggests that it has been avulsed (Fig. 3).

Fig 3 Intense lightning-like pain was felt in the dermatomes of C5, C6 and C7 in this patient on the day of injury. At surgery three nerves were seen to be avulsed from the spinal cord.

4 Pain was at its worst ≈3 months after injury.

5 In general, drugs were ineffective, whereas distraction by work, study or normal social intercourse was effective (Table 2).

Table 2 Response to drugs and other methods of treatment in 198 patients (2000–2004)

	Good	Fair	Poor	Total
Amitriptylline	5	28	23	56
Other antidepressants	1	2	2	5
Pregabalin	7	1	2	10
Gabapentin	13	31	15	59
Anticonvulsants	3	7	11	21
Anti-inflammatory	3	33	12	48
Minor opioids	11	33	11	55
Major opioids	24	39	6	69
Diazepam and otheranxiolytics	0	0	4	4
Cannabis	4	4	3	11
Physiotherapy, transcutaneous nerve stimulation (TNS)	27 2	2	3 1	32 3
Social activity/study	33	1	2	36
Work	30	2	1	33

6 Pain was usually alleviated by re-innervation of muscle. Improvement often occurred suddenly, some days before clinical signs of muscle recovery. Relief was not associated with recovery of skin sensation or sympathetic function (Fig. 4).

Fig 4 Left-sided lesion in a 28-year-old female. Preganglionic C5 and C6. Pain was severe. Surgery at 8 weeks: accessory to suprascapular transfer, and intercostal T3 and T5 with deep branches of T4 to musculocutaneous nerve. Function at 22 months; she could abduct to 120°. Her pain disappeared with recovery of elbow flexion at 9 months.

7 Pain relief was more certain after repairs undertaken within 4 weeks of injury.

There can be no justification for delays in exploration and repair in severe lesions of the brachial plexus unless the patient has such severe associated injuries that these must take priority. Unfortunately, this is the case in about 1 in 3 of our patients. Most referrals come from orthopaedic surgeons, about half within 7 days of injury.

Table 3 illustrates the outcome in 228 patients who underwent repair in 2000–2004, by which time repair of the ventral root (usually by nerve transfer) was regularly undertaken. The success rate by function gain and pain relief was higher in those who had surgery within 7 days of injury (Fig. 5).

Table 3 Results of repairs in 585 elements in 228 patients who underwent surgery between 2000 and 2004 by interval between injury and procedure

Interval (days)	Number of patients	Results of repairs good/total	%	Results (excluding ventral root repairs) good/total	%	Average number of elements repaired in each patient	Average number of functions regained in each patient
0–7	52	114/175	65.1	86/140	61.0	3.4	5.4
8–14	25	41/72	57.0	21/45	46.7	2.9	3.8
15–28	31	48/87	50.1	34/73	46.6	2.8	3.3
29–56	32	25/74	33.8	21/68	30.1	2.3	1.6
57–84	31	31/67	46.2	30/65	46.2	2.2	1.8
85–112	16	13/35	37.1	12/33	36.4	2.2	1.9
113–182	22	8/34	23.5	7/33	21.2	1.5	1.1
>182	19	12/41	29.3	11/39	28.2	2.2	1.0
	228	288/585	49.2	222/496	44.8		

The average numbers of repairs for each patient was 2.6. The average number of functions regained in each patient was 2.9. The total of functions regained was 658.

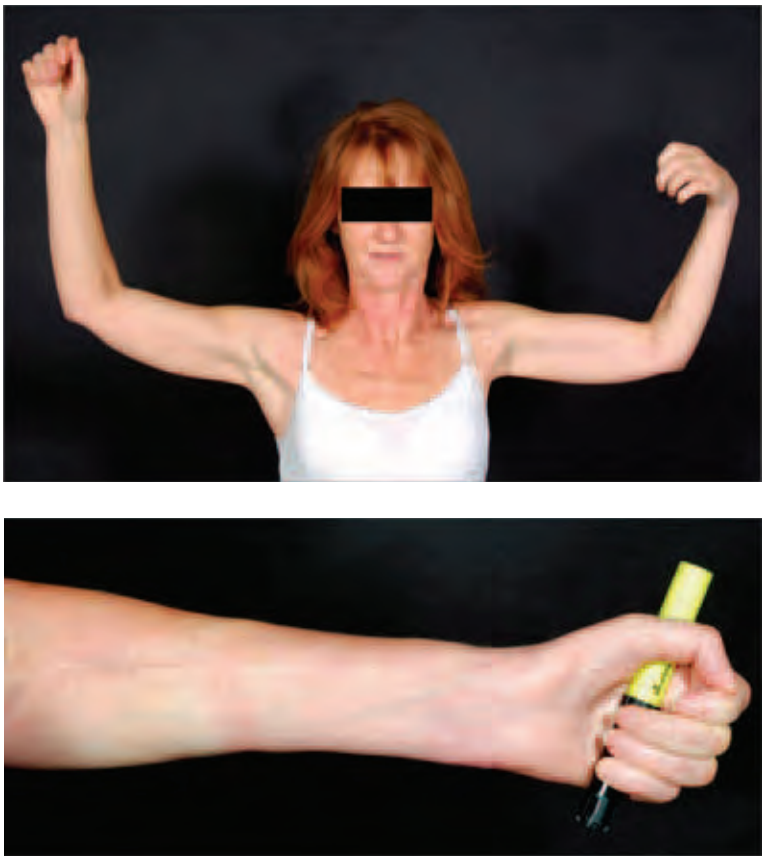


Fig 5 Left-sided lesion. Rupture C5, C7, C8 T1, avulsion C6. Surgery at 6 days. Function at 96 months after repair in a nurse aged 28 years at the time of injury. Wrist extension was regained by late transfer of FCU to ECRB.

