Tailoring immune suppression following liver transplantation

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Declaration

All of the work in this thesis is original unless otherwise acknowledged in the text or by references and has been undertaken during the registered period of research.

None of the work has been submitted for another degree to this or any other university.
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Chapter 1. Introduction

Liver transplantation

Liver transplantation was first performed in 1963 (1) as an experimental treatment for end stage liver disease. Three patients were transplanted, all of whom died within 3 weeks. Since then it has become an established therapy resulting in improved quality of life (2), with 675 transplants from cadaveric donors taking place in the UK in 2001 and 706 in 2002 (3). This level of activity compares with 10 years ago when 502 liver transplants were performed in 1992. Figures released for survival up to the year 2000 show that early (1 year) survival has improved to 88% for patients transplanted from 1998 – 1999, with 3 year survival for the period 1996 – 1997 being 73% and 5 year survival for the period 1994 – 1995 being 64% (3). This improvement is probably due to a combination of factors such as improved surgical and anaesthetic technique, changes in medical management after transplantation, the improved recognition of other harmful factors like hypertension, choice of immune suppression and better prediction of patients in whom liver transplantation is not likely to be appropriate such as those with cholangiocarcinoma or multiple large hepatocellular carcinomas.

Indications for transplantation are chronic liver failure due to most chronic parenchymal liver disorders which result in cirrhosis or hepatocellular carcinoma and acute liver failure. This includes viral hepatitis (B and C), primary biliary cirrhosis, primary sclerosing cholangitis, cryptogenic cirrhosis, metabolic liver disease, alcoholic cirrhosis, autoimmune hepatitis, biliary atresia, alpha-1-antitrypsin deficiency, haemochromatosis and acute liver failure due to paracetamol overdose, autoimmune hepatitis, hepatitis A, B and E or drugs (see figure 1.1).
Figure 1.1. Indications for liver transplantation with percentages in the calendar year 2002. Total number of transplants performed was 665.

Because of its success in treating liver failure due to these conditions, there is a mismatch between donor and recipient numbers which is worsening with demand. There is now a shortage of organs available for transplantation (there were 158 patients on the liver transplant waiting list at the end of 2001, 163 at the end of 2002) and there is therefore much debate about who should receive this scarce resource. Re-transplantation already accounts for some of the transplant activity (but less than 5 years ago, see figure 1.2) and as long term survival improves in transplant recipients, graft failure due to recurrent disease will also rise. This is
particularly true for hepatitis C which is now the commonest indication for liver transplantation worldwide. Re-transplantation is well known to be associated with higher complication rates and lower patient and graft survival for all conditions (4). In patients with graft failure due to recurrent hepatitis C, transplantation after the development of hepatic decompensation gave a 1 year survival rate of 41% (5, 6). This has resulted in much research being aimed at preserving graft function. As survival improves (7) and the number of available treatment options increases (such as new immunosuppressive and anti-viral drugs), the long term effects of the different available therapies and their side-effects, such as renal failure (8, 9), become more apparent and more important.
Figure 1.2. Indications for liver transplantation with percentages in the calendar year 1997. Total number of transplants performed was 639.

There are several approaches which can be taken to address the shortage of donors. The best approach for the long term is to try to improve donor rates. There is currently much discussion about the possibility of making all people who are brain dead potential donors unless they have previously opted out of the scheme. This would increase donor numbers by removing the need to gain permission from the relatives of patients to harvest their organs although this is a contentious issue. A second approach to increase the donor pool is to include non-heart beating donors. These are patients who do not fulfil the criteria for brainstem death but who are
expected to die in the very near future. They are taken to the operating theatre immediately after death for harvesting of organs. A small number of extra organs are gained in this way at Addenbrooke’s hospital (personal communication, Dr. G. Alexander) and an active study is underway at Kings College Hospital, London. A third approach is to encourage more living related organ donation. This is possible for liver transplantation, but if the liver is to be transplanted into another adult, the success rates are much higher for transplantation of the right lobe of the liver. This is however a higher risk operation for the donor and carries a small mortality risk which is currently estimated to be between 0.4% and 1% (10, 11).

**Hepatitis C**

Hepatitis C is an increasingly common chronic viral infection affecting the liver. Estimates of the number of people infected in the UK range from 200,000 to 400,000 although the true number remains unknown (12). It is mostly spread by blood transfer and is therefore common amongst people who have used intra-venous drugs (13). It is also prevalent in patients who have received blood transfusions or other blood products before blood was screened for hepatitis C infection. This includes patients with haemophilia who have often received many transfusions or blood products (14). There is also a small risk of transfer by sexual intercourse which has been quantified at less than 5% to spouses of infected patients (15) although the precise magnitude of this risk has not been fully established. An audit of 1000 patients performed in Cambridge of intra venous drug users with hepatitis C found that no patient had transmitted it to their spouse (personal communication, Dr. G. Alexander).
Once infected, most people become chronic carriers and the virus affects the liver (16). It can cause liver fibrosis, with the median time from infection to cirrhosis estimated to be 30 years (17). The biggest determinants of the rate of fibrosis progression are the age at infection, with older ages being associated with more rapid progression of disease, alcohol consumption greater than 50g per day, male sex (17) and viral genotype 1 (18).

When infection has been established by the presence of the hepatitis C antibody and viral RNA, a liver biopsy is usually performed to assess the degree of liver damage. Any patient with significant fibrosis or inflammation is usually offered treatment with interferon-α and ribavirin. For types 1 and 4 hepatitis C virus this is continued for 1 year and for types 2 and 3 it is continued for 6 months (12). Overall cure rates are 30 – 40% of patients treated (19, 20).

When cirrhosis has developed in an infected liver, the disease can progress to decompensation and there is also a risk of development of hepatocellular carcinoma (21). These situations carry a poor prognosis and the only effective treatment is liver transplantation.

**Recurrent disease**

Most liver disease has the potential to recur in the graft after transplantation and some factors have been noted to alter the course of this recurrent disease.

After transplantation for hepatitis C, graft infection is almost universal (22) with treatment for acute rejection and higher cumulative steroid dose known to have a detrimental effect on the graft. Sheiner et al demonstrated a clear correlation between time to recurrence of biopsy proven hepatitis C and treatment for acute rejection (23). They found that patients without acute rejection had the lowest levels
of recurrent inflammation in their graft (18%), patients with 1 episode of rejection had a risk of recurrent inflammation of 42% and patients with multiple episodes of rejection had a recurrence risk of 70%. Patients with steroid resistant rejection treated with OKT3 had a risk of recurrent inflammation of 71%. Berenguer et al also found a link between treatment of acute rejection and recurrence of hepatitis C infection in the graft (24). Although they found that there was no relationship at 1 year after transplantation, a significant relationship had developed by 2 years. This was stronger in patients who had had multiple episodes of acute rejection. They also found that cumulative steroid doses were higher in patients who developed chronic graft hepatitis. Only 4 patients were treated with OKT3 in this series, but 3 of them developed moderate or severe hepatitis by 1 year post transplantation and 2 had died by 2 years post transplantation.

The drugs used for immune suppression also play a role in the progression of graft disease (25, 26). Initial immunosuppression and longer duration of steroids have been shown to correlate with fibrosis progression in the graft (25). Papatheodoridis et al found that immunosuppression with a single agent correlated with higher levels of hepatitis C RNA at 3 months but that at 12 months high hepatitis C RNA levels correlated only with longer duration of steroid treatment (27). The hepatitis C RNA level at 12 months also correlated with the severity of fibrosis at 12 months. A second study from the same group found that initial immunosuppression using multiple agents resulted in more severe graft fibrosis 3 years after transplantation (25). The agents used were cyclosporin or tacrolimus with azathioprine and prednisolone. Patients having 2 or 3 drugs had the calcineurin inhibitor and prednisolone with or without the azathioprine. The patients with the heavier immunosuppression are therefore those who also had steroids as part of their initial
treatment. Indeed it is possible that the detrimental effect of steroids has had an effect on all of these trials. They do not use steroid free arms and the steroids given to the patients may swamp any benefit of other drugs.

The use of the IL-2 receptor antagonist daclizumab together with mycophenolate mofetil (MMF) has been shown to cause more rapid progression of hepatitis C and fibrosis (26). In a study by Nelson et al of 41 patients (21 with hepatitis C) treated with MMF and daclizumab they found that there was a detrimental effect on the patients with hepatitis C. Patients with hepatitis C had a shorter time to histological recurrence of hepatitis and jaundice and greater histological activity at 1 year post transplantation, with 45% having developed advanced disease by this stage.

Regimens including azathioprine have been associated with reduced histological recurrence (28). Hunt et al studied 65 patients with hepatitis C treated with calcineurin inhibitor and prednisolone who were transplanted over a 15 year period. 17 of them also had azathioprine included in their treatment regimen. They found that regimens including azathioprine had a lower rate of histological recurrence and disease progression. There was no difference seen between the two calcineurin inhibitors used.

This progression of hepatitis C related disease may be more rapid than that seen in native liver (29, 30) with fibrosis rates increasing in recent years (5). Feray et al in a study of 79 patients with hepatitis C cirrhosis found that the progression to established hepatitis took place in 72% of patients by 4 years post transplantation (29). In another study, Boker et al found that 24% of patients had some fibrosis present in their graft biopsy and that 1 patient had progressed to cirrhosis in less than 2 years (30). Berenguer et al in a study of 284 patients transplanted with hepatitis C estimated the fibrosis progression rate to be 0.3 stages per year (cirrhosis
was stage 4) with a median time to cirrhosis of 10 years (5). They also found that the rate of fibrosis progression was increasing over the time period studied with time to cirrhosis falling from 9.8 – 13 years in 1990 – 1991 to 1.6 years in 1996. Fibrosis development can therefore progress much more rapidly in some patients. 2 further studies reported that some patients developed cirrhosis within 2 to 5 years (31). Feray et al in a multi-centre European study found that 10% of patients developed cirrhosis in 5 years (32). Berenguer et al found the proportion of patients developing cirrhosis at 5 years to be much higher than this at 44%. Wali et al found that median time to cirrhosis was 7.7 years but in a subset of patients with older donors, time to cirrhosis may be only 2.2 years (33).

Donor age has recently been recognised to be important in the course of graft disease after transplantation for hepatitis C cirrhosis. The study by Wali et al (33) estimated that with a donor aged under 40 years old fibrosis would progress at 0.6 stages per year (cirrhosis was stage 6) with a median time to cirrhosis of 10 years. If the donor was aged 50 or over, the progression rate was 2.7 stages per year with a median time to cirrhosis of 2.2 years. Berenguer et al confirmed the finding that increasing donor age was associated with more rapid progression to cirrhosis but did not estimate the rate for different donor ages (34). A third study concluded that donor age was the most significant predictor of graft failure in their series of 93 patients (35). For each decade increase in donor age, the relative risk of graft failure rose significantly.

Drugs aimed at reducing the viability of the hepatitis C virus such as interferon-α and ribavirin have been disappointing. It is now accepted that a combination of interferon-α and ribavirin is the treatment of choice for hepatitis C infection in native liver and several studies have been performed to see if viral clearance is
possible after liver transplantation. This combination of drugs has been shown to be effective in some studies, but the results are mixed. In a pilot study, Bizollon et al reported that 10 out of 21 patients (48%) cleared the hepatitis C virus and had a sustained response (36). In a subsequent study the same group reported on 54 patients treated with interferon-α and ribavirin in whom the sustained viral clearance rate was 26% (37). This was associated with lower transaminase levels and a reduced inflammatory score on biopsy. De Vera et al reported 32 patients who had been treated with interferon-α and ribavirin. Although they found that there was a good biochemical response to treatment as defined by a normalisation of serum transaminases, no patient had sustained viral clearance and biopsies at 1 year demonstrated no histological improvement. Saab et al treated 21 patients with ribavirin monotherapy (38). They found that there was a decrease in serum transaminase levels but that this did not correlate with an improvement in histology either in terms of inflammatory activity seen on biopsy or fibrosis progression. Nair et al treated 33 patients with interferon-α and ribavirin (39). They found that there was a trend towards improvement in the inflammatory score seen on biopsy, but that treatment had no effect on established fibrosis or fibrosis progression. Another study by Ahmad et al found that there was a biochemical improvement seen in patients treated either with interferon-α alone or with a combination of interferon-α and ribavirin but that fibrosis continued to progress despite this (40). Samuel et al performed a randomised controlled trial of treatment, treating 28 patients and having 24 controls (41). The patients had a 21% sustained viral response but no improvement in histology. 43% had to discontinue therapy because of severe side effects, primarily anaemia. Firpi et al found that 30% of 54 patients had a sustained
response to treatment and that those who did respond showed no progression of fibrosis (42).

Many studies have found that the combination of interferon-α and ribavirin is poorly tolerated (38, 39, 41, 43, 44) with 1 of these studies finding that 66% of patients needed dose modification or cessation of therapy (44).

The accelerated fibrosis seen after transplantation in patients with hepatitis C makes it a useful condition in which to study fibrosis, rather than studying fibrosis in patients with hepatitis C in native liver, where Poynard et al estimated that development of cirrhosis may take 30 years (17). Fibrosis in this group of 2235 patients progressed at a rate of 0.133 stages per year (cirrhosis was stage 4).

Transplantation for hepatitis B cirrhosis used to be hampered by graft hepatitis which could be rapidly fatal, but survival has improved with the administration of hepatitis B immunoglobulin (45) and the nucleoside analogue, lamivudine (46, 47). Current standard practice is to treat all hepatitis B positive patients with hepatitis B immunoglobulin for at least 2 years and lamivudine indefinitely. Administration of a new agent, adefovir dipivoxil has also been used with success in the treatment of resistant disease caused by the YMDD mutation. There are 2 case reports of patients who had developed graft failure while on treatment with lamivudine in whom rescue therapy with adefovir dipivoxil was successful (48, 49) As a result of this new drug, many centres are now stopping treatment with hepatitis B immunoglobulin after 2 years because there is no proven efficacy after this point, with a view to using adefovir in cases where the YMDD mutant develops.

Tacrolimus is used for immunosuppression at Addenbrooke’s hospital rather than cyclosporin because of in vitro experiments which have demonstrated an increase in
viral replication in cell lines infected with hepatitis B virus when cyclosporin was present, but no effect when tacrolimus was present (50).

Primary biliary cirrhosis was first reported to recur in the liver graft in 1982 (51). It has also been noted to occur more frequently in tacrolimus than cyclosporin based immunosuppression regimes (52) and occurs in a minority of patients in the short term. Recent evidence looking at 485 patients suggests that median time to recurrence is 123 months in patients taking cyclosporin and 62 months in patients taking tacrolimus. Time to recurrence is also lengthened from 93 to 120 months in patients taking azathioprine (53).

Primary sclerosing cholangitis is thought to recur in 6 – 20% of liver grafts (54-56). Because of a lack of clear diagnostic markers, however and the fact that most of these patients have a Roux loop biliary re-construction, it can be difficult to distinguish from other biliary problems related to transplantation such as graft ischaemia causing strictures. Re-transplantation in one series was 3.6% with follow up ranging from 1 – 19 years (57).

Autoimmune hepatitis can occur in the liver graft after transplantation (58, 59), with diagnostic features similar to disease arising de novo. It has been suggested that recurrence of autoimmune hepatitis can coincide with reduction of immunosuppression (58). In a multi-centre review of cases in Spain, all recurrences of autoimmune hepatitis in the liver graft occurred while the patient was taking only a calcineurin inhibitor with no cases in patients who were on 2 or more immunosuppressive drugs (60). As immunosuppression is usually reduced over time, this could be a confounding factor because the length of follow up in the recurrence group was significantly longer than in those patients in whom hepatitis had not recurred. However all patients responded to increased immunosuppression
leading the authors to recommend weaning immunosuppressive therapy with extreme caution. At Addenbrooke’s hospital, patients receiving a liver graft for this indication remain on long term azathioprine in addition to a calcineurin inhibitor to reduce the risk of disease recurrence.

**Fibrosis**

Fibrosis results from all liver disease, the end result being cirrhosis. As such, the measurement of fibrosis is important because its stage affects prognosis and management of all patients. Cirrhosis is particularly important because of the potential to develop liver failure, hepatocellular carcinoma and portal hypertension with its complications, especially variceal haemorrhage, which may be life threatening. It is therefore important to assess the stage of fibrosis in all patients presenting with chronic liver disease and to consider its reassessment periodically.

*Measurement of fibrosis*

Currently the only way to assess fibrosis is by histological assessment of a liver biopsy. A special stain (reticulin) is usually used and the stage assessed by one of the accepted scoring systems (61-63). This allows comparison between groups of patients with a condition by producing a numerical semi-quantitative score and also allows standardisation of management plans. Thus the treatment of newly diagnosed hepatitis C is dependent on the assessment of a liver biopsy and its fibrosis stage in addition to its histological grade and certain other factors such as genotype and co-existent pathology (12).

There are however some problems with this approach. The first is the risk associated with liver biopsy. In an audit of 1500 patients in British centres performing liver biopsy, mortality was found to be 0.13 – 0.33% (the authors were
not sure whether 3 deaths were caused by the liver biopsy) with significant bleeding seen in 1.7% and transfusion required in 0.7% (64).

The other problems are of inter- and intra-observer variation among pathologists and that of sampling error. Clearly a liver biopsy is small in relation to the size of the liver and the disease may be patchy, but due to the risks associated with liver biopsy, usually only 1 or 2 biopsies are taken at any one time. It is therefore possible to biopsy a part of the liver that is more or less severely affected by disease than the rest of the liver. These problems have been assessed for liver biopsies in patients with hepatitis C (65). In this study, 124 patients underwent simultaneous laparoscopic guided needle biopsy of the left and right lobes of the liver. 33% of patients had a difference in fibrosis of at least 1 stage between the left and right lobes. In 18 patients cirrhosis (Scheuer stage 4) was reported in 1 lobe while stage 3 fibrosis was reported in the other. In 3 patients a difference of 2 stages was reported between the lobes and in 10% a different stage was reported when the biopsies were re-assessed 3 months later by the same person.

Ideally a quantitative method to measure fibrosis in the whole of the liver would be used, but unfortunately no such method exists. No radiological method of imaging the liver is yet able to demonstrate the degree of fibrosis although there are promising early MRI data (66). As a result of this, research has been conducted into serum markers of fibrosis.

*Serum markers of fibrosis*

Research into serum markers of fibrosis aims to address the problem of sampling error and variation of histological interpretation amongst pathologists by providing a quantitative assessment of a marker which reflects the amount of fibrosis in the
whole liver. The process of liver fibrosis results in turnover of the liver matrix and the production of breakdown products and may alter the function of the remaining liver. All of these factors provide potential to measure markers which may reflect the stage of fibrosis which is present. They may also be able to address the question of whether fibrosis is ongoing and progressive or old and no longer progressing.

Although a set of markers which could predict the stage of fibrosis would be ideal, a set of markers which could reliably exclude cirrhosis would also be useful. This would identify the subset of patients in whom liver biopsy would have a higher diagnostic yield and those in whom it may not be necessary. This differentiation would be useful to identify those at risk of the complications of cirrhosis. One system of quantifying hepatic fibrosis is the Fibrotest. This was established in hepatitis C and was found to be a reliable alternative to liver biopsy (67, 68). The markers used are alpha2 macroglobulin, haptoglobin, gamma globulin, apolipoprotein A1, gamma glutamyltranspeptidase and total bilirubin.

Various trials have been conducted looking at the effectiveness of different markers at predicting the stage of fibrosis in different conditions, some looking at combinations of markers (67) and others looking at just 1 or 2 markers. In hepatitis C, serum hyaluronic acid level has been shown consistently to correlate well with fibrosis (69-71) and can exclude cirrhosis with 99% certainty or significant fibrosis with 93% certainty (72). The studies by Nimoya et al and Yamada et al (69, 71) looked at patients with hepatitis C being treated with interferon-α. Both groups found that the serum concentration of hyaluronic acid correlated with fibrosis before treatment and that the level fell in responders in whom fibrosis improved. The study by Wong et al looked only at a single time point and found that serum hyaluronic acid was 85% sensitive and 88% specific for predicting stage 4 or 5 fibrosis.
(cirrhosis was stage 5). Several studies have investigated the levels of matrix metalloprotease 2 (MMP-2) and tissue inhibitor of metalloprotease 1 (TIMP-1). Boeker et al (73) found that a raised level of MMP-2 in patients with hepatitis C was correlated only with cirrhosis and that TIMP-1 levels correlated well with amount of fibrosis. They concluded that a raised level of TIMP-1 suggested developing fibrosis and that if the level of MMP-2 was also raised, this suggested cirrhosis. Kasahara et al also found a positive correlation between the fibrosis score and levels of MMP-2 and TIMP-1 (74). They also found that the ratio of MMP-2 to TIMP-1 predicted response to interferon-α treatment. Murawaki et al looked only at serum MMP-2 levels. They found that it was markedly increased in cirrhosis (75). Walsh et al looked at MMP-2 and TIMP-1 levels in patients with hepatitis C and found that only the TIMP-1 level correlated with fibrosis (76). MMP-2 did not correlate with fibrosis but the authors did not state whether it was predictive of cirrhosis. No studies have however been done in liver transplant recipients.

Immunosuppressive agents

Immunosuppression is essential following organ transplantation to prevent the host immune system rejecting the organ. There are now many different drugs used including calcineurin inhibitors (cyclosporin A, tacrolimus), sirolimus, anti-proliferative drugs (azathioprine, mycophenolate mofetil), steroids and monoclonal antibodies.

Immunosuppression is usually started during the transplant operation and continued thereafter, usually for life. Because of the long duration over which the patient takes the drugs, any side effects are important and may only become apparent after
several years. The effect of the drug on the liver graft is also important, particularly now that patients survive longer.

*Calcineurin inhibitors*

Cyclosporin A was the first calcineurin inhibitor to become available in the late 1970s. In combination with improved operative and anaesthetic techniques it created a milestone in transplantation by allowing much improved graft and patient survival compared to previous treatments which had relied on a combination of prednisolone and azathioprine (77-79). It acts by binding cyclophilin, the resulting complex inhibiting the activity of calcineurin phosphatase. This in turn prevents dephosphorylation of nuclear factor of activated T cells (NF-AT) resulting in reduced production of IL-2 by T cells. IL-2 stimulates B-cells and natural killer (NK) cells to become activated, thus damaging the allograft.

Many studies have been done looking at how best to use this drug and for several years it was the mainstay of treatment for all solid organ transplants. Unfortunately there are some difficulties using cyclosporin because of the side effects which it is known to cause. There is currently some debate about how to monitor patients, with trials underway on monitoring the drug level at 2 hours post dose compared with the more standard pre dose trough level. The aim of this approach is to try and minimise the side effects, but different pharmacokinetics between patients are likely to cause problems because time from dosing to peak level is variable and not predictable.

The main side effects of cyclosporin are renal impairment, hypertension, diabetes mellitus, tremor, headache, hirsutism and gingival hyperplasia.

Tacrolimus, also a calcineurin inhibitor, was first used in the late 1980s and binds FK-binding protein 12 within the cell. This complex acts on the enzyme calcineurin
phosphatase, resulting in a similar mode of action to cyclosporin. The side effects are similar, causing renal impairment, hypertension, tremor and headache, although it does not cause hirsutism or gingival hyperplasia.

Tacrolimus was initially promoted as a rescue therapy for failed treatment with cyclosporin but is now often used as first line therapy. There have been several studies comparing the efficacy of the 2 drugs in liver transplantation. In a European multi-centre study of 545 patients randomised to either tacrolimus or cyclosporin, tacrolimus was found to be significantly better in reducing acute rejection, refractory acute rejection and chronic rejection (80). There was a trend to improved patient and graft survival although this was not significant. In a similar American study of 478 patients, there was a significantly lower rate of acute rejection and steroid resistant rejection in the tacrolimus group (81). Graft and patient survival at 1 year was comparable, with no significant difference. The tacrolimus group suffered from a higher rate of side effects and withdrawals from the study (14% vs. 5%), mainly due to neurotoxicity and nephrotoxicity. In a third, much smaller study of 64 patients randomised to receive either tacrolimus or cyclosporin, there were no significant differences between the groups for patient or graft survival or acute or chronic rejection (82).

A study examining the differences in blood pressure, cholesterol levels and weight after switching from cyclosporin to tacrolimus found that there was a benefit to all of these parameters after 11 months (83).