It is likely that the constant crushing pain arises from spontaneous activity in nociceptor neurones in the dorsal horn of the spinal cord, and that shooting pains represent sudden, convulsive discharges of larger mechanoreceptor neurones in more deeply placed laminae [29, 31]. The close relationship between pain relief and reinnervation of muscles suggests a possible role for the deep afferent pathway in which the afferent fibres in the ventral root may play a part [33]. The extent of recovery of function and pain relief is closely related to the urgency of repair [10, 19, 24].

Pain from peripheral lesions

Two important lessons are revealed by the study of the pain of lesions in the brachial plexus compared with the pain from more peripheral lesions. Spontaneous firing in different populations of neurones within the dorsal horn causes constant or convulsive pain. The central response to a peripheral lesion may be reversed by correcting that peripheral event. However, there is one common finding after peripheral injury: the spread of pain beyond the distribution of the affected nerve. This is rare in avulsion pain and signifies that there is a progressing lesion of the spinal cord [28]. The events which follow an injury to a peripheral nerve are summarised below [9].

1 Lowering of the threshold to stimulation as well as spontaneous firing in nociceptor fibres and neurones [27, 31, 40].

2 Central sensitisation of neurones in the dorsal horn so that pain is experienced beyond the territory of the injured nerve [31, 41, 42] (Fig. 6).

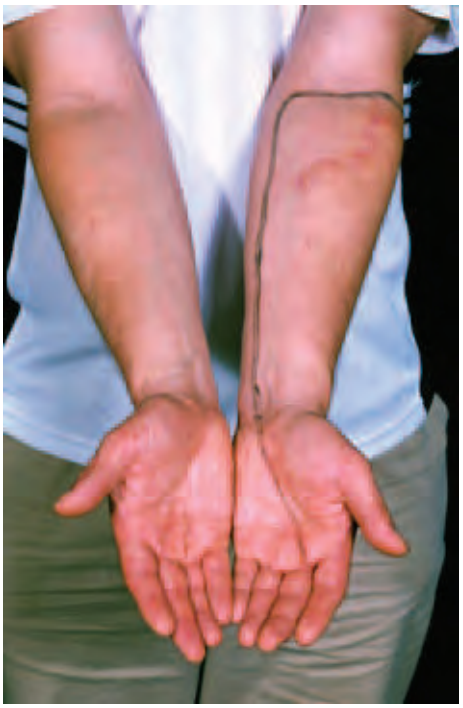


Fig 6 Central sensitisation in post-traumatic neuralgia. Injury to a branch of the lateral cutaneous nerve of forearm in a 34-year-old male. He developed severe mechanical allodynia and allodynia initially in the distribution of the nerve and then over the course of the next 6 months into the territories of the median and radial nerves.

3 Sensitisation of mechanoreceptor fibres and neurones because of the changing expression of receptors and of ion channels so that stimuli which do not normally evoke pain begin to do so [41, 43].

4 Involvement of afferent and efferent sympathetic fibres [34].

War experience

More than 900 nerve injuries in 350 servicemen and servicewomen injured in Iraq or Afghanistan have been followed up at the War Nerve Injury Clinic. A study of the first 100 consecutive patients outlines the characteristics of war wounds [11]. Most are caused by explosive devices; they are multiple and complex. Avulsion, laceration, blast and crush combine to tear and shred tissues of all compartments. Heavy contamination with dirt and debris is usual. Meticulous debridement is the essential first step in time-limited resuscitative surgery. The field-hospital policy of emergency restoration of arterial flow and extensive decompression has led to a remarkably low incidence of ischaemic fibrosis; the sole case occurred in a patient in whom the brachial artery was ligated.

The severity of damage at the level of the nerve lesion is probably unparalleled: fracture in 50%, arterial injury in 32%, moderate or severe loss of muscle in 28%, and moderate or severe loss of skin in 50%. Two of more nerves were injured in 70% of patients. Massive fibrosis and skin loss are inimical to the regeneration and function of nerves. Damaged target tissues (notably in the hand and foot) diminish function. Thirty-six patients underwent secondary procedures because of persistent, severe neuropathic pain. Thirty experienced such relief post-operatively that the requirement for analgesic medication was considerably reduced or abandoned. Some patients experienced relief when they awoke from the anaesthetic. The procedures included 6 revision repairs, 11 neurolyses of repaired nerves, and neurolysis in the other 19 patients. The causes of persistent pain included displaced bone fragments, heterotopic bone, retained metallic fragments or suture material and, most commonly, scar tissue which enveloped and constricted the nerve. Resurfacing by free fasciocutaneous flaps were used in 15 patients to relieve pain and enhance nerve regeneration. False aneurysms or arteriovenous fistulae were not encountered.

The diagnosis of neuropathic pain

History-taking is essential, and the patient must be given ample time to tell his/her story. In some cases, there may be diffidence about expressing symptoms which seem bizarre and which have often been dismissed by others. It may prove necessary to put leading questions which indicate that the clinician is listening to the patient, believes what he/she is saying, and that the clinician understands what is being said. Five features are particularly important. The first featire is onset. The immediate onset of pain after a wound or on awakening from a procedure implies that the lesion has already been inflicted whereas delayed onset suggests a later event such as haematoma. Distribution is the second feature. The patient is asked where the pain started and where it went to. Did the pain spread beyond an earlier, well-defined area and, if so, over what period of time? The third feature is quality. Was the pain there continuously, was it episodic, and was it constant or intermittent? Was the pain on the surface or deep? Many terms (e.g., burning, bursting, crushing, compressing) and phrases ("the hand was in a vice", "the bones of the foot were coming out of my skin", "a hot needle or a hot file was rasping on the skin") are used. Episodic or convulsive pain is often described as "lightning-like", "electrical", "shooting" or "lancinating". The fourth feature is aggravating and relieving factors. Many patients describe allodynia. It is common to hear of intolerance of a sheet on the leg and the foot after compression of the sciatic nerve by haematoma in the thigh or buttock. The effects of changes in temperature or weather or of associated illness are important. Finally, the effect of pain upon work, study, social activities and sleep provides an insight into pain severity.

Physical examination must be gentle and, in some patients, no more than inspection is possible. Important features include trophic changes, vasomotor/sudomotor abnormalities, and spontaneous movements. Allodynia, in its various forms, must be sought before deep palpation, which may reveal hyperpathia. Three characteristics of pain are extremely important: dysaesthesiae, allodynia, and Tinel's sign. With the evidence provided so far, the clinician ought to be able to arrive at an accurate diagnosis as to which nerve has been injured, where it was injured, and have a view about the cause of that injury and of the underlying mechanisms.

Causalgia

The severity of this terrible pain is matched only by that caused by malignant infiltration of nerves or by the worst examples of avulsion pain. Barnes' definition [4] is good, and states that it is: "(i) severe spontaneous and persistent; (ii) usually has a burning quality; (iii) may spread beyond the territory of the injured nerve or nerves; (iv) invariably aggravated by physical and emotional stimuli". It is this aggravation by examination or distraction which distinguishes causalgia from other neuropathic pain syndromes. The first clear description came from Guthrie [21] of a soldier wounded in the back of the thigh by a musket ball at the Battle of Waterloo. Pain became intense. "It seemed to increase by paroxysms, during which the man was in agony; the pain not only being permanently intense in the foot, but darting down to it, and accompanied by spasms of the whole extremity. No kind of medicine had any effect upon it; and at these periods the poor fellow found relief only by putting his foot on the cold stone, or by enveloping it in cloths wet with water or the liq. plumbi subacet. dilutes...from habit he became less sensible of the pain, and more accustomed to the necessity of jumping out of bed, and placing his foot on a stone or keeping it constantly wet and cold".

Barnes [4] blocked the somatic nerves to the lower limb by low spinal anaesthetic. He stated that pain disappeared minutes before or even in the absence of paralysis of the sympathetic efferent fibres. It is a great pity that subsequent researchers did not study

this essential article for this might have avoided the blind alley of "sympathetically maintained pain". As Schott [34] said: "despite the more eloquent effects of efferent sympathetic dysfunction which occur perhaps as epiphenomena it is the afferents which subserve those pains that appear "sympathetic dependant".

There is strong evidence for the efficacy of sympathectomy [25] but I abandoned sympathectomy 20 years ago as a result of the case illustrated in Figure 7. The association with arterial injury was recognised by Stewart [36]. Expanding haematomas, aneurysms or fistulae were encountered in 20 of 48 patients with causalgia who underwent surgery over the last 30 years. Sympathectomy was undertaken in the first 16 of these cases; pain was relieved in 13 subjects. Pain was relieved in all but one of the 32 patients where the lesion was corrected, but sympathectomy was not done. Two of the 3 children were subjected to weeks of futile treatment by drugs and by blocks on a presumed diagnosis of "complex regional pain syndrome".