There seems, therefore, to be little difference between the two drugs in terms of graft and patient survival although rejection rates are lower with tacrolimus. Patients sometimes switch between the drugs because side effects vary from patient to patient with each drug.
**Mycophenolate mofetil**

Mycophenolate mofetil (MMF) is an ester pro-drug of mycophenolic acid, an anti-proliferative agent which predominantly affects lymphocyte proliferation. It inhibits the action of inosine monophosphate dehydrogenase in a non-competitive manner (this is the same enzyme which is competitively inhibited by ribavirin). It has been used predominantly as an add-on therapy with a calcineurin inhibitor to try and improve outcome or as a means of reducing calcineurin inhibitor dose in patients with renal dysfunction. It has also been used as monotherapy in patients with renal failure. A retrospective study comparing 2 immunosuppression protocols using tacrolimus with and without MMF in 130 patients found a lower rejection rate in the MMF group with similar graft and patient survival (84). A prospective randomised study of 97 patients comparing cyclosporin and MMF with tacrolimus and MMF reported good graft and patient survival in both study arms but a high overall infection rate of 48% (85). MMF was thought to be both safe and effective with both calcineurin inhibitors. Another randomised trial compared cyclosporin with either MMF or azathioprine (86). The 2 drugs were found to be similarly effective and safe with equivalent patient and graft survival although the MMF treated patients had lower rates of rejection in the first 6 months.

In a prospective non-randomised study of 22 patients with progressive renal dysfunction, Barkmann et al substituted MMF for the calcineurin inhibitor which the patients were taking (87). They documented an improvement in renal function in 17 of the patients but had to stop the MMF in 4 patients because of severe side effects. The same group reported a subsequent randomised controlled trial of substitution of calcineurin inhibitor with MMF monotherapy (88). They randomised 28 patients to calcineurin inhibitor withdrawal and replacement with MMF. In the
MMF group there was a significant improvement in renal function over the control group although there were 3 episodes of acute rejection when changing drugs. A second report of a pilot study from another group of calcineurin inhibitor dose reduction then withdrawal with MMF introduction, followed by a randomised trial of the same treatment did however sound a cautionary note (89). Calcineurin inhibitor dose reduction with MMF was found to be safe and effective but calcineurin inhibitor replacement with MMF resulted in rejection and graft loss in 3 of the first 9 patients. This was thought to be an unacceptably high risk and the study was stopped.

MMF has also been trialled for use in hepatitis C (85, 86) because of a possible antiviral effect since it is known to inhibit the same enzyme as ribavirin (although by a different method). In a randomised controlled trial involving 106 patients, MMF was added to tacrolimus and prednisolone therapy (90). 50 patients received MMF in addition to tacrolimus and prednisolone at standard doses. The outcome for patients was no different to the control group taking only tacrolimus and prednisolone in terms of graft and patient survival, viral recurrence (based on biochemical criteria) and histological recurrence on biopsies taken when the liver function tests were abnormal, although protocol biopsies were not done. These findings led the authors to suggest that any antiviral effect of MMF is weak and is overcome by the drug’s immunosuppressive effects. A second study by Smallwood et al. compared the response to interferon-α and ribavirin in patients taking tacrolimus and prednisolone with or without MMF (91). They found that there was no difference in outcome of patients taking MMF when compared to those taking only tacrolimus and prednisolone and that the MMF did not alter the response to
anti-viral treatment. The main side effects are bone marrow suppression, nausea, diarrhoea and low immunoglobulins (personal communication, Dr. G. Alexander).

**Azathioprine**

Azathioprine used to be the most commonly used immunosuppressant before the development of cyclosporin and works by inhibiting the differentiation and proliferation of B and T lymphocytes by interfering with RNA and DNA synthesis. Its main problem is dose limiting bone marrow toxicity (although this is rare at the doses used) and it has been largely superseded by the calcineurin inhibitors as the mainstay of immunosuppressive therapy post transplantation. It is however often used in the first few months after transplantation in addition to a calcineurin inhibitor and may improve outcome when used in combination therapy after transplantation for hepatitis C related cirrhosis (28). It is also used in the longer term in patients who have received a transplant for autoimmune hepatitis. These patients seem to need stronger immunosuppression than that provided by a calcineurin inhibitor alone and this is usually provided by the addition of azathioprine (58, 60).

**OKT3 and ATG**

OKT3 is an anti-CD3 murine monoclonal antibody which is directed against the CD3 complex expressed on mature T-cells. It has been used as primary immunosuppression following transplantation but is now generally used to treat steroid resistant rejection. It is associated with a poor outcome and severe recurrence of hepatitis if used in a patient with hepatitis C (24, 92). Rosen et al retrospectively analysed 19 patients who received OKT3 for steroid resistant rejection and compared them to 33 patients who received steroids for acute rejection.
but no OKT3 (92). They found that there was a shorter time to hepatitis C recurrence and a more severe hepatitis in those patients who received OKT3 and that these patients were more likely to develop cirrhosis during follow up.

There is no evidence to suggest that patients treated with OKT3 have a worse outcome for conditions other than hepatitis C as demonstrated by 2 studies using OKT3 as prophylaxis in the immediate post transplantation period. McDiarmid et al randomised patients to receive OKT3 and compared the outcome to patients receiving cyclosporin, prednisolone and azathioprine (93). There was a trend to worse graft and patient survival in the OKT3 group but this was not statistically significant. There was also no advantage to renal function in avoiding cyclosporin around the time of transplantation. Wall et al analysed the results of treating 199 consecutive patients with OKT3 (94). They found that this resulted in good graft and patient survival but there was no control group to compare with.

ATG is a polyclonal antibody to all human thymocytes but is generally thought to be much safer to use in steroid resistant acute rejection (personal communication, Dr. G. Alexander). This is used in preference to OKT3 at Addenbrooke’s hospital.

Clearly OKT3 and ATG have a role to play in patients in whom pulsed steroids cannot control acute rejection, but there appears to be no advantage to their routine use and they should be used with extreme caution in patients with hepatitis C.

_Corticosteroids_

Corticosteroids are potent anti-inflammatory drugs which act through intracellular receptors to regulate gene transcription. They were the first drugs used in combination with azathioprine to control rejection. Until the discovery of cyclosporin this combination provided the mainstay of treatment for the prevention
of rejection. The main problem with steroids is their numerous side effects including hypertension, diabetes mellitus, osteoporosis, adrenal suppression, cushingoid appearance, thin skin, bruising, proximal myopathy, weight gain and psychosis. Many of the side effects are the result of longer term use and they are therefore still useful drugs, particularly if they are used sparingly. They are therefore used as part of the initial immunosuppression after transplantation and in short courses of large doses to treat acute rejection. Specific problems with steroids after transplantation have been documented with hepatitis C. The rate of fibrosis progression in the graft has been shown to be faster with higher cumulative doses of steroids (5, 27) and there is some evidence that the rate of withdrawal of steroids may have an effect on the graft. Brillanti et al studied a retrospective cohort of patients with hepatitis C and found that those who were on higher doses of prednisolone at 12 months and were therefore having their dose tapered very slowly had less histological recurrence of hepatitis (95). This finding is controversial and contradicts the findings of other most studies which have demonstrated a link only to total steroid dose and treatment of acute rejection. The result could have arisen because the endpoints used are biochemical rather than histological (96, 97).

Corticosteroids also increase hepatitis B virus replication (98) but are safe in other liver conditions except for the well documented side effects from which any patient can suffer.

**Sirolimus**

Sirolimus is a macrolide immunosuppressant derived from *Streptomyces Hygroscopicus*. It was initially investigated as a chemotherapeutic agent and for its anti-fungal properties but caused too much immunosuppression to be clinically
useful. It was subsequently investigated as an immunosuppressive agent and was first used in liver transplantation in the late 1990s (99). It acts by binding FK-binding protein 12 which in turn binds the mammalian target of rapamycin (mTOR) protein (rather than binding calcineurin as tacrolimus does), blocking cytokine mediated and ligand binding mediated signal transduction, by inhibition of the p70 S6 kinase enzyme. It causes arrest at the G1/S phase of the cell cycle. It has no effect on calcineurin phosphatase activity and therefore has a different side effect profile to the calcineurin inhibitors (100-105). Known side effects include lymphocele development, impaired wound healing, abdominal and bony pains, diarrhoea, oedema, oral ulceration, anaemia and leucopenia. It has recently been licensed as monotherapy for use in renal transplantation but remains unlicensed for use after liver transplantation.

Sirolimus has a long terminal elimination half life of over 60 hours. It is therefore necessary to administer sirolimus only once daily, although if a loading dose is not used it may take 1 – 2 weeks to reach a steady state. It binds extensively to red blood cells and also other blood components, with less than 0.2% of the drug in whole blood being free (106). It is therefore assayed as a whole blood level because levels in plasma or serum would be too low to measure.

Sirolimus has been used increasingly at Addenbrooke’s hospital in several trials, some of which are currently underway. As a result of this increased experience with the drug, it has also been used off license when patients have been unable to tolerate calcineurin inhibitors due to toxicity, side effects or progressive renal impairment. Initially there were many side effects and the drug was poorly tolerated probably as a result of high doses being used, some patients having whole blood trough levels from 50 – 100 ng/ml. As experience of its use has increased it is being used at much
lower doses, most patients having trough levels of 5 – 10 ng/ml and is much better tolerated.

The first study done at Addenbrooke’s hospital using sirolimus for immune suppression after liver transplantation used 3 different regimens (99). Sirolimus was used initially with cyclosporin and prednisolone, then with cyclosporin alone and finally as monotherapy from the day of transplantation. All regimens aimed to achieve sirolimus monotherapy by 3 months post transplantation. 4 patients with hepatitis C were transplanted as part of the study and also 2 other patients who were not part of the study (personal communication, Dr. G. Alexander). Of these 6 patients it was noted that fibrosis in their liver biopsies did not progress as rapidly as expected when compared to contemporaneous controls. There were also major problems noted with wound healing when sirolimus was used in the immediate post-operative period, some patients taking several months for their wounds to heal and large incisional hernias persisting after wound closure. All of these factors suggest that sirolimus may exert an anti-fibrotic effect in addition to its immunosuppressive effect.

A study done on a rat model of hepatic fibrosis also suggested that sirolimus exerts an anti-fibrotic effect (107). Rats were administered carbon tetrachloride and it was found that when sirolimus was administered in addition to carbon tetrachloride hepatic fibrosis did not develop as it did in the control group. It was also found that sirolimus inhibited the proliferation of hepatic stellate cells stimulated by platelet derived growth factor in a cell culture model, leading the authors to suggest that this could be a possible mechanism of action. A previous study performed by the same group investigating the effect of tacrolimus on the development of liver fibrosis in
rats found that administration of tacrolimus with carbon tetrachloride caused an increase in the amount of fibrosis (108).

Two other studies performed in culture models have also found that sirolimus may have an anti-fibrotic effect. A study using a fibroblast cell culture model found that in the presence of platelet derived growth factor or basic fibroblast derived growth factor, fibroblast proliferation was reduced (109). A second study using a tissue slice growth model demonstrated that collagen deposition and fibrosis development in the slice of cultured liver tissue was reduced in a dose dependent fashion in the presence of sirolimus (110).

Various other studies have looked at the effect of sirolimus on cytokines in animal and cell models. It is known that sirolimus reduces vascular endothelial growth factor (vEGF) production in a mouse model (111) and that it has an effect on other cytokines such as interleukin 10 (IL-10), matrix metalloproteases (MMP) 2 and 9 and haem oxygenase-1 (112-114).

**Current practice at Addenbrooke’s hospital**

Immunosuppression following liver transplantation at Addenbrooke’s hospital usually comprises 3 types of drugs. Steroids (methyl prednisolone) are given both intra-operatively and post-operatively, with azathioprine and tacrolimus added post-operatively from day 2. These are given on a daily basis according to weight and trough drug level in the case of tacrolimus. The steroid dose is rapidly tapered so that all steroids should have stopped by 3 months post transplantation. The azathioprine is stopped 6 months post transplantation (except in the case of transplantation for autoimmune hepatitis) and the patients continue on tacrolimus monotherapy with a target trough dose range of 5 – 10 ng/ml.
Acute rejection is treated with methyl-prednisolone 500mg bolus intra-venous injections for 3 sequential days. This can be repeated if necessary before using specific antibodies such as ATG for biopsy proven recurrent acute rejection.

**Summary**

The improvements seen in survival following liver transplantation have made it a highly effective treatment for end stage liver disease of all aetiologies but recurrent disease presents an ongoing challenge, particularly for hepatitis C where recurrence is almost universal. Immunosuppression is probably needed for all patients after transplantation and should be tailored to the individual patient, with particular problems being noted for those with hepatitis C. The longer term effects of immunosuppression, particularly renal failure and the adverse effects of certain treatments on the liver graft, have become more important as survival improves and results are studied for 5 or 10 years rather than just 1 year post transplantation. Studying the outcome of liver grafts may become easier with the development of serum markers of liver fibrosis which might reduce the need for liver biopsy and increase the diagnostic yield where it is performed.
Hypothesis

- An immunosuppressive agent with possible anti fibrotic activity would improve outcome in patients with hepatitis C who are developing fibrosis.

- There may be other beneficial effects (such as improvement in renal function) or detrimental effects (such as increased incidence of sepsis).

Outline of thesis

During the period of research, the aim was to investigate transplant recipients with hepatitis C to see if switching their immunosuppression to a different agent affected the progression of fibrosis as assessed by liver biopsy. Blood was also taken for analysis of serum markers of fibrosis both prior to switching drugs and 3 months and 1 year later. Further data has been collected on the patients entered into the first published study on the use of sirolimus after liver transplantation to see if there were any long term side effects or beneficial effects (particularly renal function) which could be identified.

Some laboratory based projects were also undertaken to look at the effect of sirolimus on cell culture and tissue culture models of fibrosis. The effect on neutrophil function has also been studied because of the higher incidence of bacterial infections that have been seen with sirolimus use.
Chapter 2. Update on the 1999 Addenbrooke’s study

Introduction

The first study using sirolimus as the primary immunosuppressive drug after liver transplantation was published in 1999 (99). The study was commenced originally in transplant recipients with primary liver tumours who did not meet accepted criteria for liver transplantation as defined by Mazzaferro (115). Subsequently, patients were included who did meet such criteria and another group that did not have tumours, with a view to gaining further experience with this agent and to identify an effective and safe dose. The primary aim of that study was to be using sirolimus monotherapy within 3 months of transplantation and assess the outcome of these patients. The study was done because sirolimus was known to be an anti-proliferative drug and it was thought that it may have a beneficial effect on tumour outcome. It was the first study performed in humans on liver transplant recipients and at the time, no other published data was available on other solid organ transplant recipients. At the outset of the study initial immune suppression comprised sirolimus, cyclosporin and prednisolone; the latter 2 drugs were tailed off and withdrawn within 3 months. After early experiences some patients were then transplanted using sirolimus and cyclosporin without prednisolone and a third group using only sirolimus from the time of transplantation. The authors concluded that sirolimus was a useful immunosuppressive drug which may have a role to play after transplantation and that it was as effective as monotherapy with calcineurin inhibitors. They concluded that at the time of publication the follow up time was too short and the numbers too small to fully assess the anti-tumour properties of sirolimus.
They also encountered some unexpected problems using sirolimus. The main side effect published in the study was of major bacterial and opportunistic infections, particularly when using sirolimus in conjunction with cyclosporin and prednisolone. This group included 1 patient with *Pneumocystis carinii* pneumonia despite a normal lymphocyte count – an exceptional observation – who was being treated with both sirolimus and cyclosporin at the time. Sirolimus has been noted in subsequent studies to have an increased incidence of bacterial infectious complications when used at appropriate concentrations (100) with a trend for this seen in a second study (116). In contrast CMV infections have been noted to occur at lower rates in some studies (117-119).

Wound healing was also noted to be a problem with some abdominal wounds taking several months to heal although this was not reported in the published paper at the time (personal communication from Dr. Alexander). 6 of 27 patients subsequently needed repair of an incisional hernia and there were 4 others in whom their incisional hernia was deemed too large for surgery. Delayed wound healing has been reported subsequently (104) and other studies have noted a higher incidence of lymphocele when using sirolimus after renal transplantation (120-122) although no problems with wound healing were reported in one study which specifically addressed this question (123).

One further problem which has resulted in discontinuation of sirolimus in some patients and which was not reported at the time was the high incidence of mouth ulcers. This has been reported subsequently by another group (105) although they postulated that it could be the result of over-suppression of the immune system or the fact that the patients were not on steroids rather than a direct side effect of sirolimus.
No patient in the Cambridge series taking sirolimus developed hepatic artery thrombosis. This is a complication which has recently been associated in the United States with sirolimus use immediately after transplantation although American data are conflicting. A recent international study was stopped because of a higher incidence of hepatic artery thrombosis in patients taking sirolimus although no mechanism has been suggested (124). Other investigators have found no increase in this complication (117, 125).

It is possible that some or all of these side effects could be dose related. Sirolimus was given as a loading dose then at a fixed dose based on body surface area. The dose of sirolimus prescribed to those patients (2 – 4 mg/m²) was higher than we would now use and only a syrup was available which had variable absorption. Mean trough levels at 3, 6 and 12 months were 20, 20 and 19 ng/ml respectively with a range of 1.4 – 66.2 ng/ml at 6 months. Some interim levels were even higher than this. This resulted in extremely variable whole blood trough levels ranging from 1 ng/ml in a patient also on phenytoin to greater than 80 ng/ml in a patient also taking cyclosporin. It is now known that a drug interaction with cyclosporin increases sirolimus levels and that induction of the cytochrome P450 IIIA4 (CYP3A4) enzyme system reduces sirolimus levels. This compares to the current situation where therapy is monitored and adjusted according to whole blood trough levels, aiming for 5 – 10 ng/ml, although firm evidence that this is the most appropriate dose is lacking.

Further data on the patients included in this first study have now been collected and analysed to examine the longer term effects of sirolimus.
Methods

Survival

The long term outlook of patients who survived for more than 3 months after the operation has been examined with figures calculated for 5 year survival. This has been compared with survival of other patients transplanted in the years 1996 and 1997 for all indications.

Fibrosis rate

The policy at Addenbrooke’s hospital for patients with hepatitis C after transplantation is to biopsy the graft annually. Consequently there are biopsies for 6 patients dating back to the earliest transplant in the study series in mid 1997. These have been reviewed by a single pathologist and scored for fibrosis and activity on the Ishak scale (63). A subjective assessment of the adequacy of the biopsies was made at the time although the length of each biopsy and the number of portal tracts has not been recorded. Fibrosis rates have been calculated for the biopsies and compared to other patients transplanted for hepatitis C related cirrhosis treated with other immunosuppressive drugs (also reviewed by the same pathologist). Two different rates have been calculated. The first uses only the most recent biopsy and calculates the mean rate over the time since transplantation. The second rate is the rate calculated from all of the biopsies taken since transplantation. The rate of progression between annual biopsies has been calculated and the mean rate derived from this. This method of calculating fibrosis progression rates in hepatitis C was first reported in native liver using the METAVIR scoring system on a fibrosis scale of 0 – 4 (17). We have used the same method but used the Ishak scale for scoring both fibrosis and inflammation. Kaplan-Meier plots have been constructed for the
time to graft fibrosis stage 5 or 6 for each of the different drugs used for immunosuppression.

Renal function

Many patients treated with calcineurin inhibitors develop renal dysfunction over time (8) which may require dialysis or renal transplantation. In the published study (99), although the intention was that all patients should be on sirolimus monotherapy by 3 months post transplantation there were some patients who did not achieve this. The reasons were: didn’t like the taste (1), recurrent chest sepsis (1), acute rejection and bony pains (1), acute rejection (1) and no tumour found on explant (1). Of these there are follow up data over 5 years on the renal function of 5 patients. This has been compared to the patients who continued on sirolimus monotherapy from 3 months.

Since many patients were taking cyclosporin in the first 3 months after transplantation and the 2 drugs are synergistic in impairing renal function (126, 127), possibly due to a pharmacokinetic interaction which raises renal tissue levels of cyclosporin more than whole blood levels (128), the baseline for renal function has been taken as the creatinine at 6 months after transplantation. The change in renal function from baseline over time has been calculated for the 2 groups.

Some patients stopped sirolimus early on due to side effects and took a calcineurin inhibitor instead. These immediate side effects have been documented in other studies but there are no data on any long term side effects of patients remaining on sirolimus.

Because of the small size of the group of patients on tacrolimus a second control group was found. This was a contemporaneous group to avoid any effect seen by
taking patients from a different decade and should therefore control for any time
related factors. The control group was generated by taking the 2 patients
transplanted before and the 2 patients transplanted after the study patients, provided
they survived more than 1 year. Creatinine change over time was compared to a 6
month baseline in the same way as for the study group and compared to the patients
taking sirolimus.

_Tumour survival_

As part of a separate study, an analysis has recently been undertaken to examine the
effects of various factors on outcome predictors of survival and tumour recurrence
at Addenbrooke’s hospital in all patients undergoing liver transplantation for
hepatocellular carcinoma (Dr. Aileen Marshall). One of the variables included in the
analysis was the immunosuppression used in these patients. Univariate and
multivariate analyses were performed looking at tumour size, vascular invasion,
drugs used and some of the cell cycle markers.

Results

_Survival_

A flow diagram of the patients included in the trial is shown in figure 2.1.

Because of the extended indications for liver transplantation in these patients,
mortality was high. The study aimed to assess whether tumour recurrence was lower
in patients after transplantation and some patients were therefore transplanted as
part of the trial when they would not otherwise have met criteria for transplantation
in place at that time (115).
Thus, 3 patients with cholangiocarcinoma were included, although liver transplantation is contraindicated in this group except as part of a trial – a recent review of transplantation in patients with cholangiocarcinoma found that 5 year survival was 29% in European centres as a whole and was similar or worse in other single centre studies (129). In addition 3 patients with large hepatocellular carcinoma, which is thought to have a poor outcome (115, 130), were also included. A patient with an angiosarcoma of the liver on a background of HBV related cirrhosis was also included. Prior to liver transplantation the tumour was thought to be a large hepatocellular carcinoma based on radiology alone because the prothrombin time pre-operatively precluded biopsy. The diagnosis only became clear when the histology of the explant became available.
27 Patients entered into trial

No tumour

8 patients

Sirolimus

4 patients

4 patients

3 alive and well

1 died after re-transplantation for recurrent PSC after 4 years

3 alive and well

1 died after chronic rejection after 9 months

Tumour

15 patients

Sirolimus

4 patients

HCC

11 patients

4 patients:
3 cholangiocarcinoma (died at 9 months, 15 months and 3 years)
1 angiosarcoma (died at 6 months)

Other tumour

Vascular invasion

6 patients

Sirolimus

3 patients:
1 alive and well
1 died after 21 months from recurrent HCC
1 died at 4 years from other primary carcinoma

3 patients:
1 alive, severe renal failure
2 died from recurrent HCC at 9 and 11 months

Tacrolimus

11 patients

No vascular invasion

5 patients

Sirolimus

2 patients:
1 alive and well on sirolimus
1 alive and well after re-transplant and switch to tacrolimus

1 died at 6 months from graft versus host disease

Tacrolimus

3 patients:
2 alive and well
1 died at 6 months from recurrent HCC

Figure 2.1
Six of the 11 patients with hepatocellular carcinoma had vascular invasion seen in the tumour at explant. Only 2 of these 6 patients are now alive (33%) at 6 years. Three died between 8 months and 2 years from recurrent tumour and one patient died from a de novo metastatic adenocarcinoma (the primary was never found) after 4 years.

Four of the 5 patients without vascular invasion within the HCC at explant are alive (80%) after 7 years. 1 patient died from graft versus host disease after 6 months.