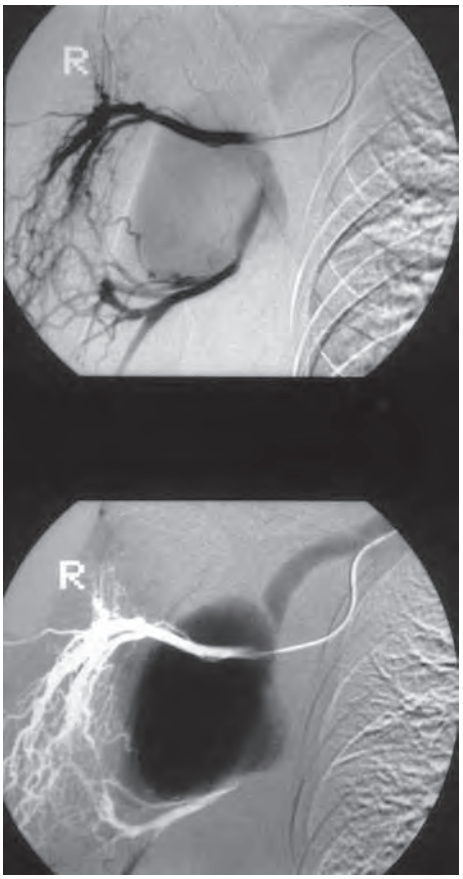


Fig 7 Causalgia. A 27-year-old male suffered a bullet wound in the right arm. There was immediate and intense burning pain in the arm, forearm and hand. Sweating was profuse, there was severe allodynia and he kept the hand and forearm wrapped in a cool, moist cloth. He was in right-heart failure from a large axillary arteriovenous fistula. Surgery in the neck was not possible. The fistula and a partial wound of the median nerve were repaired. Pain relief was early, complete and enduring.

Causalgia usually arises from an incomplete wound of a main trunk, such as the median, ulnar or tibial nerves. The wound is untidy and often contaminated. Some fibres have been divided, some have undergone Wallerian degeneration, and some have become demyelinated while others remain intact. These are subjected to the actions of local chemical agents and neurotransmitters which lower the threshold of intact and lesioned fibres. The nerve is subjected to ischaemia, to continuing irritation by retained foreign bodies, or by the pulsatile compression of an expanding haematoma or fistula. These agents continue to work unless and until the wound has been treated appropriately by debridement, bleeding has been controlled, axial flow restored, and the nerve repaired. Prolonged local anaesthetic block through an infusion proximal to the level of lesion is essential.

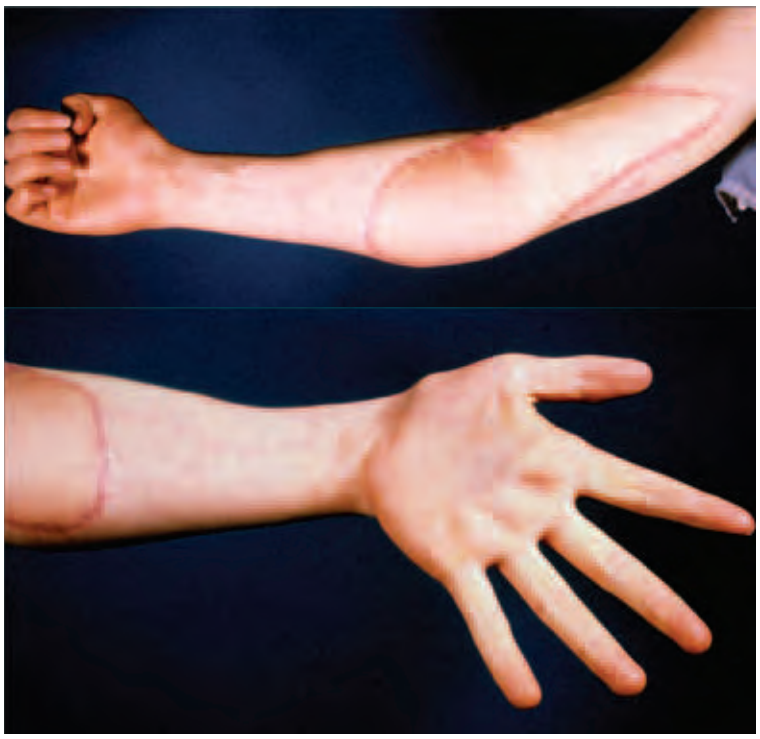
Neurostenalgia

Neurostenalgia was defined in 1998 by George Bonney [7]: "We have called this pain neurostenalgia from στενοσ (stenos) a strait as in stenosis added to νευρον (neuron) a nerve or tendon. It is agreeable that the verb στενζυ means to 'moan' or 'groan', so that there is a dual reference to the idea of pain. This group undoubtedly contains many examples of Seddon's 'irritative' lesions".

Neurostenalgia was recognised at least two centuries ago. The first Lord Nelson, who suffered constant stump pain after amputation of his right upper limb through the arm, was relieved of his pain, quite suddenly, when the ligature around the median nerve came loose and was discharged through the wound [16]. Guthrie [21] reported several similar cases. Denmark [18] described neurostenalgia in a patient injured by a musket ball in the arm at Badajoz in 1812: the pain was intense, but the radial nerve was working and Denmark proposed to the patient (Henry Croft) that the limb might be saved by removing part of the nerve, above the wound: "which he willingly consented to; but observed that he would rather have the arm amputated at once, than run the risk of a second operation". Amputation was done. A small portion of the musket ball was seen embedded within the nerve which was "blended with and intimately attached" to the adjacent structures.