In total 27 patients (17 with tumours, 10 without) were entered into the trial although only 15 of these were reported in the paper. Survival at 1 year in the 15 patients reported was 67% (10 out of 15) and in the 27 patients entered into the trial was 67% (18 out of 27). Of these 27 patients, 16 continued on sirolimus either in the long term or until death. Sirolimus was discontinued in 11 patients for various reasons including didn’t like the taste (n = 1), acute rejection (n = 4), bacterial chest sepsis (n = 2), no tumour found in explant (n = 2), biliary anastamotic breakdown with wound infection and severe hyperlipidaemia (n = 1) and Pneumocystis carinii pneumonia (n = 1). The patient with Pneumocystis carinii pneumonia was not lymphopenic at the time of infection but was being treated with a combination of sirolimus and cyclosporin. The 1 year survival of the 20 patients who did fulfil the Mazzaferro transplant criteria in place at the time was 90%. One year survival of 77 patients undergoing liver transplant for more conventional indications at Addenbrooke’s hospital was 85% in 1996 and 88% in 1997 (78 patients transplanted).

At 5 years after transplantation 13 patients are alive (48%). Of these, 6 were still taking sirolimus as their primary immunosuppressive drug. 1 patient switched to tacrolimus while having a hernia repair then back to sirolimus and 1 further patient
had taken sirolimus for 3 years before being listed for re-transplantation for a biliary stricture with bacterial liver abscess formation, when he switched to tacrolimus. After 6½ years he developed a single lung metastasis (biopsy proven) which was very slow growing and has recently been resected. The histology was identical to the primary tumour. The other 7 patients switched to tacrolimus within 6 months of transplantation (see table 2.1). One of these patients, who discontinued sirolimus 10 weeks after transplantation, developed an adrenal metastasis which has been resected recently. The histology was identical to the primary tumour. Causes of death in patients who died are shown below in table 2.2. 5 year survival of the 20 patients who did fulfil the transplant criteria in place at the time was 65%. These figures compare to 5 year patient survival figures following liver transplantation for more conventional indications in Cambridge of 72% for the years 1996 and 1997 (chronic disease only, fulminant liver disease excluded).
<table>
<thead>
<tr>
<th>Age</th>
<th>Tx indication</th>
<th>Comments</th>
<th>Current drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>Wilson’s disease. HCC with vascular invasion</td>
<td>Subphrenic abscess. Recurrent chest sepsis. Sirolimus stopped at 10 weeks post transplantation</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>47</td>
<td>Alcoholic liver disease. HCC</td>
<td>Stopped sirolimus at 1 month. No tumour found in explant</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>52</td>
<td>HCV cirrhosis. 3 x 2.5cm HCC</td>
<td>Biliary anastamotic stricture, stented.</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>53</td>
<td>Hepatitis C cirrhosis. Multi-focal HCC (5 under 1cm, 2 over 1cm – 1.8 &amp; 2.2cm). Vascular invasion</td>
<td>Florid recurrent hepatitis at 1 year. Oedema improved after reducing dose of sirolimus</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>44</td>
<td>Hepatitis C cirrhosis. 1cm HCC well differentiated</td>
<td>Sirolimus stopped at 3 weeks – initial histology suggested no tumour. Patient unwilling to re-start when definitive histology available</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>58</td>
<td>HCV cirrhosis</td>
<td>Post operative sepsis and fit. Stopped cyclosporin at 2 weeks. Anaemia</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>Age</td>
<td>Tx indication</td>
<td>Comments</td>
<td>Current drug</td>
</tr>
<tr>
<td>-----</td>
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<td>--------------------------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>49</td>
<td>Alcoholic cirrhosis. Thrombosed portal vein</td>
<td>Stopped sirolimus at 2 weeks. Didn’t like taste</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>31</td>
<td>Gaucher’s disease. Hepatopulmonary syndrome</td>
<td>Incisional hernia – repaired</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>56</td>
<td>PSC</td>
<td>Incisional hernia</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>59</td>
<td>PBC</td>
<td>Raised creatinine 2 – 3 months, resolved on stopping cyclosporin. Incisional hernia repair at 3 years. Converted to tacrolimus for this then back to sirolimus</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>62</td>
<td>PBC</td>
<td>Acute rejection day 7 and at 5 months. Sirolimus discontinued</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>62</td>
<td>PBC</td>
<td>Acute rejection at 5 months. Converted to tacrolimus. Incisional hernia repair at 2 years</td>
<td>Tacrolimus</td>
</tr>
</tbody>
</table>

_Table 2.1. Patients surviving at 5 years after transplantation. Age is age at transplantation._
## Table 2.2. Causes of death

<table>
<thead>
<tr>
<th>Age</th>
<th>Tx indication</th>
<th>Cause of death</th>
<th>Time</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>68</td>
<td>HBV cirrhosis. 3 x 2.5cm hepatomas</td>
<td>Fatty donor liver - poor initial function. Staphylococcal pneumonia</td>
<td>Day 6</td>
<td>Sirolimus,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cyclosporin</td>
</tr>
<tr>
<td>42</td>
<td>HCV cirrhosis. 5cm hepatoma</td>
<td>Sepsis and recurrent tumour</td>
<td>Day 227</td>
<td>Cyclosporin</td>
</tr>
<tr>
<td>67</td>
<td>CAH. 4cm hepatoma</td>
<td>Adenocarcinoma, unknown primary</td>
<td>4 years 10 months</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>60</td>
<td>Alcoholic cirrhosis. 2.5cm hepatoma</td>
<td>GvHD</td>
<td>Day 191</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>64</td>
<td>Alcoholic cirrhosis. 7cm hepatoma</td>
<td>Recurrent tumour</td>
<td>1 year 10 months</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>53</td>
<td>Cryptogenic cirrhosis. 8cm hepatoma</td>
<td>Recurrent tumour</td>
<td>Day 332</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>45</td>
<td>PSC. Cholangiocarcinoma</td>
<td>Recurrent tumour</td>
<td>Day 257</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>34</td>
<td>PSC. Cholangiocarcinoma</td>
<td>Recurrent tumour</td>
<td>1 year 4 months</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>38</td>
<td>Glycogen storage disease. Cholangiocarcinoma</td>
<td>Recurrent tumour</td>
<td>3 years 9 months</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>36</td>
<td>Large HCC. Thought to be neuro-endocrine</td>
<td>Hepatic artery thrombosis, sepsis, multi organ failure</td>
<td>Day 30</td>
<td>Sirolimus,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cyclosporin</td>
</tr>
<tr>
<td>54</td>
<td>Angiosarcoma of liver (thought to be HCC pre-operatively)</td>
<td>Recurrent tumour</td>
<td>Day 189</td>
<td>Cyclosporin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>initially then</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sirolimus</td>
</tr>
<tr>
<td>Age</td>
<td>Tx indication</td>
<td>Cause of death</td>
<td>Time</td>
<td>Drug</td>
</tr>
<tr>
<td>-----</td>
<td>------------------------</td>
<td>----------------------------------------------------------</td>
<td>-------</td>
<td>-----------------</td>
</tr>
<tr>
<td>56</td>
<td>Alcoholic cirrhosis</td>
<td>Pneumonia (paralysed right hemi diaphragm)</td>
<td>Day 95</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>55</td>
<td>Alcoholic cirrhosis</td>
<td>Chronic rejection</td>
<td>Day 236</td>
<td>Sirolimus to day 111. Tacrolimus</td>
</tr>
<tr>
<td>54</td>
<td>Alcoholic cirrhosis, COPD</td>
<td>Sepsis (pneumonia, biliary anastamotic breakdown)</td>
<td>Day 83</td>
<td>Sirolimus</td>
</tr>
</tbody>
</table>

Table 2.2. *Causes of death in patients entered into the trial. Age is age at transplantation. Time is time from transplantation to death.*

**Effect of sirolimus on survival of patients with HCC**

Use of sirolimus was found to have no effect on survival in the univariate analysis looking at all patients transplanted with hepatocellular carcinoma (p=0.12).

**The effect of sirolimus on hepatitis C as assessed by annual biopsies**

During and after the study period, 7 patients with hepatitis C were commenced on sirolimus as their primary immune suppression. One of these has always refused to have a graft biopsy. There are therefore biopsies for 6 patients dating back to the earliest transplant in the study series in mid 1997. Immune suppression in these patients has been comparable to other patients except for the use of sirolimus. Patients received intravenous steroids, azathioprine and calcineurin inhibitor post-operatively and switched to oral drugs as soon as possible after transplantation. The steroids were reduced and stopped after 4 months and azathioprine after 6 months. Calcineurin inhibitor was titrated according to trough drug level.
Data are presented in table 2.3 for these biopsy results from the most recent biopsy and in table 2.4 and figure 2.2 for all biopsies combined.

**Table 2.3.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fibrosis</th>
<th>Donor age</th>
<th>Age at transplant</th>
<th>Steroid</th>
<th>Grade</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin median</td>
<td>1.25</td>
<td>50</td>
<td>53</td>
<td>1480</td>
<td>5.00</td>
<td>18</td>
</tr>
<tr>
<td>Tacrolimus median</td>
<td>1.00</td>
<td>46</td>
<td>52</td>
<td>1219</td>
<td>4.50</td>
<td>33</td>
</tr>
<tr>
<td>Sirolimus median</td>
<td>0.82</td>
<td>52</td>
<td>51</td>
<td>1308</td>
<td>7.50</td>
<td>6</td>
</tr>
</tbody>
</table>

*Table 2.3. Medians are presented for each group, calculated from the most recent biopsy. Fibrosis is the median fibrosis rate in stages per year (Ishak scale) for each group of patients. The fibrosis rate was calculated for each patient as the fibrosis stage at time of biopsy divided by the time between transplantation and biopsy in years, giving a mean fibrosis rate in stages per year. Cirrhosis is stage 6 on the Ishak scale. The differences between the groups are not statistically significant. Steroid is median of the cumulative steroid dose in milligrams given to the patients in each group in the first 4 months and includes treatment for acute rejection. Grade is the median grade of activity seen (Ishak scale) in the most recent biopsy for each group. Number is the number of patients in each group. The differences between the groups are not statistically significant.*
Table 2.4.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fibrosis</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin mean</td>
<td>1.05</td>
<td>37</td>
</tr>
<tr>
<td>Cyclosporin median</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Tacrolimus mean</td>
<td>0.88</td>
<td>51</td>
</tr>
<tr>
<td>Tacrolimus median</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Sirolimus mean</td>
<td>-0.11</td>
<td>12</td>
</tr>
<tr>
<td>Sirolimus median</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.4. Means and medians are presented for each group, calculated from all biopsies available. Fibrosis is the mean or median fibrosis rate between biopsies calculated from patients who had more than 1 biopsy. Number is the number of patients with more than 1 biopsy. The differences between the groups are not statistically significant but the numbers are small.*
Figure 2.2. Distribution of fibrosis rates between paired biopsies for patients on each of the different immunosuppressive drugs used.

To investigate further whether there might be a link between the rate of progression of fibrosis and the immunosuppression used, Kaplan-Meier plots were constructed for the time to fibrosis stage 5 or 6. These are presented in Figure 2.3 with the statistics below.
Figure 2.3. Kaplan-Meier plot of time to stage 5 or 6 fibrosis with table for test statistics. Number of cases is the number of patients in each group. Number of events is the number of patients who developed stage 5 or 6 fibrosis. Log rank tests demonstrated that the differences between the groups are not statistically significant.

There is a difference in mean time to fibrosis stage 5 or 6 however this difference is not significant which may be due to the small number of events in each of the
groups (there are either 1 or 6 events in a group). However, there is also a difference in time to biopsy between the groups which is almost significant (Figure 2.4). This may be causing the difference in mean time to fibrosis stage 5 or 6, so this combined with the small number of events mean that the results need interpreting with care.
Figure 2.4.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of cases</th>
<th>Number of events</th>
<th>Mean time to biopsy (95% CI)</th>
<th>Kruskal-Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin</td>
<td>18</td>
<td>18</td>
<td>2.9 (0.0, 6.6)</td>
<td>$\chi^2 = 5.30, \text{df}=2, p=0.071$</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>33</td>
<td>33</td>
<td>1.7 (1.2, 2.3)</td>
<td></td>
</tr>
<tr>
<td>Sirolimus</td>
<td>6</td>
<td>6</td>
<td>2.9 (1.6, 4.3)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.4. Kaplan-Meier plot of time to biopsy for each group of patients with table for test statistics. Number of cases is the number of patients. Number of events is the number of biopsies. Mean time to biopsy is the time between transplantation and graft biopsy.

There is almost a significant difference between the times to biopsy because of a change in prescribing policy over time. Cyclosporin used to be the drug of choice after liver transplantation and was the main drug being used at the time of the trial.
which used sirolimus. Tacrolimus became the drug of choice after several trials demonstrated a benefit over cyclosporin, with lower rates of acute, refractory acute and chronic rejection (80). This difference is therefore to be expected but it is not possible to determine whether, or to what degree it confounds the first Kaplan-Meier plot relating to the rate of fibrosis.

The difference seen between the fibrosis rates calculated from the raw data could therefore have arisen by chance and further studies will need to be performed to ascertain whether a real difference exists.
The effect of sirolimus on renal function

The median age of patients in the sirolimus group was 57 and in the group taking a calcineurin inhibitor was 49. Change in renal function from the 6 month baseline is shown below in Figure 2.5.

Figure 2.5.

![Graph showing median creatinine differences from 6 months post-transplantation.](image)

**Figure 2.5. Median creatinine difference in sirolimus and tacrolimus treated patients from the 6 months post-transplant creatinine.** Sirolimus treated patients, n = 10 at year 1, n = 5 at year 5. Tacrolimus treated patients, n = 5 at year 1, n = 3 at year 5

Because the means and medians are different the data have been analysed using non-parametric tests. The distribution of the data can be seen in figure 2.6 (the columns labelled sirolimus and tacrolimus).

There was no significant change from baseline in the creatinine of the group of patients taking sirolimus at 3 and 5 years (p = 1.00, sign test). There is also no change from baseline and 3 years (p=0.50, sign test) and baseline and 5 years
(p=0.25, sign test) in the patients taking tacrolimus, although this may be due to the very small numbers (n = 5 at 3 years and n = 3 at 5 years).

There is a significant difference between the medians of the 2 groups only at 2 years from transplantation (p = 0.021, median test). The distribution of creatinine in the 2 groups differs at 2 and 3 years (Mann-Whitney U test). 1 patient taking tacrolimus was on haemodialysis by 5 years after transplantation due to renal failure secondary to biopsy proven calcineurin inhibitor toxicity.

Clearly in this small group of patients there has been a clinically significant long term advantage to renal function of taking sirolimus rather than tacrolimus. There is statistical significance only at 2 and 3 years, but this could be due to the very small size of the groups, particularly the group taking tacrolimus.

A second control group was therefore found (see methods). 33 controls were obtained with follow up data for at least 1 year, some having been lost to follow up during this time. 24 of these patients had follow-up data available at 5 years post transplantation (see figure 2.6). Median age was 49.

In this control group, creatinine levels at 3 and 5 years were compared to the 6 month baseline level. At 3 years the levels were significantly higher (p=0.001, sign test) and also at 5 years (p=0.035).
Figure 2.6. The creatinine level for patients in each group over time. Tacrolimus is the group of patients in the study who could not tolerate sirolimus. Control is the control group of 33 patients generated from taking the 2 patients transplanted before and the 2 patients transplanted after the patients in the sirolimus group. Sirolimus is the group of patients in the study who continued taking sirolimus in the long term.

The median creatinine levels between the sirolimus and the calcineurin inhibitor group were compared with the median test (this test compares the median figures of 2 groups but does not take into account the distribution of the data). It was found that there was no significant difference between the medians of the groups at any time point. The distribution of creatinine values was then compared using the Mann-Whitney U test. This showed that the distribution differed significantly between the sirolimus group and the control group at 1 year ($p = 0.049$) and at 2
years (p = 0.05). There was borderline significance at 3 years (p = 0.057) but this was lost at 4 years (p = 0.12) and 5 years (p = 0.64).

Interpreting this data suggests that the creatinine did not rise for the majority of patients in the control group (so the median did not change significantly) but that a minority of patients did have a rise in creatinine (so the distribution of values changed). The change of creatinine concentration over time is significant at 2 and 3 years, despite small patient numbers in the group of patients taking sirolimus.

**Long term side effects**

Of the 10 patients remaining on sirolimus, 1 patient had to switch to a calcineurin inhibitor after the initial transplant period when he was re-listed for transplantation because of chronic abscess formation secondary to multiple intra-hepatic biliary strictures. He re-started sirolimus a few months after the second transplant operation and developed multiple liver abscesses a second time. He then switched back to tacrolimus and has had no further problems. There were no other major adverse events related to the immunosuppression which required switching to a calcineurin inhibitor. No patients developed chronic rejection while on sirolimus and after the initial period around the time of the operation only the patient described above has been admitted to hospital with a systemic infection. Of the patients treated only with sirolimus from the time of transplantation, 3 out of 4 (75%) developed acute rejection. This compares to 4 out of 7 (57%) who were treated initially with a calcineurin inhibitor and 4 out of 11 (36%) of those treated with a combination of sirolimus and a calcineurin inhibitor. None of the 5 patients treated with prednisolone, sirolimus and a calcineurin inhibitor developed acute rejection. 1 patient also had persistent peripheral oedema which improved on lowering the dose.
of sirolimus. Only 1 of the patients takes a statin for a raised cholesterol level and also anti-hypertensive drugs. The hypertension has responded better to standard treatment while being immunosuppressed with sirolimus than when on a calcineurin inhibitor. The other 4 patients had normal cholesterol, triglycerides and blood pressure at 5 years.

**Current doses and side effects**

The way in which sirolimus is now used at Addenbrooke’s hospital has changed since the original study was performed. Because of the problems encountered with wound healing it is not now used until the operation site has fully healed. This is usually after a minimum of 1 month post transplantation. Tacrolimus can then be switched to sirolimus at any stage after this if indicated by the patient’s clinical condition. Sirolimus is started at 1 or 2 mg per day without a loading does and the tacrolimus stopped either the day before starting sirolimus or after a brief overlap period if prevention of acute rejection is imperative (usually in a patient with hepatitis C). Whole blood sirolimus trough levels are assayed weekly aiming for a level between 5 – 10 ng/ml.

The side effects seen using this method are usually mild but include mouth ulcers, skin rashes and oedema. The mouth ulcers usually settle after a few weeks although sometimes dose reduction is necessary. Occasionally patients stop sirolimus due to these side effects being particularly troublesome, however this is unusual. Generally the side effects are much milder than seen when using the drug at high doses in the original study.
Summary

- The first study reported on sirolimus use after liver transplantation found it to be a safe and effective immunosuppressive drug.
- There was a high early mortality, probably due to the conditions for which patients were offered transplantation, such as cholangiocarcinoma.
- Survival of patients with HCC was no better in patients taking sirolimus.
- Survival of patients who did fulfil transplant criteria in place at the time was similar to patients who were not entered into the trial.
- There was no statistically significant difference in fibrosis progression rate in patients with hepatitis C was for any immunosuppressive agent.
- Renal function was significantly better 3 years after transplantation compared with a contemporaneous control group.

Discussion

The original study on which these further follow up data are based was reported as a pilot study to assess the safety and efficacy of sirolimus after liver transplantation and also to evaluate its effects in patients with primary liver tumours undergoing transplantation. As a pilot study, the follow up was only short in some of the patients, but longer in the initial patients enrolled as recruitment was deliberately slow. There was a high early mortality in the patients, mainly because of the indications for transplantation and the fact that many of the patients would not normally have been eligible for transplantation for reasons such as the presence of a cholangiocarcinoma or a large HCC. There was also a learning curve using the drug, with 3 different regimes being described. The paper concluded that further trials
were needed to assess the long term effects on chronic rejection and tumour growth compared to other immunosuppressive agents.

Survival after transplantation for hepatic tumours is currently a problem. The presence of cholangiocarcinoma is a contra-indication to liver transplantation outside a clinical trial setting because of the inevitable recurrence of the tumour and poor outcome. The patients transplanted in this trial with cholangiocarcinoma have all died and although there were only 3 patients with this condition, there is no suggestion that sirolimus may be beneficial. Transplantation for HCC is also a problem and is currently only contemplated if there is a single tumour of less than 5 cm diameter or multiple tumours of which the largest is less than 3 cm diameter on radiological imaging. The results for HCC demonstrate that sirolimus is no better at preventing disease recurrence in this small series than any other immunosuppression. One group, however is currently using sirolimus as primary immunosuppression after transplantation for larger tumours because of its known anti-proliferative effects due to causing cell cycle arrest in G1 phase (131).

Another drug which is an ester of sirolimus is also in use clinically as an anti-tumour agent. CCI-779 is a chemotherapeutic agent which is being trialled for breast carcinoma and renal cell carcinoma, particularly where these are resistant to other treatments. Claims have also been made that it may be useful in treating several other cancers (such as cervical, ovarian, prostate, lung, head and neck, pancreatic, colon, lymphoma and melanoma) (132, 133). It is, however, used at much higher doses, either up to 20 mg per day or 140 mg once per week for the maximum anti-tumour effect.

The effect of sirolimus on hepatitis C is an issue that is of major importance. Currently there is nothing that can be done to prevent fibrosis progression from
hepatitis C after transplantation, so any agent which is shown to slow fibrosis progression will be of major importance to this group of patients. The patients described here who have hepatitis C and were treated with sirolimus have generally done very well after transplantation. Of the 6 patients switched, none have developed cirrhosis in their latest biopsy, with follow up being more than 5 years in some patients. Looking at the fibrosis rates shown in figure 2.1, although there is no significant difference between the groups, it can be seen that there are no patients taking sirolimus with very rapidly progressive fibrosis as there are in the patients taking a calcineurin inhibitor. The median rate is no different but the distribution of rates is different, suggesting that some patients may have benefited. No other patients at Addenbrooke’s hospital have developed cirrhosis whilst on sirolimus as their primary immunosuppression (personal communication Dr. G. Alexander). Although the results of fibrosis rates calculated from protocol biopsies shown above are not significantly different, it is possible, therefore, that sirolimus does slow the rate of fibrosis progression caused by hepatitis C.

The main limitation of the data in this study is the small numbers of patients who have been taking sirolimus. In the Kaplan-Meier analysis, the confounding factor of time to biopsy for the different immunosuppressive drugs is a problem which has been created historically. Cyclosporin was the main immunosuppressive drug used after liver transplantation for many years and was in use at the time of the study to examine the effects of sirolimus. Around the time of the study, tacrolimus was starting to be used in some patients and following the study, tacrolimus became the drug of choice as the results of several large multi-centre studies were published, showing a better long term outlook with tacrolimus. It is not possible to tell how much this difference in time to biopsy confounds the other results, making them
very difficult to interpret, particularly because the development of fibrosis assessed with a numerical scoring system is probably not linear.

The other concern raised by this study is the tolerability of the drug. 11 out the 27 patients switched to sirolimus were unable to tolerate it for various reasons. These patients switched to a calcineurin inhibitor early after commencing sirolimus. It is possible that if they had persevered with the drug the side effects may have settled. The availability of a tablet form of sirolimus is also beneficial, particularly as 1 patient discontinued it because of the taste of the liquid preparation of the drug. Clearly the number of patients discontinuing the drug is high, but other series also quote significant numbers of patients discontinuing sirolimus due to side effects (134, 135).

The effect of using sirolimus instead of a calcineurin inhibitor for immunosuppression clearly has an important effect on renal function. The main side effect of the calcineurin inhibitors is renal impairment and any intervention which may improve this will be important. The initial analysis of renal function looking only at the 2 groups of patients entered into the study showed a significant difference at 2 years, but as the groups became very small due to patient death and being lost to follow up, no significant difference was seen between the groups at 4 or 5 years, however 1 patient taking tacrolimus was dialysis dependent after 5 years. When the second control group was examined (controls transplanted around the same time as the patients) the median creatinine level rose significantly at 3 and 5 years but did not change between the groups. Examining the graph however, it can be seen that the distribution of the creatinine levels is different between the groups. The statistics also show that when distribution is examined, there is a significant difference between the patients taking sirolimus and those taking a calcineurin
inhibitor. This suggests that there is a small group of patients who will benefit from treatment with sirolimus rather than a calcineurin inhibitor.

There is always a concern about the long term effects of drugs which have only been studied over short periods and the data on the patients who have taken sirolimus for 5 years is therefore important. There were very few long term problems in the patients taking sirolimus apart from the one patient who had raised lipid levels which are being treated successfully with a statin. His hypertension has also responded well to treatment with standard anti-hypertensive agents, rather than being resistant to treatment as is sometimes the case in patients taking a calcineurin inhibitor. The peripheral oedema which was attributed to sirolimus improved with dose reduction.

Although the mortality in the study was high, particularly in the first year, this was attributable to the conditions for which the patients were transplanted. Cholangiocarcinoma is known to have a very high mortality from tumour recurrence in all patients and patients with a large HCC are not transplanted for the same reason. If the 7 patients transplanted for these reasons are excluded, 13 out of 20 (65%) were alive at 5 years (of whom 6 are on sirolimus), a figure comparable to the 72% survival seen in other patients transplanted in the years 1997 and 1998.

In this small group of patients, therefore, sirolimus at a long term trough level of 5–15 ng/ml has been a safe alternative to calcineurin inhibitor immunosuppression.
Chapter 3. The effect on renal function of switching to sirolimus

Introduction

Liver transplantation with calcineurin inhibitor immunosuppression has been associated with renal failure (defined by a creatinine greater than 125 μmol/l) in up to 83% of patients at 3 years (136). Estimates of the prevalence of end stage renal failure vary from 3.4 and 5% at 10 years (8, 137) up to 9.4% at 13 years (138). Renal replacement therapy for end stage renal failure after liver transplantation has a mortality of up to 44% (8). Since virtually all patients are now maintained on calcineurin inhibitor immunosuppression there will be a significant number of patients in any liver transplant centre who are developing renal failure, some of whom will require renal replacement therapy and consideration for renal transplantation.

Renal impairment is thought to be caused in the short term by cyclosporin causing vasoconstriction of the renal afferent arteriole and withdrawal of the drug usually leads to a rapid rise in renal plasma flow. There are several possible candidates for mediating this vasoconstriction including endothelin, thromboxane A₂, reduction of vasodilator prostaglandins, activation of renal sympathetic nerves, angiotensin II, platelet derived growth factor and reduced nitric oxide production. It is not known which of these is responsible. The chronic lesion of striped interstitial fibrosis develops over time, but the mechanism is not known.

The only treatment for calcineurin inhibitor toxicity is withdrawal of the calcineurin inhibitor, which often results in an improvement in creatinine but may precipitate graft rejection. A calcineurin inhibitor is therefore usually substituted for an
alternative drug such as azathioprine, prednisolone or mycophenolate mofetil or often the alternative calcineurin inhibitor. As sirolimus has been used increasingly at Addenbrooke’s hospital, patients who have been having troublesome side effects on a calcineurin inhibitor have had their drug substituted with sirolimus. This course of management was based on the clinical impression seen in patients who were on sirolimus as part of a clinical trial that their renal function was generally better than those on a calcineurin inhibitor and that no patients appeared to be developing end stage renal failure. There were also no data in the published literature suggesting that sirolimus may be nephrotoxic.

The diagnosis of calcineurin inhibitor toxicity is made definitively by renal biopsy however a pragmatic approach has been taken in these patients. All patients had been reviewed regularly in the out patient clinic and their renal function optimised by reduction of calcineurin inhibitor dose, control of blood pressure and diabetes and withdrawal of drugs which are toxic to the kidneys (particularly NSAIDs). Underlying calcineurin inhibitor toxicity has therefore been assumed rather than biopsy proven.

Rising creatinine level was used as the marker for renal dysfunction. The reason for this is that it was thought to be the most reliable and reproducible index. Alternatives would be the creatinine clearance (requiring 24 hour urine collection which is intrinsically unreliable), glomerular filtration rate (GFR) measurement (which is time consuming and expensive) and a calculated creatinine clearance using a method such as the Cockroft-Gault equation or MDRD formula (used in the study of Modification of Diet in Renal Disease). These methods take into account the weight and age of the patients and are thought to give a reasonable estimate of GFR however a steadily rising creatinine was thought to be a sufficiently reliable indicator of renal dysfunction to warrant intervention. A calculated GFR may give a more accurate idea
of the degree of improvement of renal dysfunction but is unlikely to alter the management of the patients in any way.

Method

All the notes of patients who had their immunosuppression switched to sirolimus between 1999 and 2001 were examined. The reason for changing drugs was noted and the serum creatinine concentration prior to changing drugs and at 12 and 24 months later was documented. Patients were included if there was follow up of at least 1 year. Analysis of the patients who changed to sirolimus because of renal impairment was undertaken.

For all patients switching to sirolimus, systolic blood pressure, uric acid levels, cholesterol and triglycerides were measured and compared with pre-switch levels.

In addition, all patients who had had creatinine clearance measured before changing drugs had this repeated after 1 year.

4 patients were also switched prospectively to sirolimus during the period of switching patients with hepatitis C and fibrosis described in chapter 4. Although there is a randomised trial for these patients in the hospital, it was not felt appropriate to randomise some of the patients with renal failure for various reasons.

Results

Patients switched to sirolimus between 1999 and 2001

24 patients had their immunosuppression switched from a calcineurin inhibitor to sirolimus between 1999 and 2001 at 6 – 83 months post-transplantation. 17 of these were switched because of nephrotoxicity. Other indications included calcineurin inhibitor neurotoxicity, liver fibrosis with recurrence of hepatitis C and hepatocellular
carcinoma with vascular invasion in the explanted liver. Of these 24 patients, 7 discontinued sirolimus because of side effects (rash n = 2; pruritus n = 1; lethargy n = 1; mouth ulcers n = 2; granulomatous pneumonitis n = 1) and 1 died from multi organ failure. In total 16 patients continued sirolimus for more than 1 year of whom 13 were changed to sirolimus because of renal impairment, median age 62, range 48 – 71.

Of the 13 patients who switched drugs to sirolimus for renal impairment, the creatinine of 8 patients had fallen at 1 year and 9 patients at 2 years. The median change in creatinine was -29 μmol/l (inter-quartile range -38, 12) at 1 year and -26 μmol/l (inter-quartile range -42, -2) at 2 years. At 1 year the creatinine had risen in 5 patients and at 2 years in 3 patients (1 patient had died) (see figure 3.1).

Creatinine clearance was measured in 8 patients prior to change of drugs. This was repeated in all 8 patients 1 year later. The creatinine clearance rose in 4 of the 8 patients and fell in the other 4. This did not correlate with the patients in whom creatinine fell or rose and was attributed to unreliable sample collection by the patients.
Figure 3.1. Creatinine concentrations before switching and 2 years later in patients with renal failure who switched from a calcineurin inhibitor to sirolimus.

The changes in blood pressure and other blood results are listed in table 3.1 below. 8 patients were already being treated for hypertension before switching to sirolimus. One of these was able to stop treatment 18 months after switching. 2 additional patients had to start anti-hypertensive treatment (one at 4 months and one at 8 months) after switching. 4 patients were being treated with allopurinol because of a raised uric acid level before switching to sirolimus and no new patients had to be treated after switching. 5 patients were being treated with a statin before switching to sirolimus and 6 further patients had to start a statin because of raised cholesterol, all in the first 6 months after switching.
Table 3.1. Changes in median values with inter-quartile ranges (IQR) for systolic blood pressure (mmHg), uric acid (mmol/l), cholesterol (mmol/l), triglyceride (mmol/l) and creatinine (μmol/l). Positive numbers denote a rise, negative numbers a fall. All changes are not significant because of the small number of patients.

Patients switched between 2001 and 2003

4 patients had their immunosuppression changed from a calcineurin inhibitor to sirolimus because of renal impairment. At the time of switching to sirolimus, creatinine levels ranged from 143 to 354. By 1 year after switching, 3 of these levels had fallen (see table 3.2). One patient had had a nuclear medicine glomerular filtration rate measured at 35 ml/min before switching and this had risen 1 year later to 61 ml/min. The second patient had a creatinine clearance of 30.8 ml/min which had risen to 36.5 ml/min. The third patient had several attempts at a 24 hour urine collection but was unable to return a completed collection. The fourth patient had cystic fibrosis and poorly controlled insulin dependent diabetes mellitus. His creatinine was rising rapidly despite a tacrolimus level that was below the limit of detection and he was switched to sirolimus as a last resort. Unfortunately despite this, he commenced
dialysis within 4 weeks and is currently being considered for renal transplantation. No patient developed acute rejection.

**Table 3.2.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Creatinine pre-switch (µmol/l)</th>
<th>Creatinine 1 year later (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>143</td>
<td>87</td>
</tr>
<tr>
<td>Patient 2</td>
<td>216</td>
<td>186</td>
</tr>
<tr>
<td>Patient 3</td>
<td>220</td>
<td>184</td>
</tr>
<tr>
<td>Patient 4</td>
<td>354</td>
<td>Dialysis</td>
</tr>
</tbody>
</table>

*Table 3.2. Creatinine levels pre-switch and 1 year later in the 4 patients who switched to sirolimus because of renal impairment.*

**Summary**

- Most patients who switched from a calcineurin inhibitor to sirolimus because of impaired renal function did not have further deterioration of renal function.
- There was a high rate of discontinuation of sirolimus because of drug side effects.
- In patients who tolerated sirolimus there were no long term adverse effects.
- There was a trend to fall in systolic blood pressure 2 years after switching.
- The renal function of 3 of the 4 patients switched during my time in Cambridge improved.
Discussion

In this small group of patients with renal impairment the progressive deterioration of renal function appears to have stopped in all but 1 patient. It is therefore very likely that the underlying cause of renal failure was indeed calcineurin inhibitor toxicity although this was not biopsy proven. There is no control group because all patients with deteriorating renal function had their immunosuppression changed and their calcineurin inhibitor stopped. No patients at the time were maintained on their calcineurin inhibitor because of the almost inevitable decline in renal function which was expected if they were to do this. It is not therefore possible in this cohort of patients to compare the renal function in those who changed drugs to a group who did not.

Not all of the patients improved however. 1 patient continued to have progressive deterioration of renal function and 5 patients had worse renal function at 1 year but this did not appear to be progressive over the second year of follow up. 4 patients had worse renal function at year 2 although the rise in creatinine was small.

This pattern of some patients responding to withdrawal of calcineurin inhibitor and replacement with sirolimus has been noted in other studies (139, 140). It is not clear why some patients do not respond to the withdrawal of calcineurin inhibitor but it seems that cases of worse renal function may be less likely to respond to treatment (135). As can be seen from figure 3.1, this is not always the case in these patients. 2 patients started with very similar creatinine levels over 200 µmol/l. The renal function of one patient improved while the other deteriorated.

Blood pressures in the patients switched to sirolimus were generally a little better after 2 years although 2 patients did have to start anti-hypertensive drugs at some
point after switching. 1 patient was also able to stop her anti-hypertensive drugs after switching due to a sustained fall in blood pressure over the following 18 months. The general fall in blood pressure at 2 years after switching may be because blood pressure is more responsive to pre-existing treatment which the patient is already taking when they are no longer on a calcineurin inhibitor.

3 of the 4 patients who were switched between 2001 and 2003 had an improvement in renal function soon after changing drugs. The greatest improvement was seen in the patient with the least impaired renal function (creatinine 143 μmol/l). The renal function in this patient returned to normal. The other 2 patients who had an improvement both improved by a very similar amount (creatinine falling from about 220 μmol/l to 185 μmol/l). The fourth patient had severe renal failure prior to switching drugs, an unrecordable tacrolimus level (<2 ng/ml) casting doubt on whether he was actually taking any immunosuppression, and poorly controlled insulin dependent diabetes mellitus. With this combination of problems we would not expect an improvement in renal function after switching to sirolimus but it was thought to be worth trying as it was the only way in which he might have avoided dialysis.

The number of patients studied here is small and there is no control group, so the findings should be interpreted with caution. It seems likely that the renal function of these patients would have continued to deteriorate if they had not switched to sirolimus but further studies are necessary to prove that changing immunosuppression improves renal outcome. This should take the form of a prospective randomised controlled trial of switching to sirolimus or continuing on a calcineurin inhibitor, using as the primary outcome a nuclear medicine glomerular filtration rate to measure renal function, because this is known to be more accurate than creatinine clearance in
worsening renal failure (141). A trial of this design is currently underway at Addenbrooke’s hospital.
Chapter 4. The effect of switching to sirolimus on fibrosis progression in hepatitis C

Introduction

Graft fibrosis following liver transplantation for hepatitis C virus related cirrhosis is a major problem with almost all grafts becoming infected (22). There is clear evidence of accelerated rate of fibrosis compared with pre-transplantation (29, 31-33). In an internal audit at Addenbrooke’s hospital it was found that up to 20% of patients progressed to cirrhosis or liver failure within 1 year of transplantation for this condition (personal communication Dr. G. Alexander). Another internal audit looking at paired biopsies of patients with hepatitis C and any amount of fibrosis on the first of these biopsies found that fibrosis progressed from the first biopsy to the next biopsy in 18 out of 24 patients (75%). It was unchanged in 5 (21%) and improved in only 1 (4%). Other studies have demonstrated similar high rates of progressive fibrosis due to graft infection with HCV (29, 30) with an estimated median time to cirrhosis of 10 years (5). Donor age (33, 34) and steroid doses (27) have been shown to have an adverse effect upon outcome but it is not clear whether any of the long term immunosuppressive agents are superior in delaying graft fibrosis. A recent randomised trial comparing cyclosporin with tacrolimus for initial immunosuppression in the first year after transplantation found no difference between the 2 drugs assessed by liver biopsy (142). Drugs aimed at reducing the viability of the hepatitis C virus such as interferon-α and ribavirin have been disappointing (see chapter 1) and are not used routinely.
Following the study using sirolimus at Addenbrooke’s hospital which was published in 1999 (99) it was noted that none of the patients with hepatitis C appeared to be progressing towards cirrhosis and that fibrosis in the annual biopsies was less than expected although the number of patients was small (personal communication Dr. G. Alexander). Some patients also had problems with their surgical wounds healing up, with this taking several months on occasions, suggesting a possible anti-fibrotic effect of sirolimus. Thus it was hypothesised that immunosuppression with sirolimus may be having a beneficial effect on hepatitis C virus infected liver grafts in terms of fibrosis progression. A benefit would be defined as slowing, stopping or even reversing the fibrotic process. Therefore if the stage of fibrosis became stable and did not change, this would clearly be to the advantage of any patient who previously had progressive fibrosis.

Sirolimus has previously been demonstrated to have an anti-fibrotic effect in a rat model of fibrosis (107), a tissue culture model of fibrosis (110) and in human fibroblasts cultured in vitro (109) (see chapter 1). It is therefore possible that using sirolimus in patients with graft fibrosis after liver transplantation may be beneficial and may reduce or even prevent fibrosis progression although there is an uncertainty that doses for man which are well tolerated would be clinically effective.

Various serum markers of fibrosis have been associated with liver fibrosis in native liver, but not in liver transplant recipients. Hyaluronic acid, matrix metalloprotease 2 (MMP-2) and tissue inhibitor of metalloprotease 1 (TIMP-1) have all been associated with fibrosis of varying degrees or cirrhosis (69) (see section on serum markers of fibrosis in introduction chapter) (70-76). These markers were measured in patients who were included in the study.
All cells divide by mitosis and pass through the various phases of the cell cycle (G1, S, G2 and M) before returning to the resting phase (G0). If a cell undergoes senescence and stops dividing it expresses the protein p21. This protein has been associated with cell senescence on biopsy specimens, but not assayed in serum. Because it is expressed when cells undergo senescence which may in turn stimulate fibrosis, p21 may also be a serum marker of fibrosis. The p21 protein has been measured in the serum of these patients to see whether it correlates with liver fibrosis and could therefore be used as a serum marker of fibrosis.

Quality of life is an important issue for patients undergoing a major procedure such as organ transplantation and has been assessed in many different populations using various different tools. One of the most commonly used tools is the short form 36 (SF-36) questionnaire. This can be filled in by the patient and takes about 10 minutes. It has also previously been used and validated in liver transplant recipients (143-145) and can be used to monitor changes in quality of life over time. There are 8 different modalities derived from the questions which assess different aspects of wellbeing. These include physical and mental wellbeing. The change in score can be measured over time and should reflect a person’s general health and wellbeing split into the different areas measured. The 8 scores are physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotional and mental health. The physical functioning score reflects limitations in performing all physical activities from bathing and dressing to vigorous sporting activities. Role physical reflects problems associated with work or other daily activities as a result of physical health. Bodily pain relates to pain experienced, general health to the perception of the person’s general health, vitality to how tired or full of energy the person feels and social functioning to the extent to which physical and emotional problems affect
normal social activities. The last 2 scores relate to mental wellbeing. Role emotional reflects problems with work or other daily activities resulting from emotional problems and the mental health score relates to how nervous and depressed or happy and calm a person feels.

**Study hypothesis**

We hypothesised that changing immunosuppression from monotherapy with a calcineurin inhibitor to monotherapy with sirolimus in patients with hepatitis C virus infection and progressive fibrosis in their graft would result in a reduced rate of fibrosis progression or stop or even reverse the progression of fibrosis. This may also be reflected in serum markers of fibrosis.

In summary, the rationale on which this hypothesis was based was:

1. The internal audit by Dr. G. Alexander which found a low rate of progression of fibrosis in the small number of patients with hepatitis C treated with sirolimus compared to those treated with a calcineurin inhibitor.

2. The paper published by Zhu et al (107) which showed an anti-fibrotic effect of sirolimus in a rat model.

3. Work done in Southampton by Dr. Alexander’s group in conjunction with Dr. N. Sheron’s group which showed inhibition of stellate cell proliferation and fibrosis by sirolimus in a tissue culture model with a dose response relationship (110).

4. Clinical observations made in transplant recipients who were treated with sirolimus that wounds did not heal up properly, especially if patients had high blood levels of sirolimus.
The question to be answered was whether using sirolimus at doses which patients could tolerate would have any impact on the development of fibrosis. The issue of dosing is particularly important because the original study used doses that were not well tolerated by patients and were often 10 times higher than current trough levels in use today. The in vitro studies also demonstrated a greater effect at higher doses.

Ethical approval was given by the local research ethics committee for a prospective pilot study to investigate this hypothesis.

There are also follow up data on 2 further patients who were switched to sirolimus before the start of this research project.

**Method**

The best way to investigate the hypothesis is a randomised controlled trial. Unfortunately there is not enough evidence to support a randomised controlled trial so an open label pilot study was planned comparing the outcome of patients with a group of historical controls with a view to planning a randomised controlled trial using the new data if the pilot study is successful.

All patients attending the liver transplant clinic with hepatitis C virus infection were reviewed to see if their latest biopsy had significant graft fibrosis. A policy of performing an annual biopsy on all patients with hepatitis C infection has been in force for several years, so most patients have recent biopsy data available. At the start of the study, there were some patients who had been waiting for the study to start to switch drugs but apart from these patients, biopsies were reviewed prospectively soon after any protocol biopsy. Any patient with progressive fibrosis of stage 2 or greater on the Ishak scale (63) was offered the opportunity to change their primary immunosuppression from monotherapy with a calcineurin inhibitor to monotherapy.
with sirolimus. Because of the undefined risk of acute rejection when changing drugs and the detrimental effect that treatment with corticosteroids would have had on a hepatitis C virus infected liver graft, the 2 immunosuppressive drugs were overlapped for up to 2 weeks in an attempt to reduce the risk of acute rejection. If any patients were to be changed over to sirolimus and develop acute rejection, they would need treating with large doses of steroids or other immune modulating drugs (the standard first line treatment for acute rejection is 1.5 g methyl prednisolone over 3 days and for steroid resistant rejection, ATG, both of which are well known to be detrimental to a hepatitis C infected graft). If some patients were being treated for acute rejection as part of the study, it would also make interpretation of the results extremely difficult. Sirolimus was therefore started at a dose of 1 mg daily in addition to the calcineurin inhibitor which was stopped after 2 weeks or sooner if the sirolimus level at 1 week was in the target therapeutic range. There is no known drug interaction of sirolimus with tacrolimus and the sirolimus dose was taken with the morning tacrolimus dose in a patient on tacrolimus. There is however an interaction with cyclosporin which alters the uptake and metabolism of sirolimus (causing a small rise in sirolimus levels), so a patient taking cyclosporin had to take the sirolimus 4 hours after the morning cyclosporin dose. When cyclosporin was stopped, there was often a small fall in the sirolimus level in the following week.

Blood was taken for full blood count, urea, electrolytes, creatinine, uric acid, cholesterol and triglycerides before addition of sirolimus, 2 weeks later, after 3 months and at 1 year. Blood was also taken at these intervals for storage at -80°C and later analysis of serum markers of fibrosis. Sirolimus levels were measured at 1, 2 and 4 weeks and at regular intervals thereafter. Because of the long drug terminal elimination half life (62 hours) weekly monitoring was considered to be sufficient,
and dosing is necessary only once daily. Samples were collected for analysis of epidermal growth factor (EGF) in blood and saliva and vascular endothelial growth factor (vEGF) in blood (see chapters 5 and 6). A 24 hour urine sample was collected for measurement of creatinine clearance before switch to sirolimus and 1 year later.

Liver biopsy was repeated annually as per unit protocol for patients transplanted with hepatitis C. The liver biopsies taken before and 1 year after entry into the study were compared side by side by 1 pathologist who was blinded to whose biopsies were being examined and which biopsy was taken first. The pathologist compared the amount of fibrosis seen and the overall grade of inflammation and whether these looked worse in either biopsy. This was necessary because the scoring system does not differentiate between small differences in the degree of fibrosis or inflammation. For example, the staging system is based on fibrous expansion of portal tracts and fibrous bridges forming between them, but takes no account of the thickness or age of the bridges formed, which may change with time. A fibrous bridge may look active or may appear to be an old scar. Blood was collected for measurement of fibrosis markers at the time of the biopsy.

Due to recruitment being slower than expected there were also some biopsies available at the end of the study from the patients who switched early on which were taken 2 years after switching to sirolimus. These have also been compared to the biopsies taken before switching drugs.

Quality of life was assessed before switch to sirolimus by a SF-36 quality of life questionnaire and again after 1 year of treatment with sirolimus. Scores were calculated for the different modalities and normalised to give a z score which was then converted to a t score with a mean of 50 (146).
**Inclusion Criteria**

All patients with hepatitis C virus graft infection with fibrosis stage 2 or greater on the Ishak scale were considered for inclusion in the study. If fibrosis appeared progressive then they were invited to take part. If fibrosis appeared to be stable for more than 2 years, they were not included in the study. This was to prevent inclusion of patients whose fibrosis had clearly been stable for some time, but any patient whose fibrosis was progressing 1 year after a transplant was included.

Some other patients with severe acute hepatitis (defined as jaundice with biopsy proven acute hepatitis) soon after transplantation also had their immunosuppression switched to sirolimus. This included 4 patients who had a liver biopsy soon after transplantation (3 – 6 months) for persistently abnormal liver function tests. If there was a severe hepatitis C recurrence, which has previously been noted to be a precursor for the rapid development of fibrosis (147), these patients were also offered the chance to switch immunosuppression to sirolimus. The aim was to slow the rate of fibrosis forming as a result of the severe inflammation seen in the liver.

During the trial, a further group of patients was identified who might benefit from treatment with sirolimus. This was patients undergoing re-transplantation for hepatitis C related graft failure from recurrent cirrhosis, a condition known to have poor outcome (148, 149). The policy in force at Addenbrooke’s hospital has been that patients with graft cirrhosis would not be re-transplanted because of a poor outcome in the past, many of them dying within the first year after re-transplantation. It was thought that these patients may do better if immunosuppressed with sirolimus rather than a calcineurin inhibitor because the sirolimus may prevent or slow the development of fibrosis. Any patients who may need consideration for re-
transplantation were considered for this procedure with a switch to sirolimus when the surgical wound had fully healed.

Any patients who needed to switch drugs for other reasons were monitored so that renal data and fibrosis markers could be collected, but no additional biopsies were done on these patients to assess fibrosis progression.

**Exclusion Criteria**

Patients were excluded from the study if it was thought that sirolimus treatment may cause a clinical problem. This included patients with graft failure, those who were felt unlikely to comply with follow up, patients with a prothrombin time >18, patients who had already changed their immunosuppression in the last 3 months, any patients with acute rejection in the last 3 months, patients with a wound which was not fully healed, other planned surgery, pregnancy or planned pregnancy or patients already in another study.