Neurostenalgia signifies the continuing action of the responsible noxious agent. Neurostenalgia is common in civilian practice as a sign of critical ischaemia. All too often the significance of the pain is missed and neglect leads to severe ischaemic consequences, amputation or even death. Delorme [17], Tinel [38] and Seddon [25] emphasised that a nerve trunk or nerve repair subjected to strangulation by a scar or split skin graft will cause pain (Fig. 8). It is fortunate that neurostenalgia may be cured by surgery carried out years after pain onset [15].

Fig 8 A 28-year-old female suffered a gunshot wound to the elbow, destroying the brachial artery and median nerve. These were repaired and skin cover provided by a free latissimus dorsi myocutaneous flap. Surgery done with Professor Roy Sanders in 1981. Her pain was relieved.

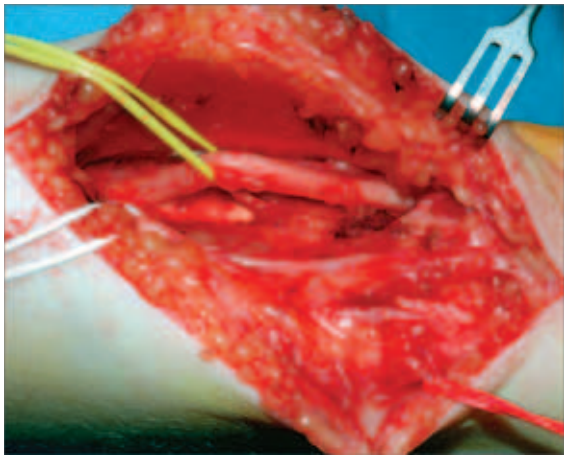


Post-traumatic neuralgia

Many of the worst cases of post-traumatic neuralgia follow damage to a terminal branch, where it lies in the superficial fascia. The only treatment with any hope of lasting success is restoration of a normal input of non-painful impulses; this usually involves successful repair of the affected nerve(s). These cases cause the most difficulty in treatment. It is a salutary reflection that many of these injuries are caused by surgeons and physicians in the course of treatment.

The initial characteristics of the pain reflect the qualities of the nerve fibres involved. Injury to a nerve of cutaneous sensation causes spontaneous pain which is often intermittent or even convulsive against a background of constant dysaesthesia. Mechanical allodynia is common, as are warm and cool allodynia. By contrast, the pain after section of the spinal accessory nerve or nerve to the serratus anterior (neither of which innervate skin) is characterized by a boring, deep-seated, dull, aching pain which is poorly localised around the shoulder girdle. The pain caused by injury to these two nerves is quite characteristic yet its significance is rarely recognized. Early relief of pain after repair of the spinal accessory nerve was an important finding in the study by Camp and Birch [14]. The propensity of the pain caused by injury to cutaneous nerves to evolve so that it spreads to adjacent skin with extension of the area of allodynia associated with excessive sweating and vasomotor instability often leads to a diagnosis of "complex regional pain syndrome". This error regularly consigns patients to fruitless (and even harmful) treatment by infusions, blocks, powerful drugs, even to the idea that the patient has some form of psychological disturbance (Figs 9 and 10).

Fig 9 A 12-year-old female awoke in severe pain after arthroscopy of the wrist. A diagnosis of CRPS type 1 was made. There followed a prolonged trial of drugs which impaired her intellect and interfered with schoolwork. There was a measurable loss of non-verbal reasoning, spatial skills, information processing, auditory and working memory. The child was discharged from hospital care and referral made by her general practitioner. Surgery confirmed the diagnosis made from her history of partial division of the dorsal branch of the ulnar nerve (left). The pain was relieved by neurolysis and prolonged nerve block (right).