**Fibrosis markers**

Hyaluronic acid, MMP-2 and total TIMP-1 (free and bound) were measured before each patient was switched to sirolimus and again at 12 months or at 3 months where 12 month follow up samples were not available. The levels pre switch and at 12 months have been correlated with the liver biopsies that were taken at that time. Hyaluronic acid was measured with an ELISA protein binding assay (Corgenix), MMP-2 with an ELISA assay (R & D systems) and TIMP-1 by an in house ELISA assay. p21 was also measured by a novel in house ELISA developed by Claire Newman and Andrew Trull.
Liver biopsies from before and after the switch to sirolimus were scored by the Ishak system by 1 consultant pathologist. All biopsies were then compared in pairs (before and after switch to sirolimus) by the same pathologist who was blinded to patient identity and which biopsy was taken first.

Results

Patients switched for hepatitis C virus related graft fibrosis after 1 year

On 30 September 2004, a total of 32 patients had been switched to sirolimus. Of these, 21 had been switched at least 1 year before for fibrosis due to hepatitis C virus recurrence with progressive fibrosis and therefore had 1 year follow up data except for 2 patients who had limited information available and did not return for their repeat biopsies (these were Italian patients who live in Italy but had their liver transplants in Cambridge). The 19 patients for whom complete data were available were therefore analysed. 6 of these patients also had biopsies available 2 years after switching.

To ensure that a group of patients had not been missed when including patients in the trial the results of all biopsies performed in this period were re-examined. After the patients with hepatitis C had their biopsies re-scored (see chapter 2), all patients with stage 2 fibrosis or above on their most recent biopsy had their notes reviewed to see if they should have been switched to sirolimus under the study inclusion criteria. There were 27 further patients with stage 2 or greater fibrosis. Of these, 8 had died and 6 had been lost to follow up in Italy. 2 patients did not attend for their appointments or for protocol liver biopsy on repeated occasions, 3 had fibrosis which was stable at stage 2 or 3 (there were 5 biopsies in 1 patient and 3 in another with fibrosis at stage 2 and 3 biopsies in the patient at stage 3), 3 patients already had graft failure (1 was re-
transplanted and died, 1 was re-transplanted and switched to sirolimus under the new protocol described above and the third did not want re-transplantation), 1 patient had a lymphoma and trial of a new drug was not thought to be appropriate, 1 had already tried sirolimus and discontinued it because of a persistent mouth ulcer and 1 had biliary strictures needing an operation for Roux loop construction (his fibrosis was also thought to be due to steatohepatitis rather than hepatitis C because it had a rather different pattern). 2 patients were reported incorrectly on their initial report as having no significant fibrosis on their biopsies and were not therefore identified at the time of biopsy reporting. Out of a total of 47 patients with hepatitis C who were assessed for inclusion in the study, 21 out of a possible 23 were included. Thus only 2 patients were missed for inclusion into the study.

Of the 19 patients whose biopsies from 1 year after switching to sirolimus were available, the median age at switching was 57 (range 38 – 70). 2 had had second liver transplants at 8 and 9 months (1 for hepatic artery thrombosis, the other for biliary necrosis) and 2 had hepatocellular carcinomas in the explanted liver (2.5cm and 3cm). Median time from transplantation to switching to sirolimus was 4.6 years (range 1 – 15.8 years). Median donor age was 49 (range 14 – 70). On review of the biopsies, the pathologist reported that 5 (26%) patients had a similar amount of fibrosis and that the biopsies 1 year apart were no different. 7 (37%) patients had got worse and 7 (37%) had improved. 12 out of 19 patients (63%) had not therefore progressed over 1 year. Looking at the other components of the Ishak score, only 1 patient had a change of greater than 1 point in the lobular inflammation score (0 – 4) (where it fell by 2 points) and there was no change in the median score for the group as a whole (see table 4.1). Steatosis score (0 – 3) changed by more than 1 point (where it fell by 2 points) for only 1 patient (this is an additional component which was not part of the
original Ishak score but may be important to fibrosis progression) and there was no change in the median score for the group. Median fibrosis score fell by 1 point after 1 year.

Table 4.1.

<table>
<thead>
<tr>
<th></th>
<th>Pre-switch</th>
<th>After 1 year of sirolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lobular</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Portal</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Steatosis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4.1. Median lobular inflammation, portal inflammation, overall grade, steatosis and fibrosis before switch to sirolimus and 1 year later. There were no significant differences in the medians before and after switching.

These figures have been compared to a historical control group of patients with hepatitis C infection post transplantation using the control biopsies described in chapter 2. In patients with more than 2 biopsies, the most recent 2 biopsies were blinded as a pair and the pathologist ranked them as best and worst. In patients who had switched to sirolimus, the 2 most recent biopsies prior to switching were also used. There were 35 pairs of biopsies of whom 31 patients were taking monotherapy with a calcineurin inhibitor for immunosuppression. Of these biopsies from 31 patients, the baseline level of fibrosis was lower in the control group, \( p = 0.05 \) (Mann-Whitney U test). The fibrosis stage of 16 (51.6%) got worse from one biopsy to the
next, the stage of 13 (48.4%) did not change and 2 (6.5%) improved. The grade of 16 (51.6%) got worse, 10 (32.3%) did not change and 5 (16.1%) got better. There was a significant difference in the distribution of change in fibrosis between the group of patients changed to sirolimus and the historical controls (See Figure 4.1, Chi-square test $\chi^2 = 7.40$, df = 2, $p = 0.025$). There was no significant difference seen in inflammatory grade between the 2 groups (Fisher's Exact Test $\text{FI}(x) = 4.26$, $p = 0.12$).

**Figure 4.1**

![Figure 4.1. Graph showing % distribution of change in fibrosis taken from 2 biopsies 1 year apart. Cases are those patients treated with sirolimus, controls are historical controls. The numbers on the bars are the absolute numbers of patients.](image)

Serum creatinine in this group of patients fell by a median amount of 10µmol/l over the year. Median creatinine clearance fell by 7 ml/min (despite the small fall in serum creatinine). Only 8 patients managed to complete 2 creatinine clearance tests satisfactorily 1 year apart and in some patients there were concerns about the completeness of urine collection.
In the patients who switched to sirolimus there were changes in some of the blood parameters measured (see table 4.2).
Table 4.2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Change at 3 months</th>
<th>IQR at 3 months</th>
<th>Change at 12 months</th>
<th>IQR at 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>-0.7</td>
<td>-4.6, 1.7</td>
<td>-1.6</td>
<td>-6.1, 1.1</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>5</td>
<td>-13, 12</td>
<td>6</td>
<td>-9, 19</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>-7</td>
<td>-13, 9</td>
<td>-10</td>
<td>-22, -6</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>-1</td>
<td>-3, 2</td>
<td>-4**</td>
<td>-6, 0</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>-4**</td>
<td>-8, 1</td>
<td>-7**</td>
<td>-13, -3</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>6</td>
<td>-20, 56</td>
<td>-2</td>
<td>-26, 31</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>8</td>
<td>-26, 50</td>
<td>-5</td>
<td>-29, 28</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>-0.3</td>
<td>-1.2, 0.5</td>
<td>-0.5</td>
<td>-1.6, 0.9</td>
</tr>
<tr>
<td>Total white cell count (x 10^9/l)</td>
<td>-0.7**</td>
<td>-1.4, 0.1</td>
<td>-0.1</td>
<td>-1.2, 0.3</td>
</tr>
<tr>
<td>Lymphocyte count (x 10^9/l)</td>
<td>-0.2</td>
<td>-0.5, 0.1</td>
<td>-0.2</td>
<td>-0.4, 0.1</td>
</tr>
<tr>
<td>Neutrophil count (x 10^9/l)</td>
<td>-0.4</td>
<td>-0.9, 0.3</td>
<td>-0.2</td>
<td>-0.7, 0.2</td>
</tr>
<tr>
<td>Platelets (x 10^9/l)</td>
<td>-13</td>
<td>-39, 17</td>
<td>-15</td>
<td>-36, 3</td>
</tr>
<tr>
<td>Prothrombin time (seconds)</td>
<td>-0.7*</td>
<td>-1.3, 0.2</td>
<td>-0.3</td>
<td>-1.1, 0.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>0.9**</td>
<td>0.6, 1.6</td>
<td>0.7**</td>
<td>0.3, 1.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.3</td>
<td>0.1, 1.1</td>
<td>0.3</td>
<td>0.1, 1.1</td>
</tr>
<tr>
<td>Uric acid (mmol/l)</td>
<td>-0.06**</td>
<td>-0.11, -0.02</td>
<td>-0.09**</td>
<td>-0.11, 0.01</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>-7</td>
<td>-14, 24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 4.2. Median changes in weight, systolic blood pressure and blood parameters at 3 and 12 months with units of measurement and inter-quartile ranges (IQR) for patients who switched to sirolimus for hepatitis C related fibrosis. A negative number denotes a fall. * significant at 0.05 level, ** significant at 0.01 level*
Only one of the patients with 1 year follow up had acute rejection, which was detected 2 months after switching to sirolimus. It which was treated with intra-venous methyl prednisolone 1.5g over 3 days. None developed chronic rejection or hepatic artery thrombosis.

There was no significant difference in donor age between the patients whose fibrosis progressed and those whose did not. The median age for those whose stage improved was 45, those whose stage did not change, 39 and those who got worse, 51.

Median sirolimus level at 3 months was 6.8 ng/ml (range 3.0 – 16.1) and at 1 year was 6.0 ng/ml (range 2.6 – 13.7). The patient with the lowest level at 1 year had reduced the dose of sirolimus because he complained of mouth ulcers while living in Italy. When next reviewed in Cambridge he had 1 very small ulcer but it was thought that this could be the result of dental problems. There was no difference in the sirolimus levels at 1 year in patients whose biopsies got better or worse or did not change (p = 0.87, median test).

The median peak ALT level in the first 12 months after switching was significantly higher in those patients whose fibrosis got worse than in those whose did not change (Mann-Whitney U test, p = 0.018) but there was no difference in inflammatory grade (p = 0.279). There was no significant difference in the median peak ALT of those whose fibrosis did not change and those whose got better (p = 0.43). There were insufficient patients and samples for multinomial regression to see if peak ALT predicts how fibrosis will change.

Quality of life scores are available for 11 patients. These generally showed a slight improvement although some patients experienced a deterioration of their health over
the year studied. Median differences in the t scores for each modality are shown in table 4.3.

Table 4.3.

<table>
<thead>
<tr>
<th></th>
<th>Median rise</th>
<th>Inter quartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical functioning</td>
<td>2.8</td>
<td>0, 8.3</td>
</tr>
<tr>
<td>Role physical</td>
<td>0</td>
<td>0, 16.7</td>
</tr>
<tr>
<td>Bodily pain</td>
<td>0</td>
<td>0, 10.2</td>
</tr>
<tr>
<td>General health</td>
<td>0</td>
<td>-5.0, 5.0</td>
</tr>
<tr>
<td>Vitality</td>
<td>2.5</td>
<td>-5.1, 5.1</td>
</tr>
<tr>
<td>Social functioning</td>
<td>0</td>
<td>-5.7, 5.7</td>
</tr>
<tr>
<td>Role emotional</td>
<td>0</td>
<td>0, 10.5</td>
</tr>
<tr>
<td>Mental health</td>
<td>2.3</td>
<td>-4.6, 4.6</td>
</tr>
</tbody>
</table>

Table 4.3. Median rise in the t scores from the different modalities of the SF-36 with inter-quartile ranges. A negative figure denotes a fall in t score. p = NS

Fibrosis markers measured were hyaluronic acid, MMP-2 and TIMP-1. Each of these markers was measured before and after switching to sirolimus. Patients with 1 year follow up had levels assayed 12 months after switching and patients with less than 1 year follow up had levels assayed 3 months after switching. Results are shown in tables 4.4 – 4.6. Patients switched for all indications are included.
<table>
<thead>
<tr>
<th></th>
<th>HA pre switch</th>
<th>HA 3 months</th>
<th>HA 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median</strong></td>
<td>61</td>
<td>647</td>
<td>361</td>
</tr>
<tr>
<td><strong>Inter-quartile range</strong></td>
<td>28, 313</td>
<td>215, 1878</td>
<td>103, 407</td>
</tr>
<tr>
<td><strong>Number</strong></td>
<td>29</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 4.4. Hyaluronic acid levels (ng/ml) pre switch to sirolimus, 12 months later in patients who had 12 months follow up and 3 months later in patients who had 3 months follow up but not 12 months follow up. See text for correlation coefficients.

<table>
<thead>
<tr>
<th></th>
<th>MMP-2 pre switch</th>
<th>MMP-2 3 months</th>
<th>MMP-2 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median</strong></td>
<td>268</td>
<td>332</td>
<td>307</td>
</tr>
<tr>
<td><strong>Inter-quartile range</strong></td>
<td>246, 308</td>
<td>299, 359</td>
<td>291, 348</td>
</tr>
<tr>
<td><strong>Number</strong></td>
<td>28</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 4.5. MMP-2 levels (ng/ml) pre switch to sirolimus, 12 months later in patients who had 12 months follow up and 3 months later in patients who had 3 months follow up but not 12 months follow up. See text for correlation coefficients.
Table 4.6. TIMP-1 levels (ng/ml) pre switch to sirolimus, 12 months later in patients who had 12 months follow up and 3 months later in patients who had 3 months follow up but not 12 months follow up. See text for correlation coefficients.

<table>
<thead>
<tr>
<th></th>
<th>TIMP-1 pre switch</th>
<th>TIMP-1 3 months</th>
<th>TIMP-1 12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>394</td>
<td>406</td>
<td>309</td>
</tr>
<tr>
<td>Inter-quartile range</td>
<td>336, 485</td>
<td>245, 444</td>
<td>248, 346</td>
</tr>
<tr>
<td>Number</td>
<td>24</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

There was a moderate correlation between serum MMP-2 and hepatic fibrosis assessed by liver biopsy (Spearman’s rank correlation coefficient, \( \rho = 0.49 \), 95% confidence interval 0.059, 0.77) before switch to sirolimus, a weak correlation with serum TIMP-1 (\( \rho = 0.227 \), 95% confidence interval -0.204, 0.585) and a very weak correlation with serum hyaluronic acid level (\( \rho = 0.045 \), 95% confidence interval -0.43, 0.5). After switch to sirolimus most MMP-2 (77%) and hyaluronic acid (92%) levels rose and most TIMP-1 (67%) levels fell. There was no correlation seen with any of the levels and fibrosis score 1 year after switching. There was also no correlation with the change in level of these markers with the change in fibrosis.

Levels of p21 protein in serum were also measured by ELISA (by C. Newman and Dr. A Trull). Because this was a new assay, no normal range or reference range was available. Thus 2 sets of controls were identified, the first being patients with hepatitis C virus infection who had not received a transplant and the second being normal
blood donors. Most of the patients with hepatitis C had a liver biopsy done at the same time as their serum was stored.

There was no correlation between the level of p21 protein and stage of liver fibrosis assessed at liver biopsy in either of the groups of patients with hepatitis C. The levels of p21 were not significantly different between the 3 groups. Using this assay therefore, there is no correlation between p21 and fibrosis stage.

**Viral load**

The hepatitis C viral load of all patients switched to sirolimus was measured before switching and 1 year later. There was no change in the viral load seen before and 1 year after switching to sirolimus, suggesting that there is no anti viral effect associated with sirolimus use. The change in viral load did not correlate with change in fibrosis for patients whose biopsies got better or worse or did not change ($p = 0.1$, median test). The viral loads were much higher than those normally seen by the laboratory (as expected for immune suppressed patients post liver transplant) and were in the region of 100 to 1000 times higher than HCV carriers on no immune suppression both before and after treatment with sirolimus.

**Results from biopsies at 2 years after switching to sirolimus**

Of the patients for whom a biopsy was available 1 year after switching to sirolimus, 6 also had a biopsy available taken 2 years after switching. The stage of fibrosis compared to baseline pre-switch improved in 4 of these patients and did not change in the other 2. Median fibrosis score fell from 4 to 2 although this was not significant ($p = 0.125$, sign test) due to the small numbers of patients. In 1 patient the fibrosis score fell from 5 to 2. In this patient interface hepatitis score fell from 3 to 1 and portal inflammation score fell from 4 to 2. Other results are shown in table 4.7. Pictures of
some of the biopsies from patients are also shown below (figures 4.2 – 4.4). Median sirolimus level in these patients at 2 years was 6.1 ng/ml (range 3.7 – 16.6).

Of these 6 patients 2 had got worse 1 year after switching, 2 were the same and 2 were better. This was not therefore a subgroup who were already doing better than the rest of the group of patients who were switched, but in some cases it took 2 years for a benefit to become evident.
Table 4.7. Median changes in weight, systolic blood pressure and blood parameters at 2 years with units of measurement and inter-quartile ranges (IQR) for patients who switched to sirolimus for hepatitis C related fibrosis. A negative number denotes a fall. Insufficient data to calculate p values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Change at 2 years</th>
<th>IQR at 2 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>-3.6</td>
<td>-6.8,-1.8</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>-1</td>
<td>-2,8</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>-4</td>
<td>-8,5</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>-2</td>
<td>-3,2</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>-8</td>
<td>-18,-5</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>-15</td>
<td>-50,23</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>18</td>
<td>-73,28</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>-1.5</td>
<td>-2.4,0.2</td>
</tr>
<tr>
<td>Total white cell count (x 10⁹/l)</td>
<td>0.4</td>
<td>-0.5,0.9</td>
</tr>
<tr>
<td>Lymphocyte count (x 10⁹/l)</td>
<td>0.1</td>
<td>-0.1,0.2</td>
</tr>
<tr>
<td>Neutrophil count (x 10⁹/l)</td>
<td>0.2</td>
<td>-0.7,0.6</td>
</tr>
<tr>
<td>Platelets (x 10⁹/l)</td>
<td>24</td>
<td>-23,59</td>
</tr>
<tr>
<td>Prothrombin time (seconds)</td>
<td>-0.5</td>
<td>-1.3,-0.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.2</td>
<td>0.7,1.4</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.9</td>
<td>0.5,1.8</td>
</tr>
<tr>
<td>Uric acid (mmol/l)</td>
<td>-0.1</td>
<td>-0.1,-0.1</td>
</tr>
</tbody>
</table>
Figure 4.2. Reticulin stain of biopsies from pre switch showing stage 4 fibrosis (left) and 2 years later (right) showing stage 2 fibrosis.

Figure 4.3. H & E stain of biopsies from pre-switch (left) and 2 years post switch (right) showing reduced portal inflammation (same patient as Figure 4.1).
Results – patients switched for early recurrent hepatitis C

4 patients were switched to sirolimus because of early (less than 6 months after transplantation) biopsy proven severe recurrent hepatitis C with jaundice (peak bilirubin levels in these patients were 68, 71, 173 and 223 µmol/l). These patients were not included in the original hypothesis. Clinical experience of these patients at Addenbrooke’s hospital has been that they have a very poor prognosis with rapid progression to cirrhosis and liver failure, usually within 1 year of transplantation. In the past, there have been no interventions that have made any difference to their clinical course, but it may be possible that any anti-fibrotic effect of sirolimus could reduce the formation of fibrosis as a response to the liver injury which is seen. If switching immunosuppression delayed or prevented the occurrence of cirrhosis in this high risk group, the prognosis of these patients may improve. The first patient with this problem switched to sirolimus just over 2 months after transplantation when he was found to have a severe recurrent hepatitis and a persistently raised bilirubin at 166 µmol/l. In retrospect there were also features of chronic rejection at the time. His
bilirubin did fall for a brief period and the hepatitis improved transiently but unfortunately he developed chronic rejection and went on to die 5 months after switching to sirolimus. The second patient had a severe recurrent hepatitis seen on biopsy which was performed for a raised ALT (698 U/l) 4 months after transplantation. She had an older donor (age 64) and had received 2 courses of methyl prednisolone for acute rejection after the transplant. The biopsy demonstrated severe hepatitis C recurrence with bridging collapse and was thought likely to progress rapidly to cirrhosis. She was switched to sirolimus and 12 months later was very well although her abnormal liver function tests persisted. Liver biopsy showed stage 4 fibrosis. The third patient had a severe hepatitis C recurrence seen on biopsy 3 months after transplantation and was also switched to sirolimus. He is well 12 months later but has developed a caval anastamotic stricture, making interpretation of his liver biopsy difficult although it shows only stage 2 fibrosis. The fourth patient developed acute hepatitis 3 months after transplantation with a bilirubin of 260μmol/l. Biopsy showed fibrosing cholestatic hepatitis and he was immediately switched to sirolimus. Liver biopsy 1 year later shows no fibrosis, no lobular hepatitis and minimal inflammatory change.

Results – patients re-transplanted using sirolimus immunosuppression

Only one patient has been considered during this period for re-transplantation for recurrent hepatitis C virus related cirrhosis. This indication was not included in the original hypothesis. He was accepted onto the waiting list with the intention of avoiding corticosteroids altogether and using sirolimus as the primary long term immunosuppression. He was initially given tacrolimus and azathioprine after transplantation as per unit protocol and when the surgical wounds were thought to be healed up at 1 month, the tacrolimus was switched to sirolimus. He switched drugs
without event. As intended he had no steroids post operatively and the azathioprine was withdrawn after 12 months. He remained well over the following year and put on 31.6 kg weight (body mass index rose from 23 to 34). His blood pressure rose by 31 mmHg (probably as a result of his weight gain) and required treatment. Creatinine fell by 33 \( \mu \text{mol/l} \), bilirubin by 11 \( \mu \text{mol/l} \), ALP by 310 U/l, ALT by 32 U/l, and platelets by 44 \( \times 10^9 \)/l. Haemoglobin rose by 2.6 g/dl, lymphocyte count by 0.3 \( \times 10^9 \)/l, neutrophil count by 0.8 \( \times 10^9 \)/l, albumin by 2 g/l, cholesterol by 1 mmol/l and triglycerides by 0.2 mmol/l. His liver biopsy at 1 year after transplantation showed features in keeping with recurrent hepatitis C but only stage 1 fibrosis. At 2 years a further biopsy showed no progression of fibrosis which remained at stage 1 and only minimal inflammation.

Clearly the biggest problem in this patient at the moment is his weight gain which is likely to be causing the rise in blood pressure. Otherwise he remains very well with no significant fibrosis in his liver.

*Follow up data on 2 further patients*

2 further patients were switched to sirolimus just before the study period started, but independent of this study. These data are included for completeness. The first patient with mild haemophilia A had hepatitis C cirrhosis and received a liver transplant at the age of 33. His donor was aged 59 and he was treated with a standard regimen of tacrolimus, prednisolone and azathioprine post transplantation. He had an episode of acute rejection on day 7 which was treated with 3 bolus doses of 1g methyl prednisolone. 5 months later he became jaundiced with a severe recurrence of biopsy proven hepatitis C and was switched over to sirolimus. His levels were a little higher than we currently use (17.8 ng/ml) and he had an episode of streptococcal septicaemia.
a month later; his dose was reduced. He had a protocol biopsy 1 year after switching and was noted to have some pericellular fibrosis which was thought to be caused either by alcohol or non-alcoholic steatohepatitis (his weight was 134 kg). He admitted to drinking a small amount of alcohol. He had a further biopsy 2 years after switching to sirolimus which showed some portal and peri-sinusoidal fibrosis. Unfortunately he also developed an anastamotic stricture and has recently had a roux loop biliary reconstruction for which he had to stop sirolimus because of the risk of poor wound healing. He had a further liver biopsy intra-operatively only 4 months after the previous biopsy, having been off sirolimus for 1 month. This showed that his fibrosis had progressed from stage 2 to stage 4 in that short space of time. He will be re-starting sirolimus soon and is generally in good health. Having had a severe early recurrence of hepatitis C, he has exceeded the early expectations of his outcome. The acceleration of his fibrosis in the short period in which he was taking tacrolimus rather than sirolimus is a concern and it will be interesting to see how this changes at his next annual biopsy.

The second patient received a liver transplant at the age of 41 for hepatitis C related cirrhosis with a 5cm hepatocellular carcinoma. His donor was 56 and he was treated with tacrolimus, azathioprine and prednisolone post operatively. He had a fit while on tacrolimus and switched to cyclosporin. He also had an episode of acute rejection on day 6 which was treated with 3 bolus doses of 1g methyl prednisolone. 7 months after transplantation he was switched to sirolimus. He remained well and was free of tumour recurrence 29 months after transplantation when he was switched to cyclosporin for a total hip replacement because of the potential for wound related problems. Pre-operative chest radiograph was normal. Within 3 months of switching from sirolimus he became unwell and a chest radiograph showed lesions consistent
with multiple metastases. These were biopsied and histology demonstrated metastatic hepatocellular carcinoma. He also became jaundiced. He was then switched back to sirolimus and his jaundice has now settled. This recurrence of disease is related temporally to stopping sirolimus after 29 months of apparently disease free survival. It seems possible that the sirolimus was having an effect on any residual disease until it was stopped for his operation.
Summary

- Immunosuppression can safely be switched from a calcineurin inhibitor to sirolimus with a 2 week overlap period to minimise the chance of acute rejection.

- There is a significant reduction in fibrosis seen in the biopsies after 1 year of sirolimus treatment compared to historical controls.

- There is no significant difference seen in inflammation or viral load after 1 year.

- In some of the patients in whom biopsies are available 2 years after switching to sirolimus there has been a dramatic improvement in appearances with a fall in fibrosis stage and reduction in inflammation.

- There is no correlation between drug level and reduction in fibrosis.

- Sirolimus is now used at much lower doses than previously and provides adequate immunosuppression in this dosage range.

- None of the serum markers of fibrosis measured correlate with the degree of fibrosis seen in transplant recipients and the levels change unpredictably after switching immunosuppression.

- Quality of life was not affected by switching to sirolimus.

- There are sufficient data to plan a randomised controlled trial of switching patients with hepatitis C related graft fibrosis to sirolimus.
Discussion

This study has shown that the immunosuppression used by patients after liver transplantation can safely be switched from monotherapy with a calcineurin inhibitor to sirolimus and that there are few side effects if a short overlap period of 1 – 2 weeks is used. In particular there were no toxic effects seen with this drug combination. The overlap period is not standard practice in liver transplantation but was used because there was a concern about the risk of using low doses of sirolimus and causing acute graft rejection. The main outcome measure used was the change in biopsy findings 1 year after switching drugs compared to the biopsy before switching. In terms of fibrosis stage, there is a difference in what happened over a 1 year period compared to historical controls which is statistically significant. The fact that the group who were switched to sirolimus were worse in terms of fibrosis at the outset suggests that we had selected a group who were to be expected to have a worse outcome, making the finding even more impressive. There is no significant difference in grade of inflammation seen after switching to sirolimus. This is an important finding in a group of patients who were chosen because their fibrosis was progressive and in whom no other intervention has been shown to be effective. It was also independent of the viral load and lobular inflammation which did not change, suggesting that the effect is independent of any effect which the drugs may have on the virus.

At 2 years after switching to sirolimus some patients have seen a dramatic improvement in their liver biopsy. All 6 patients are either better or stable. Inflammation has improved and fibrosis has reduced. This too is an important finding because it suggests that in some patients there may be a sustained improvement over time. It also suggests regression of fibrosis, not just inhibition of laying down new tissue.
It is not clear why the current analysis of the control group biopsies reached different conclusions from those in an earlier audit (which in combination with the 1999 publication prompted the study) where fibrosis progressed in 75% of patients from one year to the next rather than 51%. The earlier audit was not blinded which could have biased the results and was done by a different pathologist. This analysis of the historical control patients and those in the conversion study was undertaken by the same pathologist who examined the biopsies simultaneously and under the same blinded conditions and the analysis of these two groups is therefore comparable. The most likely explanation for the different conclusions is that the earlier audit was flawed by not being blinded. Most of the biopsies reviewed were the same biopsies, although some of the more recent biopsies reviewed were not available to the first pathologist. A change in practice could therefore have influenced the results, with lower doses of immunosuppression being used and clinicians now have a higher threshold for treating acute rejection. Steroids are now stopped earlier after transplantation which will also improve outcome. It is also noteworthy that since hepatitis C virus positive patients with significant fibrosis after transplantation have been changed to sirolimus, no patient in the Cambridge series has developed cirrhosis.

Another interesting observation in the study is the low drug dose that was used. In the original study performed at Addenbrooke’s hospital high trough levels were seen, many in excess of 50 ng/ml. The *in vitro* experiments performed in Southampton have demonstrated a dose response relationship, so it is reassuring that the trough levels of 5 – 10 ng/ml used in this study are sufficiently high to exert an anti-fibrotic effect over the period studied and to give adequate immunosuppression.

All of the patients who were switched to sirolimus were thought to be in a group who were likely to have a poor outcome in the future due to progressive graft fibrosis. This
is therefore likely to bias the study against detection of a favourable outcome in the group of patients who switched drugs. If the 2 groups were comparable in terms of change in fibrosis with no change seen on biopsy over 1 year, this in itself would be a significant finding because the intervention group has been selected because it is thought that they will have a worse outcome. A significant improvement in fibrosis however is unprecedented.

The use of serum markers of fibrosis to reduce the need for liver biopsy has been suggested as a possible monitoring strategy in native liver. The results from this study suggest that measuring serum hyaluronic acid, MMP-2 and TIMP-1 would not be useful in transplant recipients with hepatitis C, particularly if they are changing immunosuppression from a calcineurin inhibitor to sirolimus. Although the numbers here are small, there was a poor correlation with these markers, which disappeared completely after switching drugs. It may be the switch of drugs or a specific effect of sirolimus which was responsible for the change, but in view of the poor correlation initially, it is unlikely that these markers of fibrosis will replace liver biopsy as a method of quantifying fibrosis after transplantation. The interaction between the host immune system, immunosuppressive drugs and the donor liver has altered the expression of these markers such that no useful correlation exists as it did in hepatitis C infection in native liver. The level of p21 protein has also been found to be unhelpful in the assay that has been developed because the levels are generally too low to measure reproducibly.

Measurement of renal function by serum creatinine is universally accepted, but has poor sensitivity for mild or moderate dysfunction. I therefore attempted to measure creatinine clearance in these patients. The creatinine clearance did not correlate well with improvement in renal function in the patients switched to sirolimus for renal
impairment and actually fell in those switched without renal impairment. Several problems were encountered while measuring this parameter, including poor patient compliance (one patient failed to produce a 24 hour sample after 3 attempts) and an inability to collect a 24 hour sample while patients were in hospital in some cases. It is likely that there were problems in collecting these samples in more patients than those ones which we identified and that the results are unreliable.

One side effect of sirolimus was a small fall in haemoglobin, white blood cell count and platelets although this was not statistically significant. There may be a mild degree of bone marrow suppression and these cytopenias are a well recognised side effect listed in the product information. There would obviously be a concern if sirolimus were to be used at higher doses that this effect would be more pronounced.

It is also known that a proportion of the myofibroblast cells seen in the liver of transplant recipients (up to 22%) originate from bone marrow (150) and it is possible that any anti-fibrotic effect is mediated by an effect on stem cells in the bone marrow rather than locally in the liver.

The use of sirolimus for immunosuppression in patients with early recurrent hepatitis C after transplantation is another area where there could be a significant benefit to patient survival. Historically these patients have a poor outcome, often developing rapidly progressive deterioration of liver function and dieing within 1 year of transplantation. In one of the patients in whom sirolimus was used for this indication there was an initial improvement in liver histology but he then developed chronic rejection which was not amenable to treatment. On review of the histology chronic rejection was definitely present at the time of switching to sirolimus, although it had not been reported at the time. He died 8 months after transplantation. The other 3 patients switched to sirolimus for this indication have had no early problems since
switching and are well 12 months later from the point of view of their livers although 1 patient has developed an anastamotic caval stricture. Without changing their immunosuppression, past experience in Cambridge suggests that these patients would all have developed liver failure within 12 months of transplantation. Because of these favourable outcomes, patients with an early recurrence of hepatitis C with jaundice in Cambridge are switched to sirolimus at an early stage.

Re-transplantation for any condition generally yields less satisfactory results, but this is particularly the case for re-transplantation for hepatitis C related graft cirrhosis. As a result of previous poor outcome of these patients, re-transplantation for recurrent hepatitis C cirrhosis is not currently offered at Addenbrooke’s hospital. The main problem is the rapidly progressive fibrosis that is seen. If sirolimus does reduce fibrosis progression after transplantation, it may be useful in patients with hepatitis C needing re-transplantation. To date, only 1 patient has been re-transplanted using sirolimus for immunosuppression after the transplant. He is very well 2 years later with no significant fibrosis seen on his liver biopsy. Any further patients with hepatitis C being considered for re-transplantation will be having sirolimus as their primary immunosuppression after wound healing and the effect of this approach studied over time.

With regard to the quality of life measurements, the changes in the SF-36 t-scores are small, with median values being less than 5 points. The number of patients in the study is however small and would only be expected to detect a change of 20 points or more (151). To detect a significant change of less than 5 points would need between 515 and 1826 patients, depending on which modality is being examined (some are more sensitive to change than others) which is clearly not feasible in the transplant population. It is unlikely that there is a very large change (of 20 points or more on
average) but a smaller change could be missed by a type 2 error. The lack of sensitivity to detect change could also be a result of the fact that quality of life after liver transplantation is so good that any improvements can only be small.

This study is limited in 4 main ways, the first being the fact that there is no randomised control group. It is however generally accepted that graft fibrosis in these patients is progressive and does not reverse or stop, as can be seen from the analysis of the biopsies in the historical cohort of patients, where only 7% improved from one biopsy to the next. Change in fibrosis progression would probably only occur if the hepatitis C virus were cleared from the liver which is not likely to happen in this situation, particularly as no specific treatment for hepatitis C virus was given – nor is available. The current situation with regard to hepatitis C infected grafts is poor and appears to have got worse in recent years (5, 31, 32). This literature suggests a worse outcome for patients than the data presented here from Cambridge.

The second limitation is the small number of patients enrolled. It was envisaged that this study would be a pilot study and that if the results suggested that patients may benefit from switching immunosuppression to sirolimus that it could lead on to a randomised controlled trial. To achieve sufficient numbers of patients, this would have to be multi-centred, particularly as most patients at Addenbrooke’s hospital with graft fibrosis have already changed their immunosuppression to sirolimus.

The third limitation is that it is an open labelled study. All patients are aware of the new drug which they are taking due to the practical difficulties of blinding when drug levels are monitored. This is however unlikely to alter the interpretation of liver histology, particularly as the pathologist examining the slides was blinded as to treatment, but may have a bearing on other endpoints such as quality of life which could improve or deteriorate simply by virtue of a patient taking part in the study.
The fourth limitation is the short follow-up time available to study these patients, with the majority having data available for only 1 year. Further analysis in the future will give useful data on the longer term outlook for patients in whom the development of fibrosis is likely to become a problem, particularly in view of the favourable 2 year biopsy data that is available.

This is therefore a useful pilot study to assess the effect of changing immunosuppression from a calcineurin inhibitor to sirolimus in patients with hepatitis C related graft fibrosis, but is not sufficient to change international practice. It does however provide sufficient data on which to base a randomised controlled trial. If successful this would lead to a change in practice which could improve graft survival for these patients. A randomised controlled trial would need to be multi-centred because of the numbers of patients involved and because most of the patients at Addenbrooke’s hospital with hepatitis C and graft fibrosis have now already been switched to sirolimus. A power calculation can be performed on the data available from the biopsies available from 1 year, but with only 6 available at 2 years, this is too small a number for a useful power calculation. If the patients behaved in the same way as the 2 groups which we examined, 72 patients would need to be included into the trial (36 randomised to each arm) to give 80% power of detecting a difference between the 2 arms, at the 5% significance level. The subgroup of patients whose biopsies are available after 2 years suggests that the outcome of patients in the longer term is better and as more 2 year biopsies become available it may become clear that a smaller trial over a longer period would be sufficient to demonstrate a favourable response. 2 possible trials could be proposed. The first would be to switch patients as we have done in the present trial when significant fibrosis has been demonstrated by liver biopsy and assess by annual biopsy. The second would be to switch all patients
with hepatitis C to sirolimus between 3 and 6 months after liver transplantation and assess with liver biopsies after 2 years and again at 4 years. This would need to be a larger trial because it would include some patients who would never progress to significant fibrosis but would remove the subjective element of the initial liver biopsy reporting for inclusion into the study and probably speed up recruitment if clinicians in other centres agreed with the strategy. Patients could be switched to sirolimus with a 2 week overlap period with the calcineurin inhibitor and a therapeutic range of 5 – 10 ng/ml would be the target. From the data presented here, there should not be concerns about the safety of this approach with monotherapy in terms of graft rejection or other side effects and there would probably be little value in measuring viral loads or fibrosis markers.

**Conclusion**

In summary, this was a pilot study to assess whether there may be a benefit in switching the immunosuppression of liver transplant recipients with hepatitis C from their calcineurin inhibitor to sirolimus. The change in drugs was generally well tolerated with few side effects. We have demonstrated a significant change in fibrosis on liver biopsy over 1 year and no change in inflammatory grade. In the small number of patients with biopsies taken at 2 years after switching there also seems to be an effect on fibrosis and in patients who develop an early severe recurrence of hepatitis C, switching to sirolimus appears to alter the course of the disease. We have used lower doses of sirolimus than the previous study done at Addenbrooke’s hospital and a relatively short follow up time. We have found no useful correlation between fibrosis stage and the established serum markers of fibrosis, hyaluronic acid, MMP-2 and TIMP-1. A randomised controlled trial is now needed to investigate these effects further.
Chapter 5. The effect of sirolimus on EGF concentrations

Introduction

Oral ulceration has been documented as a problem in patients taking sirolimus at Addenbrooke’s hospital (personal communication, Dr. G. Alexander) but although this was noted, it was not reported in the original published study about use of the drug (99). This was because ulceration used to be attributed to either fungal or viral infection and was not related specifically to sirolimus (personal communication Dr. G. Alexander). It has since been noted that many patients suffer from this side effect, particularly when the drug is used at higher concentrations. Oral ulceration has subsequently been reported in conjunction with sirolimus use (105, 152) although it was thought by the authors to be related to the degree of immunosuppression rather than a direct side effect of the drug. It appears to happen more commonly at higher doses of sirolimus and may resolve with time and dose reduction although in some patients it can be sufficiently persistent and troublesome to necessitate stopping sirolimus. The mechanism of action is not clear.

Epidermal growth factor (EGF) is a peptide secreted by the salivary glands and present in saliva as well as by the Brunner’s glands in the duodenum (153). It is responsible for the promotion of mucosal healing and it has been suggested that it may play a role in the prevention of oesophageal ulceration (154) and peptic ulceration in the stomach and duodenum (155). The level of salivary EGF has also been related to the severity of oral mucous ulceration following radiotherapy with higher levels being thought to be protective (156).
We hypothesised that sirolimus could be acting to cause oral ulceration by a direct effect on EGF concentrations in saliva (although it could also act by blocking the receptor or by post receptor events). We have investigated the effect of switching drugs from a calcineurin inhibitor to sirolimus on the concentrations of EGF in blood (serum and plasma) and saliva.

**Method**

Liver transplant recipients who were switching their immunosuppression from a calcineurin inhibitor to sirolimus in the year 2002 as part of the trial described in chapter 4 had saliva and blood samples collected for measurement of EGF concentrations before switching and 3 months after switching.

*Collection of saliva*

It has been documented that mastication increases salivary volume but may reduce the concentration of EGF in saliva. The total EGF output however is usually raised (153, 157, 158). Saliva was therefore collected without mastication (unstimulated) and during mastication (stimulated). The patients were asked to spit all saliva that came into their mouth over a 5 minute period into a universal container. They were then given a piece of parafilm (paraffin wax) to chew and asked again to spit all saliva into a second container over a second 5 minute period. The samples were placed on ice until they were centrifuged at 3000 rpm (1880g) for 10 minutes in a refrigerated centrifuge. The volume was measured and a 0.5 ml aliquot frozen at -80°C for later analysis.
Collection of blood

EGF is present in platelets in high concentrations and it is known that it is released when blood clots. This has a marked effect on the level of EGF which is measured. Although it is thought that the free EGF in the plasma is probably the most important level, the total EGF including that which is released by platelets may be important because this is available at any site of injury where blood clotting takes place. Both plasma and serum levels of EGF were therefore measured.

Collection of plasma

Blood was collected in a heparinised syringe to which 500µl of Trasylol (aprotinin) had been added as a preservative. The tubes were kept in the fridge prior to use and placed on ice immediately after the sample had been taken. Samples were then spun in a refrigerated centrifuge at 1880g for 10 minutes. 1 ml of supernatant was removed and spun again at 13,000g for 3 minutes to ensure removal of any remaining platelets. The samples were stored at -80°C for later analysis.

Collection of serum

Samples were collected in a plain syringe and allowed to clot for 30 minutes. They were then centrifuged at 1880g for 10 minutes and the supernatant stored at -80°C.

Assay used to measure levels

EGF levels were measured in all of the samples by ELISA (R & D systems, Quantikine ELISA kit).
Statistical methods

All sample results were analysed using non-parametric methods with paired tests. There is no test available to measure the spread of the results because of the paired nature of the samples.

Results

Results are displayed in tables 5.1 – 5.4 below. For all blood results, EGF concentrations have been measured and displayed. For saliva results, the concentrations of EGF in saliva have been measured and multiplied by the volume of saliva produced during the 5 minute timed period over which the saliva was collected. This amount has been compared before and after treatment with sirolimus, as has the concentration of EGF measured. A further figure has been derived which is the difference in the amount of EGF produced by stimulation over the baseline amount before and after treatment with sirolimus.
Table 5.1. EGF in saliva (unstimulated)

<table>
<thead>
<tr>
<th></th>
<th>[EGF] pre</th>
<th>Volume pre</th>
<th>EGF amount</th>
<th>[EGF] post</th>
<th>Volume post</th>
<th>EGF amount</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>1663</td>
<td>2</td>
<td>3013</td>
<td>1725</td>
<td>2.5</td>
<td>3463</td>
<td>1.0</td>
</tr>
<tr>
<td>Minimum</td>
<td>85</td>
<td>0.25</td>
<td>85</td>
<td>240</td>
<td>0.5</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>3500</td>
<td>5</td>
<td>12000</td>
<td>3100</td>
<td>5</td>
<td>9500</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Table 5.1. Results are for unstimulated saliva. [EGF] pre and post are the concentrations of EGF (pg/ml) measured in saliva before and after treatment with sirolimus. Volume is the volume collected (ml) over the 5 minute timed period. EGF amount is the total amount of EGF collected in the sample (pg). The final column is the statistical significance of the difference.
Table 5.2. EGF in saliva (stimulated)

<table>
<thead>
<tr>
<th></th>
<th>[EGF]</th>
<th>Volume</th>
<th>EGF</th>
<th>[EGF]</th>
<th>Volume</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre</td>
<td>pre</td>
<td>amount</td>
<td>post</td>
<td>post</td>
<td>amount</td>
</tr>
<tr>
<td>Number</td>
<td>17</td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
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<td>5100</td>
<td>540</td>
<td>7.5</td>
<td>4065</td>
</tr>
<tr>
<td>Minimum</td>
<td>118</td>
<td>1.25</td>
<td>470</td>
<td>150</td>
<td>1.5</td>
<td>750</td>
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<td>Maximum</td>
<td>2200</td>
<td>17</td>
<td>10030</td>
<td>1700</td>
<td>15</td>
<td>17050</td>
</tr>
</tbody>
</table>

P=1.0 for EGF amount

Table 5.2. Results are for stimulated saliva. [EGF] pre and post are the concentrations of EGF (pg/ml) measured in stimulated saliva before and after treatment with sirolimus. Volume is the volume collected (ml) over the 5 minute timed period. EGF amount is the total amount of EGF collected in the sample (pg). The final column is the statistical significance of the difference.
<table>
<thead>
<tr>
<th>Number</th>
<th>8</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>26</td>
<td>9</td>
</tr>
<tr>
<td>Minimum</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Maximum</td>
<td>640</td>
<td>825</td>
</tr>
</tbody>
</table>

Table 5.3. Results are for plasma EGF concentration (pg/ml) before (pre) and after (post) treatment with sirolimus for 3 months. ND is not detected (<1.5 pg/ml). [EGF] dif is the change in concentration of EGF. Patients in whom the concentration of EGF was not detectable both before and after treatment with sirolimus were excluded (n = 10).
Table 5.4. Results are for serum EGF concentration (pg/ml) before (pre) and after (post) treatment with sirolimus for 3 months. [EGF] dif is the change in concentration of EGF.

<table>
<thead>
<tr>
<th></th>
<th>[EGF] pre</th>
<th>[EGF] post</th>
<th>[EGF] dif</th>
<th>Sign test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>18</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>61</td>
<td>42</td>
<td>-6</td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>11</td>
<td>18</td>
<td>-185</td>
<td>P=0.33</td>
</tr>
<tr>
<td>Maximum</td>
<td>825</td>
<td>825</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

Looking at the unstimulated saliva samples, 10 went down and 8 went up over time. The median increased and the range decreased, however, there was no evidence of a difference in the pre and post samples (p=0.82, sign test).

Looking at the stimulated saliva samples, 10 went down and 7 went up over time. The median went down and the range also went down, however there was no evidence of a difference between the two samples (p=0.63, sign test).

For the total EGF amount in saliva, 8 values are higher for stimulated measurement and 9 are higher for the unstimulated measurement, therefore there is no difference in EGF amount between the stimulated and unstimulated measurements. The median is smaller in the unstimulated measurements and the range is larger.
Looking at the plasma EGF samples, 3 observations went down and 5 observations went up. There was an increase in the range of observations. The median value went down, but this was not statistically significant (p=0.73, sign test).

Looking at the serum samples, 6 increased in EGF over time, 11 decreased and one remained the same. The median value decreased and the range decreased slightly between the two time points. However, this was not significant (p=0.33, sign test).

During the period studied, no patients developed oral ulceration. In a different study taking place at Addenbrooke’s hospital however, 4 out of 12 patients switched to sirolimus (33%) developed oral ulceration. The initial dose used was higher and there was no overlap period (personal communication Mr. C. Watson).

Summary

- EGF levels in serum, plasma and saliva were not affected by changing immunosuppression from a calcineurin inhibitor to sirolimus.

Discussion

The hypothesis examined here is based on the premise that a fall in EGF levels in either saliva or blood will make an individual more susceptible to developing mouth ulcers. During previous studies, these have been documented and were a particular problem in the original study reported from Addenbrooke’s hospital. It is surprising therefore that no patients in this study who were switched to sirolimus developed mouth ulcers which could be attributed to their drugs. There are currently 2 other studies underway at Addenbrooke’s hospital looking at the effect of sirolimus on renal function. These studies are using a different way of initiating sirolimus, with no overlap period with the calcineurin inhibitor and a higher sirolimus starting dose of 2 mg daily. Most of these patients have developed mouth ulcers which have improved
over time or with dose reduction (personal communication, Mr. C. Watson). From the point of view of this study, it is unfortunate that none of these patients developed mouth ulcers because it makes it difficult to interpret the results. It is likely that there is a dose effect, however, with higher doses predisposing to more mouth ulcers. Indeed the study which reports a high incidence of mouth ulcers started loading patients with an initial dose of 15 mg on the first day followed by 5 mg daily, aiming for a trough dose of 10 – 15 ng/ml (105).

The methods of collection used here were appropriate for the EGF measurements. Both concentration and amount produced over a fixed time have been measured in both unstimulated and stimulated saliva and the concentrations in serum and plasma have been measured. The platelets must have been effectively removed from the plasma by the method of centrifuging twice as the EGF level in many samples was undetectable.

EGF is a peptide hormone whose main function is the maintenance of mucosal integrity. Low levels have been associated with oesophageal, gastric and oral disease and these measurements were based on the hypothesis that the concentrations of salivary or circulating EGF might be influenced by sirolimus. Clearly this is not the case in these patients. There is no evidence that concentrations of EGF in saliva (stimulated or unstimulated) have changed after treatment with sirolimus or that the amount of EGF secreted over a timed period has changed. There is also no evidence of a change in either serum or plasma levels. It is not possible to say whether there would have been a change in levels in a patient with mouth ulcers although this seems unlikely, but EGF levels could be assayed prospectively in patients starting on higher doses of sirolimus as in some other studies.
It is possible that sirolimus could be acting via a post receptor event but this has not been, and could not be investigated here. EGF signalling is complex. Binding of EGF to the trans-membrane receptor causes clustering, dimerisation and internalisation of the whole receptor complex followed by a chain of intracellular events. It is not known whether sirolimus affects any of these. Another alternative mechanism by which sirolimus could cause mouth ulcers might be an alteration in the profile of viruses such as HHV-8, CMV, EBV, HPV and HSV-1 which are known to cause mouth ulcers in other situations.