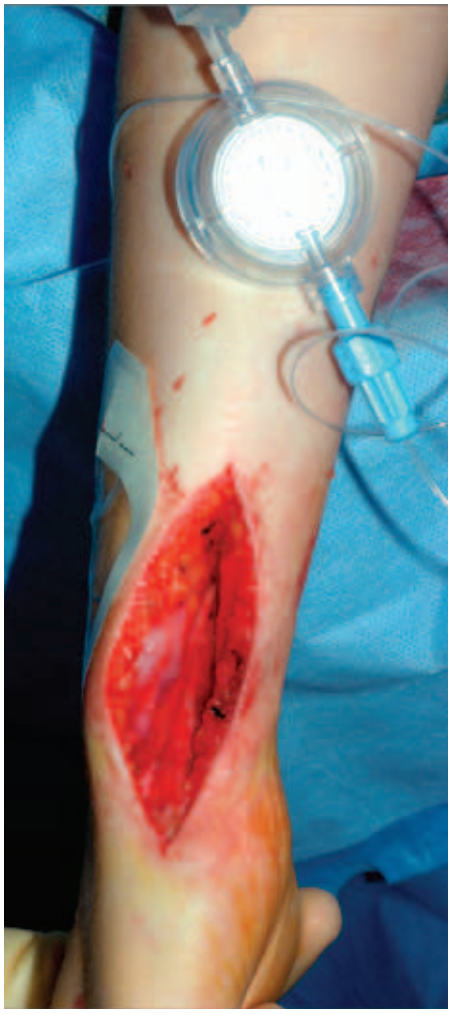
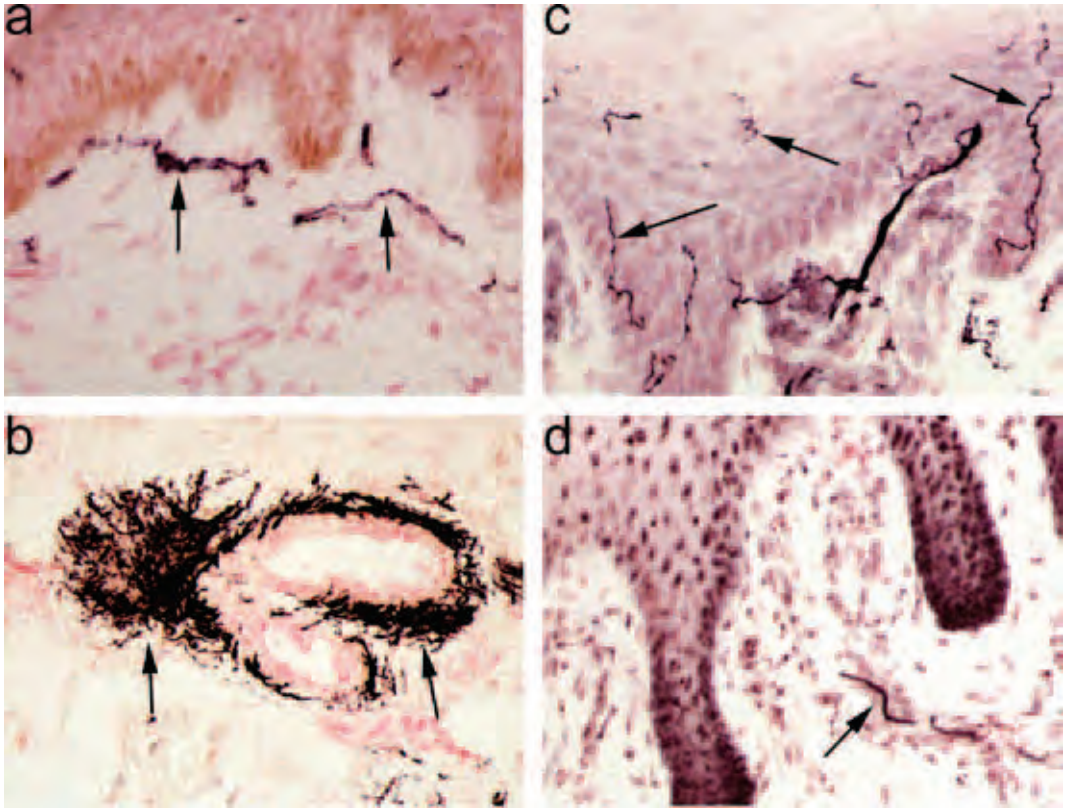
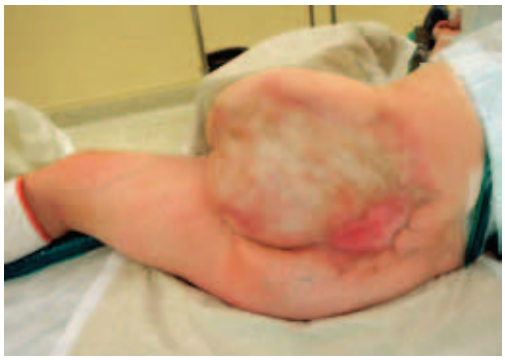


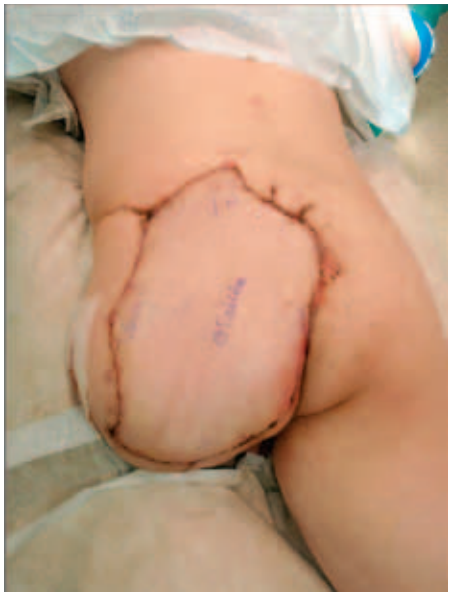
Fig 10 A 26-year-old servicewoman developed severe pain in her left foot and ankle after a seemingly trivial injury. Prolonged and extensive treatment for CRPS type 1 afforded no respite. She requested below-knee amputation because of intolerable allodynia and fixed deformity of the foot. There was abnormal expression of various nerve markers and abnormal sprouting of fine fibres in the calf skin. (a) Preserved subepithelial nerve fibres stained with the marker PGP9.5 (arrowed; ×40 magnification). (b) Unusually dense PGP9.5 fibres around the blood vessels in the skin (arrowed; ×40 magnification). (c) Increased intraepithelial TRPV1 (heat receptor) fibres (arrowed; ×40 magnification). (d) A few subepithelial TRPM8 (cool receptor) fibres (arrowed; ×40 magnification. Courtesy of Professor Praveen Anand.