In summary, therefore, we have found no evidence that sirolimus affects EGF levels in saliva or blood, but it may be that this is because of the low doses used and an effect may be seen at higher doses.
Chapter 6. The effect of sirolimus on vEGF concentrations

Introduction

Angiogenesis is the formation of new capillaries from existing blood vessels. Usually this process takes place only during wound healing and the female reproductive cycle where vEGF stimulates placental development (159, 160), but can also take place under pathological circumstances such as tumour growth and diabetic retinopathy. Vascular endothelial growth factor (vEGF) is the most studied angiogenic factor and acts on blood vessels to promote angiogenesis and vascular permeability. It has been demonstrated that vEGF must be present for hepatic fibrosis to take place in mice (161) and any change in the vEGF level could therefore have an impact on fibrosis progression. This was demonstrated in a murine model which used carbon tetrachloride as the stimulus for fibrosis (161). The authors found that blocking vEGF signalling with monoclonal antibodies to either of the two vEGF receptors inhibited the development of hepatic fibrosis. A previous study has also shown that inhibition of production of vEGF and inhibition of p70s6 kinase (a step crucial to vEGF signalling) by sirolimus reduces tumour angiogenesis in mice (111).

We hypothesised that treatment with sirolimus may cause vEGF levels to fall and also cause blocking of intracellular signalling, thereby reducing the potential for hepatic fibrosis. We have measured vEGF levels in plasma to see if there is any change after administration of sirolimus. We could not investigate post receptor signalling events.
**Method**

Liver transplant recipients who were switching their immunosuppression from a calcineurin inhibitor to sirolimus in the year 2002 had blood samples collected for measurement of vEGF concentration. Some samples were also collected from control patients to see if the levels seen in the transplant recipients were different from normal. Two control groups were chosen. The first was healthy controls who worked in the hospital. The second control group was patients with hepatitis C infection who had not received any immunomodulatory treatment (interferon-α or ribavirin).

*Collection of blood*

vEGF, like EGF is present in platelets in high concentrations and is released in large quantities when blood clots. This has a marked effect on the level of vEGF which is measured. It is thought that only plasma levels are important and that the presence of platelets probably just confounds interpretation of readings. Only plasma levels of vEGF were therefore measured.

*Collection of plasma*

Blood was collected in a heparinised syringe to which 500μl of Trasylol (aprotinin) had been added as a preservative. The tubes were kept in the fridge prior to use and placed on ice immediately after the sample had been taken. Samples were then spun in a refrigerated centrifuge at 1880g for 10 minutes. 1 ml of supernatant was removed and spun again at 13,000g for 3 minutes to ensure removal of any remaining platelets. The samples were then stored at -80°C for later analysis.
**Assay used to measure levels**

vEGF levels were measured by ELISA bought as a commercially available kit (Quantikine human vEGF assay, R & D systems). Control samples with known concentrations of vEGF were also bought to ensure that the assay worked properly.

**Results**

Results are displayed in table 6.1 below. The difference between the results before and after switch to sirolimus is very small and both levels are below the reliable limit of the assay. Although the assay should be able to quantify levels down to 9 pg/ml, this is a theoretical limit derived from extending the standard curve by 2 standard deviations below the lowest standard and it is generally agreed that levels below the lowest standard concentration (31 pg/ml) are unreliable (162). No p value has therefore been calculated for the small difference observed. There were 4 diabetic patients in the group, 2 of whom were insulin dependent and 2 of whom were taking oral hypoglycaemic agents. vEGF has been implicated in the development of diabetic retinopathy and levels could be different in diabetic patients, however their vEGF levels fell within the range of the other patients’ results.
Table 6.1.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>18</td>
<td>18</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Median</td>
<td>18.5</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Minimum</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maximum</td>
<td>38</td>
<td>42</td>
<td>25</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 6.1. Results are for vEGF concentration in plasma (pg/ml) before (pre) and after (post) treatment with sirolimus for 3 months. [vEGF] HCV is patients with hepatitis C who have not had a liver transplant and [vEGF] normal is concentrations in normal volunteers.

Summary

- Levels of vEGF seen in plasma were mostly below the reliable limit of detection of the assay.

- vEGF was detected in plasma of all transplant patients but at low levels and the median levels were higher in this group than the controls. It is not known if this difference is of biological significance.

- No change in levels was seen following conversion to sirolimus but a more sensitive assay would have to be used to test this hypothesis further.
Discussion

vEGF levels in the transplant recipients were generally low from the point of view of the assay with over 80% being below the lowest standard. This makes interpretation of the results unreliable because of the need to extrapolate the standard curve beyond the lowest standard. These levels were however generally higher than the control group of patients and volunteers whose levels were mostly undetectable.

These levels are in keeping with some of the published literature although different authors use different methods of preparing the samples. Many ranges quoted in papers are of the order of 75 – 100 pg/ml although some are quoted as being below 31 pg/ml (162). It is vital when measuring plasma levels that all platelets are removed from the plasma because they release vEGF in very large quantities which will interfere with the assay and give falsely high levels. Not all papers are clear about sample preparation and their results must therefore be interpreted with caution. A detailed review of the subject found that authors used different methods with respect to centrifugation, temperature, anticoagulant and time scale after the sample was taken (162). All of these factors have therefore been standardised in these measurements, but comparisons with other published literature must still be made with caution after a detailed appraisal of the methods used.

The method of sample preparation which we have used has clearly been effective at removing platelets, as shown by the very low vEGF levels seen in the samples. It is possible that the transplant recipients had higher levels of vEGF than controls although with this assay, it is not possible to be sure. A more sensitive assay would have to be developed to investigate this further.
In conclusion, this assay would still be useful for measuring serum levels of vEGF, but not for measuring plasma levels when platelets have been completely removed. If a more sensitive assay could be used which gave reliable results down to the levels seen here, it may be that the transplant recipients would be found to have higher levels than the non-transplant recipient controls. This would provide a possible explanation for the increased fibrotic rate seen in liver transplant recipients and a mechanism whereby sirolimus may reduce this rate. A post receptor effect is also possible, as are different local tissue concentrations, but we have not investigated these hypotheses.
Chapter 7. The effect of sirolimus on neutrophil function

Introduction

Bacterial sepsis is a recognised complication of the early post transplant period, the prevalence of which is unaffected by the use of either cyclosporin or tacrolimus at current recommended therapeutic levels (163, 164). In contrast, bacterial sepsis has been reported following transplantation with both increased severity and frequency using sirolimus, even at appropriate therapeutic concentrations (100), with a non-significant trend seen in a second study (116).

Neutrophils play a vital role in the defence against bacterial and fungal infection, so that the host is susceptible when cell numbers are low or function is compromised (165, 166). Stimulation of neutrophils activates the oxidative (or respiratory) burst with production of reactive oxygen intermediates within the cell. This process is critical to the phagocytic and bactericidal properties of neutrophils (167). Disorders of the neutrophil oxidative burst cause chronic granulomatous disease, a condition characterised by chronic fungal and bacterial infections (168, 169).

Various in vitro studies have demonstrated inhibitory effects of sirolimus on both B and T cell proliferation (170). Comparable studies on the effect of sirolimus on neutrophil activation have not been reported. We postulated that sirolimus might impair innate immunity directly and predispose to bacterial infection by inhibition of neutrophil activation. In the present study the effect of sirolimus on neutrophil activation was studied in vitro and subsequently, the effect of exposure in vivo.
Materials and methods

30 ml citrated blood was collected from 24 healthy volunteers and 3ml from 23 transplant recipients (10 liver, 13 kidney) with approval of the local research ethics committee. The latter had been recruited to a randomised controlled trial, whereby patients would either switch from calcineurin inhibitor based therapy (16 cyclosporin, 7 tacrolimus) to sirolimus monotherapy, or remain on calcineurin inhibitor based therapy. Phorbol myristate acetate (PMA) stimulated neutrophil activation was measured at randomisation and re-assessed 3 months after randomisation.

Sirolimus at 1 g/l in dimethyl sulfoxide (DMSO) was diluted with autologous plasma to yield final concentrations of 5000, 1000, 500 and 100 ng/ml. Propofol (Diprivan, Astra Zeneca), which is known to inhibit neutrophil activation (171), was used at 10 mg/ml and served as a positive control drug. The optimum time for incubation with sirolimus to maximise its effect without compromising cellular integrity or viability was 2 h (data not shown).

1 ml blood was dispensed into eight 15 ml centrifuge tubes. Volunteer blood was mixed with sirolimus. 10 μl volumes of sirolimus in autologous plasma were added slowly to each tube while mixing yielding final concentrations of 1, 5, 10 and 50 ng/ml. 6 μl propofol were added to one tube giving a final concentration of 60 μg/ml. 3 tubes had no drug added and were used as controls, 2 for stimulation with PMA and 1 for addition of the fluorescent marker dichlorofluorescein diacetate (DCFH-DA) without PMA as a measure of the background oxidative burst. Blood was incubated at 37˚ C in a shaking water bath for 2 h.

Blood from healthy volunteers or transplant recipients was washed three times in 10 ml buffer solution, which comprised 1% (v/v) HEPES (Gibco 15630-056) in 0.9%
(w/v) saline supplemented with 0.04% (v/v) human serum albumin (Zenalb 20% human serum albumin; Bio Products Laboratory) and 0.2% (w/v) glucose (50% Glucose, Phoenix pharma), adjusted to pH 7.45 at room temperature with 1M sodium hydroxide. After initial centrifugation at 200 g for 10 minutes and 2 subsequent centrifugations at 500 g for 5 minutes the cell pellet was suspended in buffer to yield a white blood cell concentration of $3 \pm 0.5 \times 10^6$/ml.

50 µl from each tube was aliquoted into separate 75 x 12 mm pre-warmed borosilicate glass tubes in the water bath at 30 second intervals. After 5 minutes the cellular viability dye, 7-Amino-Actinomycin D (7-AAD, Beckman Coulter, High Wycombe) was added at 30 second intervals. After 10 minutes, the cell suspensions were stimulated with 25 µl freshly prepared dichlorofluorescein diacetate (DCFH-DA) PMA oxidative burst reagent (Beckman Coulter #7547078) at 30 second intervals, mixing after each addition. This was performed in duplicate for patient samples. As an additional control to assess background neutrophil function, 25 µl DCFH-DA-peroxides (Beckman Coulter #7547077) was added to one tube (contains only the DCFH-DA but no PMA to activate the neutrophils). This too was performed in duplicate for patient samples.

The tubes were returned to the water bath for 10 minutes then placed in ice-cold water at 30 second intervals and incubated for 3 minutes. Red blood cells were lysed by the addition of 600 µL ice cold ImmunoPrep reagent A (Beckman Coulter 7546946) for 10 seconds with constant mixing and the reaction stopped by addition of 265 µl ice cold reagent B for 10 seconds. The tubes were placed back in ice-cold water for immediate analysis by flow cytometry. Analysis was performed by dual-colour flow cytometry using a Coulter Epics-XL. The granulocyte population was
selectively gated using parameters for forward and side scatter and the oxidative burst evaluated as the mean fluorescence signal.

**Statistical Methods**

The data were skewed, so a log transformation was applied prior to analysis. For the *in vitro* study results were compared with the maximally stimulated PMA control using a paired t-test and ANOVA test. For the *in vivo* study, pre-switch levels were compared between the 2 groups by an unpaired t-test and also compared to the PMA-stimulated neutrophil activation at 3 months within each group (switched to sirolimus or not switched) using a paired t-test. Results are expressed as percentage change in mean channel fluorescence (mean % change) with 95% confidence interval (95% CI). Spearman’s Rank correlation coefficient was used to assess correlation between PMA stimulated neutrophil activation at 0 and 3 months. A Mann Whitney U-test was used to compare the percentage change from 0 to 3 months.

**Results**

**In vitro study**

Median age of volunteers was 41 (range 23-64). 54% were male.

Propofol (60 µg/ml) inhibited (mean % change; 95% CI) PMA-stimulated neutrophil activation significantly (-5.1%; -0.4, -9.8%).

Sirolimus inhibited PMA-stimulated neutrophil activation at 50 ng/ml (-6.3%; -1.5, -11.1%) and 10 ng/ml (-4.6%; +1.3, -10.6%) but at sirolimus concentrations of 5ng/ml and below, inhibition of PMA-stimulated neutrophil activation was less than 1.5% (see Table 7.1).
Repeated measures ANOVA confirmed a linear relationship between sirolimus concentrations above 5 ng/ml and inhibition of PMA-stimulated neutrophil activation (p = 0.01).

Table 7.1.

<table>
<thead>
<tr>
<th></th>
<th>DCFH-DA control</th>
<th>PMA control</th>
<th>Sirolimus 1 ng/ml</th>
<th>Sirolimus 5 ng/ml</th>
<th>Sirolimus 10 ng/ml</th>
<th>Sirolimus 50 ng/ml</th>
<th>Propofol 60 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>18</td>
<td>24</td>
<td>24</td>
<td>23</td>
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<tr>
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<td>99,130</td>
<td>101,134</td>
<td>95,128</td>
<td>94,124</td>
<td>93,125</td>
</tr>
<tr>
<td>Mean % change</td>
<td>-1.2</td>
<td>-1.1</td>
<td>-4.6</td>
<td>-6.3*</td>
<td>-5.1*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05

Table 7.1. Mean is the mean channel fluorescence with 95% CI calculated from log transformed data and then back transformed. Mean % change is the mean of the percentage changes in PMA stimulated oxidative burst seen in individual samples from their PMA control. A negative value signifies a fall in the mean channel fluorescence. Number is the number of samples analysed in each group.

In vivo study

Median age for patients switching to sirolimus was 57 (range 36-73) and for patients not switching was 54 (range 31-70). 74% were male. 1 patient on sirolimus developed pneumonia during the study period.

There was no difference in pre-randomisation PMA-stimulated or background (DCFH-DA peroxides alone) neutrophil oxidative burst between the two groups.
(p=NS, unpaired t-test). However, the PMA stimulated oxidative burst (mean channel fluorescence) before randomisation (179) was greater than that found in the healthy volunteers from the in vitro study (116, p<0.001).

There was no difference in either the PMA-stimulated or the background neutrophil oxidative burst at 0 and 3 months in the 12 patients who were randomised to remain on calcineurin inhibitor (mean % change at 3 months +0.95%; +18.7, -16.8%; p = NS). Indeed, there was a positive correlation between results at 0 and 3 months (r = 0.776; p = 0.003) in the PMA stimulated group, compatible with a stable response to this stimulus over time in these patients.

PMA-stimulated neutrophil activation was significantly decreased (-24.4%; -7.5, -41.2; p = 0.009) in 11 patients 3 months after switching to sirolimus monotherapy (see Table 7.2). However, these changes were not correlated with whole blood concentrations of sirolimus (tandem mass spectrometry; St George’s Hospital Analytical Unit, London). There was no correlation between the PMA-stimulated neutrophil activation in these patients at 0 and 3 months (r = -0.05; p = 0.894) in contrast to the group that remained on calcineurin inhibitors.

There was a significant difference in the percentage change in PMA stimulated neutrophil activation at 3 months between the 2 groups (p=0.027, Mann Whitney U-test).

As a measure of intra-assay variability the mean coefficient of variation from all tests which were performed in duplicate was calculated as 8% (n=104). White cell viability consistently exceeded 90% for both studies.
Table 7.2.

<table>
<thead>
<tr>
<th></th>
<th>DCFH-DA pre-switch</th>
<th>DCFH-DA post-switch</th>
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<th>PMA post-switch</th>
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</thead>
<tbody>
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<td>Sirolimus switch</td>
<td>Number</td>
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<td>8</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
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<td>188</td>
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<td></td>
<td>95% CI</td>
<td>15,19</td>
<td>12,16</td>
<td>155,228</td>
</tr>
<tr>
<td></td>
<td>Mean % change</td>
<td>-25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-24&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>No switch</td>
<td>Number</td>
<td>11</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>16</td>
<td>17</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>13,18</td>
<td>15,20</td>
<td>135,216</td>
</tr>
<tr>
<td></td>
<td>Mean % change</td>
<td>+1</td>
<td>+1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>p=0.04, <sup>b</sup>p=0.009

Table 7.2. Data is divided according to whether patients switched to sirolimus therapy or not. PMA signifies PMA stimulated cells, DCFH-DA is the background control. Pre-switch and post-switch refer to before and 3 months after randomisation. Mean is the mean channel fluorescence with 95% CI calculated from transformed data and then back transformed. Mean % change is the mean of the percentage changes seen in the samples taken 3 months after randomisation expressed as a percentage of the corresponding sample taken before randomisation. A positive number signifies a rise in mean channel fluorescence, a negative number a fall. Number is the number of samples analysed in each group.
Summary

- Sirolimus causes a dose dependent reduction in the PMA stimulated neutrophil oxidative burst in vitro.
- Transplant recipients who switched from a calcineurin inhibitor to sirolimus had a reduced neutrophil oxidative burst after 3 months.

Discussion

These results demonstrate a modest, but statistically significant, dose-related effect of sirolimus on the PMA-stimulated neutrophil oxidative burst after incubation with whole blood in vitro and a larger effect in transplant recipients who switched from a calcineurin inhibitor to sirolimus therapy. This reflects a reduced capacity of neutrophils to respond to an exogenous stimulus. There was also an effect of sirolimus on the background neutrophil oxidative burst in these patients, reflecting the neutrophil activation state without exogenous stimulus. These observations may explain the increased incidence of sepsis in this context (100).

For in vitro studies, the choice of stimulant for the oxidative burst is important. Phorbol myristate acetate (PMA), an analogue of the cellular signalling molecule diacylglycerol (172), is the most effective soluble stimulus to the oxidative burst. Incubation of neutrophils or whole blood with drugs can alter the response of neutrophils to PMA stimulation measured by the oxidative burst (171, 173).

The oxidative burst can be measured by chemiluminescence or, at a cellular level, by flow cytometry (174, 175). Measurement of the oxidative burst by means of flow cytometry is technically easier and has been validated against the more time consuming method of chemiluminescence (175). Two different fluorescent markers have been used - dichlorofluorescein diacetate (DCFH-DA) and dihydro-rhodamine
(DHR). DHR is reported to be more sensitive and useful for monitoring the oxidative burst in very small sub-populations of cells (176), but the choice of these markers is probably not crucial in the detection of clinically significant inhibition of neutrophil activation.

Many studies of neutrophil activation have been performed on purified neutrophils. More recently, some studies have involved the use of whole blood for the assessment of neutrophil function (112, 177). This has the advantage that neutrophil function is unaffected by the cell purification process. The whole blood matrix also provides a physiological environment for investigating the influence of drugs on blood cell function, particularly if the drug is highly bound to plasma proteins or other cellular components in vivo as in this situation. Thus, it is the small, unbound fraction of such drugs that is regarded classically as the active component pharmacologically. At least 99.8% of sirolimus in human blood is bound either to blood cells or plasma proteins (106).

The effect of propofol seen on neutrophil function in vitro was much less than that described previously (171). This previous study was performed on separated neutrophils, which takes no account of drug binding in whole blood and is therefore likely to overestimate the effect that may be seen in vivo. Drug binding of propofol in whole blood is approximately 99% with 1.2 – 1.7% of the drug being free in the circulation (178). Therefore the level of 91% inhibition seen in that context using 60 mg/ml is not directly comparable to the level of 5.1% described herein. It is however similar to the level of between 1 and 6% inhibition (exact figure not quoted) seen for a propofol concentration of 0.6 mg/ml, an equivalent concentration when drug binding is taken into account.
It is notable that there was a positive correlation between PMA-stimulated neutrophil activation at 0 and 3 months and no significant difference in the background neutrophil activation for those patients who did not change immune suppression. This suggests that the assay is robust, and could prove useful for monitoring neutrophil function within patients over time.

The similar levels of background neutrophil activation in the transplant recipients before randomisation and the healthy volunteers demonstrated that there were no factors stimulating the oxidative burst in vivo. The fact that the PMA stimulated levels are much higher in transplant recipients may be due to neutrophil priming although this has not been described before in transplant recipients. Klein et al have found the neutrophil oxidative burst to be of similar magnitude in healthy volunteers and renal transplant recipients (179) but this experiment used a different method of stimulation.

The much greater suppressive effect of sirolimus in vivo compared to that found in vitro may be explained partly by peak levels after single dose administration being greater than 3 times trough levels (180). Furthermore when sirolimus is used in vivo, there is longer exposure of the neutrophils to the drug, which could also be responsible for the magnitude of the effect seen. Another possibility is the potential for an effect on the neutrophils during maturation as a consequence of exposure of the bone marrow to sirolimus over a period of 3 months. In one patient this effect was as great as 59% inhibition of neutrophil activation in response to PMA stimulation. The lack of a dose response relationship may be due to the small dose range used clinically, with all blood sirolimus levels except one between 6 and 14 ng/ml. Alternatively, it could reflect differences in drug binding between patients.

The mechanism by which sirolimus reduces the oxidative burst is unclear.
It has been shown that sirolimus reduces the activity of protein kinase C in A6 cells (181) and this may explain the reduced level of PMA stimulated oxidative burst which we have noted. It may also explain the reduction in the level of background oxidative burst because the oxidative burst caused by any stimulus is likely to be reduced by a reduction of protein kinase C activity since this is the main pathway of action.

These findings may partly account for the increased incidence of sepsis seen in patients treated with sirolimus and also suggests that the drug may have a beneficial effect in patients in whom neutrophil activation causes complications such as vasculitis.
Chapter 8. The effect of sirolimus on a tissue culture model of liver fibrosis

Introduction

The search for an effective anti-fibrotic agent would be aided greatly by an *in vitro* assay. A technique has been developed with this aim at Southampton University by Dr. N. Sheron for the growth of slices of neonatal rat liver tissue on a nitrocellulose membrane which is a modification of that which was used in some earlier work (182). This original work used a vibratome which cut much thicker slices and was more time consuming to use. Using the modified technique, a cube of liver is sliced into thin slices (between 60μ and 200μ thick) using a Krumdieck tissue slicer which is designed to rapidly prepare aseptic, thin slices of live tissues for *in vitro* studies. The slices are then placed on a membrane which floats in culture medium. The culture medium passes through the porous membrane, covering the tissue with a very thin layer and the tissue grows in the air-fluid interface. The cells live and retain their normal architecture but fibrosis has been shown to develop in the slice over a period of 1 – 2 weeks. Different drugs can be added to the culture medium, simulating the effect of drugs on the liver *in vivo*. The slice of liver tissue is then removed from the culture medium and processed to look for the presence of fibrosis or the collagen precursor, α smooth muscle actin (αSMA), which has been shown in the model to correlate well with fibrosis. This serves as a model for liver fibrosis *in vivo*. Other models of liver fibrosis use the whole animal or single cell cultures. Using whole animals is expensive and time consuming and also raises ethical issues about animal experimentation. Cell culture uses either an immortalised cell line, which may not be
representative of the cells from which it was derived, or primary cells which can be difficult to obtain in significant numbers. It looks only at the individual cells studied away from their normal surrounding tissue. The technique of growing slices of liver has the advantage that the cells retain their relationship to the surrounding cells and structures. Drugs can be added directly to the culture system to see their effect on fibrosis development in the liver slices. It is also much quicker than studying liver fibrosis in humans which usually takes years to develop.

The aim of the study was to culture human liver tissue in the same way in which rat tissue had been cultured previously and to see if the addition of certain drugs (sirolimus, cerivastatin, cyclosporin and pioglitazone) had any effect on the development of fibrosis. Cyclosporin was chosen because there are still many patients taking it following liver transplantation. Cerivastatin was chosen because of a suggestion that it may be antifibrotic by having an effect on fibroblast apoptosis (183) and pioglitazone as a positive control following publication of evidence that it is antifibrotic in rat models of hepatic fibrosis (184).

Normal adult human liver tissue can be obtained from liver resections being performed for metastatic colon cancer because either half of the liver is removed and the normal liver surrounding the metastases is discarded in the pathology department or individual metastases are removed with a small margin of normal tissue surrounding them. If this was not successful then the experiment could be done using rat tissue instead.