Surprisingly few of the patients seen in the War Nerve Injury Clinic have undergone surgery for post-traumatic neuralgia arising from section of cutaneous nerves, but revision surgery was necessary for some troublesome amputation neuromas. A neuroma is not a disease, rather a regenerative response to injury. There should be no criticism of the first surgeon seeking to preserve life for it is no great matter to revise an amputation if the patient's general condition permits. The trouble arises from the bed in which the neuroma lies: it may be too superficial or it may be tethered within a scar. A dramatic illustration of this problem is provided in the case of the soldier shown in Figure 11 who sustained severe injuries to the pelvis and lower limbs. A split skin graft was used to cover the great defect on the back of the thigh, buttock and sacrum. He developed intolerable pain which was accompanied by central sensitization to such a degree that the visceral nervous system became involved. Pain was exacerbated by voiding the bladder or emptying the bowel. His situation was improved greatly by excision of the scar and replacement by a massive full-thickness skin flap.

Fig 11 A 25-year-old soldier. High above-knee amputation with massive skin loss from an improvised explosive device. Pain became intense and was improved greatly by a large free full thickness skin flap. Courtesy of Mr Roderick Dunn, Odstock Hospital, Salisbury.





Conclusion

Physicians should consider pain as a symptom and not as a disease. They should never forget that persistent neuropathic pain may signify that the agent responsible for the lesion of the nerve is still at work. No patient should be sent to a Pain Clinic with persistent pain after focal injury to a nerve unless and until reasonable efforts have been made to establish the cause of that pain.

The enormous contributions made by the Royal Army Medical Corps (RAMC) in two world wars were set out in the reports from the Medical Research Council, and in the writings of Robert Jones. Much fundamental work came from the five designated hospitals during the Second World War. Seddon [26], Sanders [32], Young [44] and their colleagues in Oxford established the scientific basis of the injury, repair and regeneration of nerves. Young's reservations about grafting have been confirmed [22]. At the Gogarburn Hospital, Edinburgh, the manual *Aids to the Examination of the Peripheral Nervous System* was developed by Learmonth, Ritchie Russell and McArdle. Happily that work is now in its fourth edition under the guidance of Michael O'Brian [1].

Civilian surgeons have a very great deal to learn from the current practice of the RAMC but I put it to you that all is not well. Can we match the record of our predecessors with respect to the duration and precision of observations from prolonged study and follow-up? Can we equal a review rate of ›80% of nerve injuries over 5 years [26]? Is there a sufficient integration of later reconstructive work? Rehabilitation as an urgent process and one in which the surgeon must play an essential, and at times, leading part. The Nerve Injury Clinic embedded within Headley Court does no more than what has been done before.

Acknowledgements

Colonel John Etherington OBE (Clinical Director, Defence Medical Rehabilitation Centre (DMRC) Headley Court) invited me to set up the War Nerve Injuries Clinic 6 years ago and has staunchly supported it since. Figures and tables are from *Surgical Disorders of the Peripheral Nerves* (second edition) by permission from Springer UK.

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Rotator cuff repair: beyond arthroscopic repair

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The prevalence of adults attending primary care for new shoulder pain is 1% per annum and 2.4% for all shoulder problems. Over the last decade the number of arthroscopic subacromial decompressions undertaken in the UK has increased from 2,526 to 21,370 (746%). Likewise, the prevalence of rotator cuff repairs has increased from 1,357 to 8,735, (544%).

More than 273,000 rotator cuff surgeries are conducted annually in the USA, and 500,000 are carried out worldwide. The implant costs are ≈$1.7. Five years ago, 15% were done by open surgery, 75% were 'mini-open' and 10% totally arthroscopic. Today, the proportions are 40% arthroscopic and 60% mini-open.

The last decade has seen a shift from open rotator cuff repair (RCR) to arthroscopic cuff repair. Patients want minimally invasive surgery, less 'collateral damage', a shorter stay in hospital, and minimal postoperative pain. It seems a no-brainer to say that they get this with arthroscopic surgery.

However, numerous studies have shown that the functional outcome scores show no benefit either way. Although it is often said that post-operative pain is less after arthroscopic surgery, a recent study showed that there is no significant difference in post-operative pain related to the surgical approach (arthroscopic or open). Post-operative pain is related to pre-operative pain and the size of the tear. There is no doubt that arthroscopic repair remains more difficult, time-consuming (113 min *versus* 103 min), and more expensive (by $1,248 per case) than open repair.

Above all, patients want a procedure that works! This means a strong repair that remains structurally intact for a decade with a low re-tear rate. The re-tear rates for arthroscopic RCRs are shamefully high (20–76%). However, open repair fares little better (13–31%), albeit these are all moderate-to-large tears. The large multicentre UK study for open cuff repair (British Elbow and Shoulder Society (BESS) Panacryl study 2001) noted a re-tear rate of 14% for small/moderate/large tears, and the overall re-tear rate for all tears (including massive tears) using ethibond was 22%. This shows that we can do better, this is a challenge for the present and future.

As well as wanting the procedure to work, patients want to recover rapidly, to get out of the sling as soon as is possible, to function early, and to get back to work as soon as possible. This is far from reality even with arthroscopic repair. It has been shown that patients are worse off in every parameter 2 months after cuff repair, and that they are initially worse, recover to pre-operative level at 3 months, and by 1 year are only at 80% of the function of the other arm. This begs the question as to whether by increasing the strength of repair to that of the intact cuff, less protection and earlier function could be achieved. As surgeons we need to improve our repairs so that patients can accelerate their rehabilitation with an aim to halve the recovery time within the next 5 years.

Twenty-five years ago, early attempts at open cuff repair were a struggle. At that time we understood nothing about the pattern of cuff tearing, or the pattern of contractures that had to be released. Moreover, we understood little about the biomechanics of

cuff repair. The standard procedure was to put stay sutures in the edge of the cuff, use these to mobilise the cuff, repair the cuff and then get rid of the stays. This is when I got the first 'Eureka' moment: why get rid of the stays? Why not keep them and take them down to a screw lateral to the footprint? This was the origin of the two-row repair that I started to use from 1992, and which was illustrated in my book and demonstrated in lectures from then on. The contracted tissues need to be released. Plastic-surgery techniques were developed to close skin defects that were too tight for primary repair. A rhomboid flap is developed in the cuff by incising just behind the oblique central tendon. This rhomboid flap can now be advanced to obtain primary closure without tension.

The goal is to develop a 'bomb-proof' open repair for rotator cuff tears and then to adapt this for arthroscopic use. We looked at cuff repair as a chain, starting at the bone, then the bone anchor, the suture, the knot, knotless techniques and finally (perhaps most importantly) the suture passage and the tendon. Using a Hounsfield H20K Tensiometer we tested each link of the chain in the laboratory to determine how we could strengthen the construct. Taking the adolescent pig as a validated model we looked at bone strength and bone tunnels. We found that bone tunnels were pretty weak. A 1-cm tunnel failed at 16 N, 2-cm and 3-cm tunnels were much stronger at 114 N, and 4-cm tunnels failed at 150 N. Remember that we are trying to restore the central tendon that has a breaking strain of ≈750–1,000 N. Bone tunnels were much weaker than an anchor that failed at 200 N. We found that anchors were stronger than bone tunnels. Then I had a second Eureka moment: if a tiny screw holds 200 N, what about a decent-size screw, say 6.5 mm by 35 mm? This led to the development of my low-profile capstan screw that failed at a massive 900 N.

The forces that need to be countered in RCR are massive (750 N). Standard number-2 sutures (permanent or absorbable) fail at ≈90 N. These were found to fail at lower loads if they abraded against the eyelet of metal suture anchors. This led to the industry bringing out new 'super-sutures' that have a core of ultra-high-molecular-weight polyethylene (UHMWPE) that withstand loads of 200–300 N. The problem is that the sutures have to be tied somewhere. We looked at knots (particularly arthroscopic knots). We found that Nicky's knot, the Eezy knot and the SMC knot all failed at ≈60 N compared with the intact suture that failed at 138 N per strand. Hence, knots failed at half the strength of the intact suture. This led to a third Eureka moment: could the sutures be held within a bone tunnel using a knotless system using what we termed an 'interference fit' of the suture against the bone, similar to locking an ACL graft in its bone tunnel? This worked and we had a powerful hold at 600 N.

This left the suture–tendon interface as the critical area. I was interested to see whether we could carry out an arthroscopic grasping suture of the Mason Allen type because this had been shown to be the strongest construct by Gerber when carried out open. Our findings showed that the strength of the construct was proportional to the number of suture passes through the cuff rather than the grasping pattern of suture. Firstly, we compared two simple sutures (119 N) with a single mattress suture (134 N), both being a two-pass technique. We then compared four-pass techniques, and the two mattress sutures (170 N) were better than a single Mason Allen suture (140 N).

We have learnt a lot through experience and experimentation, in real life and in the laboratory, through 20 years of treating patients with rotator cuff tears. We have learnt about the anatomy: the fibrous framework with a strong anterior pillar that acts as a firebreak and determines the propagation of cuff tears. We have learnt that we need to assess tears pre-operatively by office ultrasound in the hands of the surgeon as a

one-stop clinic, but that we also need to assess mobility and quality. We have learnt how to release the cuff and that if primary closure is not possible, to use plastic surgical techniques such as the rhomboid flap to achieve a tensionless closure. We have learnt how to construct a repair using the Capstan screw, two-row repair with UHMWPE supersutures and knotless fixation, and with multiple suture passage spread out across the cuff. We believe that what 60-year-old patients really want is a bomb-proof repair. A bomb-proof repair works, it remains structurally intact, relieves pain, allows accelerated rehabilitation and an early return to normal function at home and at work with minimal complications. Sixty-year-olds are not worried about a mini-open scar that is virtually invisible at 6 months. Although bomb-proof repair can be achieved at open surgery, it will take another 5 years before all but small and moderate tears can be repaired arthroscopically, come out of the sling at 5 days, have a 10% re-tear rate and get on with life. However, this should be our aim and our vision: arthroscopic bomb-proof repair.