Ethical approval was granted to obtain normal liver tissue from patients undergoing liver resection for metastatic disease and for this to be grown in a culture system. It has also been obtained previously for the culture of liver slices from neonatal rat tissue in Southampton.
Method

Cubes of adult liver tissue 1cm$^3$ were obtained from 2 liver resection operations on separate occasions at Addenbrooke’s hospital and placed immediately into cold University of Wisconsin (U-W) solution (this is the solution normally used to perfuse livers being removed for transplantation). They were transported immediately to Southampton for slicing and culture.

The liver cubes were cut into slices 100µm thick and placed on the nitrocellulose membrane in the culture medium. To ascertain viability and see if this was likely to be a useful technique with adult human tissue, no drugs were added to the culture but the slices were removed and processed at 24 – 48 hour intervals over the following 2 weeks.

In both of these human liver experiments the tissue had died by 48 hours after slicing.

There are 3 possible reasons for this. The first is that because of the nature of the operation there is a prolonged warm ischaemic time of the tissue. The arterial and venous supplies must be clamped prior to dissection of the liver, making a prolonged warm ischaemic time inevitable in which damage to the liver will occur. Ethical approval to perform the operation in a different way purely to obtain better quality tissue for research would probably be very difficult to obtain. The second reason is the prolonged cold ischaemic time. Liver tissue was not available in the centre in which the slicing facilities were located and the slicing machine could not be relocated. There was therefore a much longer cold ischaemic time than when neonatal tissue was used from a local source. The third reason is that adult liver tissue was used. Experiments in Southampton have given very variable results with adult rat tissue and it may be that the technique will only ever be reliable when performed with neonatal
tissue. We felt that we were unlikely to be able to change the technique sufficiently to improve the tissue viability to an acceptable level and further experiments were therefore undertaken with neonatal rat tissue which is known to survive in this culture system.

Neonatal rat liver was removed from 8 – 10 day old Wistar rat pups. The liver was placed in cold phosphate buffered saline (PBS) until it was processed for cutting 30 minutes later. The liver was cut into small pieces about 5mm$^3$ and placed in warm agarose gel which set as it cooled. The agarose blocks with liver tissue embedded were then sliced into 100µm thick slices on a Krumdieck tissue slicer. The resulting slices were placed on the nitrocellulose membrane and into the culture medium. Culture medium consisted of Dulbecco’s modified eagles medium with glutamine which was supplemented with 10% fetal calf serum (FCS), 5% penicillin-streptomycin-gentamycin solution, 5µg/mL insulin, 0.4µg/mL dexamethasone, 10mmol/L HEPES buffer solution, 50µg/ml ascorbic acid and 20mmol sodium pyruvate. Drug solution was added to the culture medium at different concentrations (see table 8.1).

The concentrations of sirolimus and cyclosporin used were chosen to cross the therapeutic range used in humans although the degree of drug binding to FCS is not known. The cerivastatin concentration was chosen after consulting company literature on concentrations which might be seen in blood and pioglitazone concentrations from published literature.
Table 8.1.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentrations used</th>
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<tbody>
<tr>
<td>Sirolimus</td>
<td>2, 10, 50 ng/ml</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>1, 5, 25 ng/ml</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>50, 100, 500 ng/ml</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>1, 5, 25 μM</td>
</tr>
</tbody>
</table>

*Table 8.1. Drugs used in the culture system and the concentrations used.*

The liver slices were incubated at 37°C for 10 days with the medium being changed every 48 – 72 hours. After 10 days the slices were removed from culture and placed in acetone plus enzyme inhibitors (2mM phenylmethylsulphonyl fluoride and 20mM iodoacetamide) at -20°C overnight and then processed into glycol methacrylate (GMA). This was frozen at -20°C until it was sliced on a microtome into 2μm thick sections. These slices were stained with haematoxylin and phloxine (H&P) and αSMA and examined under the microscope for hepatocyte viability and αSMA positivity.

Each experiment was performed 3 times with 2 culture wells on each 6 well plate being used for control samples and 2 for the highest concentration of drug. 1 well on each plate was used for the intermediate drug concentrations.
Results

Slides were examined under the microscope for hepatocyte viability (H & P stain, figure 8.1) and the presence of αSMA (special immunostain, figure 8.2) by 2 experienced observers who were not blinded. Results for sirolimus are shown below in figures 8.3 and 8.4.

Figure 8.1

Figure 8.1. An H & P stain on rat liver. This shows the cell architecture at an early stage when most cells are viable.
Figure 8.2

Figure 8.2. An $\alpha$SMA stain on rat liver. The brown staining is positive for $\alpha$SMA.

Figure 8.3.

Figure 8.3. Mean percentage hepatocyte viability for each concentration of sirolimus (0 – 50 ng/ml) with 95% confidence interval. $N$ is the number of slices examined for each concentration.
Figure 8.4. The slices were graded 0 (low) – 3 (high) in terms of αSMA positivity. Individual bands were not counted, so these scores are on an abstract scale with 95% confidence interval for each concentration of sirolimus. N is the number of slices examined.

The first graph of viability for each concentration of sirolimus shows the effect on cell viability when sirolimus is added to the culture system. It is clear that there is a large overlap in the error bars and there is no effect seen. The second graph shows the αSMA positivity in each slice and again there is a large overlap in the error bars and there is no difference between any of the concentrations studied.

The results for cyclosporin, cerivastatin and pioglitazone were similar, with very little αSMA being generated in the control slices and consequently no inhibition of this
being seen. There was also no increase in αSMA positivity seen at any of the concentrations of any of the drugs.

**Summary**

- The technique used for culturing liver slices has improved so much that they do not develop fibrosis over 10 days as had previously been demonstrated.
- No difference was seen in the lack of fibrosis development with any of the drugs studied.

**Discussion**

This is a new technique which is still being refined and it is in its early stages. It may be promising for the future if fibrosis and αSMA can be shown to develop reproducibly, either spontaneously or by the addition of a fibrotic stimulus such as a chemical or a virus.

The initial plan was to use human adult tissue obtained in Cambridge but unfortunately this did not work. Experiments in Southampton using adult rat tissue have given variable results with lower viability after 2 weeks than neonatal tissue, suggesting that the technique may only work with neonatal tissue. There is also the problem of the prolonged cold ischaemic time before slicing (4 – 5 hours) during transport of the samples to Southampton. This compared to the cold ischaemic time before slicing of 30 minutes in the neonatal rat tissue which could be obtained locally.

It is unfortunate for these experiments that the technique using neonatal rat tissue has been developed sufficiently well that under current laboratory conditions fibrosis does not develop by day 10 as it had been shown to do previously. This is probably due to the refinement of the technique, especially the use of the tissue slicer but also finding
the best culture conditions for the liver slices, particularly the concentrations of the culture medium supplements.

It is also noteworthy that none of the drugs induced fibrosis development in the liver slices. This is to be expected for sirolimus and pioglitazone, both of which have been demonstrated to have an anti fibrotic effect in *in vitro* and animal models (107, 184), but there is no such evidence for cyclosporin or cerivastatin. Indeed tacrolimus (which binds to a different intra-cellular signalling molecule but also acts on calcineurin phosphatase in the same way as cyclosporin) has been shown to exert a pro fibrotic effect in an *in vivo* carbon tetrachloride rat model (108). It would be interesting to look at the effect of tacrolimus in this culture model where it might be expected to induce some fibrosis and αSMA expression, but none was available at the time of the experiments.

In summary this is a technique which may prove useful in the future to screen drugs for an anti-fibrotic effect, either in the liver slices as fibrosis develops over a longer time, or as fibrosis develops in response to a suitable stimulus. It will however need further development to ensure that fibrosis develops reproducibly before it can be used in this way.

Unfortunately, due to the constraints of time and the distance to Southampton, I was unable to carry out this work over a prolonged period of time. As a result of this, a cell culture model of fibrosis was investigated and is described in chapter 9.
Chapter 9. The effect of sirolimus in a cell culture model of fibrosis

Introduction

There is potential for many drugs to exert an anti-fibrotic effect in the body but it is time consuming and expensive to demonstrate this in vivo in both animals and humans. Animal models are useful in providing data in a physiological setting, but because of the cost and time involved, are not ideal for screening drugs for properties such as their effect on fibrosis development. Because fibrosis normally takes many years to develop, animal models are generally used only if the animals are given a fibrotic stimulus such as intra-peritoneal carbon tetrachloride injections. Drugs may then be given to see if this effect is modified. Cell culture is useful for the purpose of screening drugs outside a physiological setting, but its limitations must be realised. Cells are grown in isolation in a plastic flask under sterile conditions. This allows the effect of a drug on an individual cell to be studied, but the results are not necessarily reproducible in an in vivo system. Cell culture does however provide a useful method for screening for drug effects on gene expression as evidenced by specific proteins, enzymes or RNA produced by the cell in response to an exogenous stimulus.

There are 2 general types of cells which can be used in cell culture experiments. The first is primary cells which have been isolated from a human or animal and are then grown under cell culture conditions. They have a limited life span before undergoing cell senescence when they stop dividing and die. They have to be used at early passages (when the cells are divided and split into new flasks) if they are to produce consistent results. Because of their limited life span, primary cells are more difficult to
use and more expensive to obtain. The second type of cell which can be used is an immortali
cell line. This is generated by infecting cells with a virus, usually a simian virus, which prevents them undergoing senescence. The cells should then keep dividing and not die, provided they are kept under cell culture conditions. This does not always work in practice, however, and sometimes cell lines can lose their phenotype after repeated passages. It is also possible for them to become contaminated over time which may alter their gene expression. There is a wide choice of cell lines available because of their longevity.

As discussed before, there is evidence that sirolimus exerts an anti-fibrotic effect in a rat model of fibrosis (107) but the mechanism of action is not clear. It could work on the collagen matrix either by reducing its production or by enhancing its resorption.

We have used a fibroblast cell line to look at the effect of sirolimus on the expression of the collagen 1 gene and the matrix metalloprotease 1 (MMP-1) gene. These are both genes which could have an effect on the collagen matrix in the liver, the collagen gene by regulating collagen deposition and the MMP-1 gene by regulating matrix re-absorption.

**Methods**

MRC-5 cells (derived from a human fetal lung fibroblast cell line) which had been transformed with the SV40 virus to prevent the onset of senescence were used in a cell culture system. A range of cytokines were screened initially to see if collagen and MMP-1 gene expression were stimulated by their addition and if so, whether the gene expression was inhibited by a fixed concentration of sirolimus (100 ng/ml). These cytokines were epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), interleukin 1 (IL-1), transforming growth
factor beta (TGF-β) and insulin like growth factor 1 (IGF-1). EGF, PDGF and IGF-1 were all used at a concentration of 10 ng/ml. IL-1, TGF-β and bFGF were used at 1 ng/ml. These concentrations were chosen because they would be expected to give a maximal stimulation of cells if they are going to respond to the stimulus (personal communication, Dr. A. Corps). Sirolimus was used initially at 100 ng/ml because this concentration was thought sufficiently high to inhibit any actions of growth factors and could then be titrated down to lower concentrations in later experiments. Sterile cell culture conditions and reagents were used throughout.

Stock cells were cultured at 37°C in 75cm² culture flasks in Dulbecco’s modified Eagle medium (DMEM) supplemented with L-glutamine, 10% fetal calf serum (FCS) and penicillin-streptomycin. The doubling time was found to be approximately 36 hours and the cells required passaging at least once per week. Cells in the stock flasks were plated out at 2x10⁵ cells per flask and reached confluence by 1 week later.

Cells used for the experiments were taken from the passages and plated out into 6 well plates at a concentration of 1x10⁵ cells per well. This concentration was chosen to allow the cells to divide and reach confluence without becoming overcrowded. They were allowed to grow in serum free medium for 24 hours with any growth factors or drug to be studied before harvesting. The medium used was serum free because sirolimus may bind unpredictably to FCS, reducing the amount of free drug available to the cells. After 24 hours growth the cells were harvested with trizol reagent, a phenol based reagent which dissolves the cell walls. The resulting product was then processed further with phenol-chloroform separation followed by precipitation with iso-propanol and ethanol to extract the RNA, either at the same time or at a later date after freezing the trizol solutions at -20°C. The end result of harvesting was an RNA pellet which was then dissolved in water and frozen at -20°C. The RNA was analysed
in triplicate by one step RT-PCR, a method of quantifying the amount of a specific RNA protein present in a sample (185). Briefly, 2 primers are used to amplify the RNA sequence that is being examined and a probe binds to the RNA as it is generated, becoming fluorescent on binding. After each cycle of the RNA amplification the fluorescence is measured. The time (number of cycles) for the fluorescence to reach a given level is recorded and is related to the concentration of that RNA sequence in the original sample (which is then calculated) and therefore the level of gene expression in the cells over their period of culture. Gene expression was compared to GAPDH gene expression which was used to control for cell number and the amount of RNA recovered in the extraction process. This is a commonly used “housekeeping” gene because expression is not thought to be influenced by external stimuli. All primers and probes used have been described previously (186).

After the preliminary experiments, it was found that the greatest stimulation of the cells was seen in the presence of IL-1 and this was used in the subsequent experiments to stimulate the cells.

Cells were grown under 2 different conditions. The first was with a variable concentration of IL-1 and a fixed concentration of sirolimus and then with a fixed concentration of IL-1 and variable concentrations of sirolimus. Concentrations of IL-1 used were 1, 10, 100 and 1000 pg/ml with a sirolimus concentration of 1 ng/ml. Each of these experiments was performed 3 times. The experiments were then performed with the concentration of IL-1 fixed at 1 ng/ml and the concentrations of sirolimus varying. Sirolimus was used at concentrations of 0.01, 0.1, 1, 10 and 100 ng/ml.
Results

All results are expressed as a ratio of the collagen or MMP-1 RNA being studied divided by the amount of GAPDH RNA present so that variability in cell count and amount of RNA extracted from the cells is controlled for. Absolute amounts are generally considered to be meaningless, mainly because of the potential for different amounts of RNA to be extracted from the culture wells in the separation process.

The RNA extracts from experiment 1 were analysed for collagen 1 RNA and MMP-1 RNA in response to a variety of different stimuli. These stimuli were also studied in the presence of sirolimus at a fixed concentration. The results from experiment 1 are shown in table 9.1 below.
Table 9.1.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Collagen 1 RNA</th>
<th>MMP-1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.13</td>
<td>0.50</td>
</tr>
<tr>
<td>EGF</td>
<td>2.04</td>
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<td>PDGF</td>
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<td>IL-1</td>
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</tr>
<tr>
<td>TGF-β</td>
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<td>0.49</td>
</tr>
<tr>
<td>IGF-1</td>
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<td>1.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Collagen 1 RNA</th>
<th>MMP-1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>0.53</td>
</tr>
<tr>
<td>EGF</td>
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<td>PDGF</td>
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<tr>
<td>IL-1</td>
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<td>TGF-β</td>
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</tr>
<tr>
<td>IGF-1</td>
<td>1.99</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 9.1. Results from experiment 1 examining the effect of several different growth factors on SV40 transformed MRC-5 cells in the presence or absence of sirolimus at a concentration of 1 ng/ml. Each number is the amount of RNA present divided by the amount of GAPDH RNA present in the same sample.

As can be seen from the results in table 9.1, IL-1 (1 ng/ml) stimulated a rise in collagen 1 RNA production which was almost 7 times greater than the control. This rise was reduced to just greater than 2 times control in the presence of sirolimus. The other growth factors produced much less stimulation. A similar rise in MMP-1 RNA production was seen which was reduced in the presence of sirolimus. The experiment was repeated (experiment 2) and the results are in shown in table 9.2.
Table 9.2. Results from experiment 2 examining the effect of several different growth factors on SV40 transformed MRC-5 cells in the presence or absence of sirolimus at a concentration of 1 ng/ml.

As can be seen from the results shown in table 9.2, the stimulation of collagen 1 RNA production was only 3 times that of the control and this was reduced to 2 times that of the control in the presence of sirolimus. The MMP-1 RNA expression was reduced to a sixth of the level seen in the control but some of this inhibition was removed in the presence of sirolimus. Clearly these results are not consistent with the findings in the first experiment and the RT PCR step was repeated on the RNA extracts, giving very similar results therefore suggesting that the cells had behaved differently in each experiment.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Collagen 1 RNA</th>
<th>MMP-1 RNA</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.49</td>
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<td>EGF</td>
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<td>Control</td>
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<td>EGF</td>
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<td>PDGF</td>
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<tr>
<td>IGF-1</td>
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<td>2.02</td>
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experiment. A third experiment was then set up to titrate the concentration of IL-1 used in the presence or absence of sirolimus. The concentrations used were 1, 10, 100 and 1000 pg/ml. FGF at 1 ng/ml was also used to see if this had any effect. The concentration of sirolimus was kept unchanged at 1 ng/ml. The results from experiment 3 are shown in table 9.3 below. Due to a technical problem with the analysis, the MMP-1 RNA was only analysed for the control, highest concentration of IL-1 and the FGF.
Table 9.3. Results from experiment 3 examining the effect of different concentrations of IL-1 and FGF at 1 ng/ml on SV40 transformed MRC-5 cells in the presence or absence of sirolimus at a concentration of 1 ng/ml.

As can be seen from the results, there was no stimulation of collagen 1 RNA production by IL-1 and the presence of sirolimus did not alter the levels of RNA measured. IL-1 stimulated the production of MMP-1 RNA to 10 times the control level and in the presence of sirolimus this was stimulated a further 5 times. The control levels are however low, so a small variability in these would make the effect of a growth factor or sirolimus look very large. In view of the variability of the collagen 1 RNA results and the apparent loss of stimulation with IL-1 further

<table>
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<tr>
<th></th>
<th>Collagen 1 RNA</th>
<th>MMP-1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth factor</strong></td>
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</tr>
<tr>
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<td>0.03</td>
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<tr>
<td>IL-1 10 pg/ml</td>
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<td>IL-1 100 pg/ml</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>IL-1 1 ng/ml</td>
<td>1.50</td>
<td>0.30</td>
</tr>
<tr>
<td>FGF</td>
<td>1.15</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Growth factor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ sirolimus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1 100 pg/ml</td>
<td>2.53</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-1 1 ng/ml</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>FGF</td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.53</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>4.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>
experiments were conducted only on MMP-1. FGF stimulated collagen production in the presence of sirolimus but not in its absence and stimulated MMP-1 production in the absence of sirolimus but inhibited its production in the presence of sirolimus. This experiment was repeated only looking at MMP-1 RNA levels and the results are shown in table 9.4 below.

**Table 9.4.**

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>MMP-1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.19</td>
</tr>
<tr>
<td>IL-1 1 pg/ml</td>
<td>0.19</td>
</tr>
<tr>
<td>IL-1 10 pg/ml</td>
<td>0.17</td>
</tr>
<tr>
<td>IL-1 100 pg/ml</td>
<td>0.77</td>
</tr>
<tr>
<td>IL-1 1 ng/ml</td>
<td>1.82</td>
</tr>
<tr>
<td>FGF</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Table 9.4. Results from experiment 4 examining the effect of different concentrations of IL-1 and FGF at 1 ng/ml on SV40 transformed MRC-5 cells in the presence or absence of sirolimus at a concentration of 1 ng/ml.
These results show that there is a rise in MMP-1 RNA production stimulated by IL-1 of 9 times. In the presence of sirolimus this level rises by a further 6 times at the highest concentrations of IL-1 with the larger rises being seen with the higher doses of sirolimus. FGF stimulated a rise of 2½ times with no additional effect in the presence of sirolimus. A further experiment was performed using a higher concentration of IL-1 (5 ng/ml) instead of FGF. The results are shown in table 9.5 below.

Table 9.5.

<table>
<thead>
<tr>
<th>Growth factor only</th>
<th>MMP-1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.16</td>
</tr>
<tr>
<td>IL-1 1 pg/ml</td>
<td>0.20</td>
</tr>
<tr>
<td>IL-1 10 pg/ml</td>
<td>0.15</td>
</tr>
<tr>
<td>IL-1 100 pg/ml</td>
<td>0.80</td>
</tr>
<tr>
<td>IL-1 1 ng/ml</td>
<td>1.67</td>
</tr>
<tr>
<td>IL-1 5 ng/ml</td>
<td>1.73</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth factor + sirolimus</th>
<th>MMP-1 RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.21</td>
</tr>
<tr>
<td>IL-1 1 pg/ml</td>
<td>0.26</td>
</tr>
<tr>
<td>IL-1 10 pg/ml</td>
<td>0.39</td>
</tr>
<tr>
<td>IL-1 100 pg/ml</td>
<td>1.18</td>
</tr>
<tr>
<td>IL-1 1 ng/ml</td>
<td>4.57</td>
</tr>
<tr>
<td>IL-1 5 ng/ml</td>
<td>7.20</td>
</tr>
</tbody>
</table>

Table 9.5. Results from experiment 5 examining the effect of different concentrations of IL-1 on SV40 transformed MRC-5 cells in the presence or absence of sirolimus at a concentration of 1 ng/ml.
This experiment showed a 10 fold stimulation of MMP-1 RNA by IL-1 at 1 ng/ml with very little extra stimulation at 5 ng/ml. In the presence of sirolimus there was an extra doubling of MMP-1 RNA production at the 1 ng/ml concentration and a 4 fold increase at the 5 ng/ml concentration.

In summary, these experiments so far have shown quite a large degree of variability in the results. There is a suggestion in the later experiments that IL-1 stimulated the production of MMP-1 RNA and that this is further stimulated in the presence of sirolimus. In some of the experiments there is a systematic concentration effect with higher levels of MMP-1 being produced at higher concentrations of sirolimus. Because MMP-1 is related to fibrous matrix breakdown, this could provide a possible explanation for the anti-fibrotic effect of sirolimus. The other growth factors studied had much smaller effects and the results obtained looking at collagen RNA production were variable. 3 further experiments (experiments 6 – 8) were performed to examine the effect of different concentrations of sirolimus in the presence of a fixed concentration of IL-1. Sirolimus was used at concentrations of 0.01, 0.1, 1, 10 and 100 ng/ml. The results are shown in table 9.6 below.
Table 9.6.

<table>
<thead>
<tr>
<th></th>
<th>MMP-1 RNA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 6</td>
<td>Experiment 7</td>
<td>Experiment 8</td>
</tr>
<tr>
<td>Control</td>
<td>0.06</td>
<td>0.26</td>
<td>0.42</td>
</tr>
<tr>
<td>Sirolimus 0.01ng/ml</td>
<td>0.15</td>
<td>0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>Sirolimus 0.1ng/ml</td>
<td>0.09</td>
<td>0.18</td>
<td>0.55</td>
</tr>
<tr>
<td>only</td>
<td>Sirolimus 1ng/ml</td>
<td>0.10</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Sirolimus 10ng/ml</td>
<td>0.04</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Sirolimus 100ng/ml</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td>Control</td>
<td>1.83</td>
<td>0.89</td>
<td>1.24</td>
</tr>
<tr>
<td>Sirolimus 0.01ng/ml</td>
<td>2.21</td>
<td>0.83</td>
<td>1.55</td>
</tr>
<tr>
<td>Sirolimus 0.1ng/ml</td>
<td>2.24</td>
<td>1.12</td>
<td>2.17</td>
</tr>
<tr>
<td>+ IL-1</td>
<td>Sirolimus 1ng/ml</td>
<td>8.72</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>Sirolimus 10ng/ml</td>
<td>7.77</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Sirolimus 100ng/ml</td>
<td>5.55</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Table 9.6. Results from experiments 6 – 8 examining the effect of different concentrations sirolimus on SV40 transformed MRC-5 cells in the presence or absence of IL-1 at a concentration of 1 ng/ml.

Experiment 6 shows that sirolimus on its own in the absence of a growth factor has little or no effect on the MMP-1 RNA production. In the presence of IL-1 however, there is a potentiation of MMP-1 RNA production with larger amounts of MMP-1 being produced at higher concentrations of sirolimus. In experiments 7 and 8 this effect has been lost, with no effect being seen of increasing concentrations of sirolimus.
One further test was performed on the cell culture supernatants from experiment 6. It was felt to be important to ensure that RNA production related to protein production and a western blot for MMP-1 was therefore performed on 1 set of supernatants (by Dr. A. Trull). This was performed on the supernatants which had been aspirated from the cells in experiment 6 immediately prior to harvesting and stored at -20°C. The western blot is shown in figure 9.1 and densitometry figures derived from the blot are shown in table 9.7.

**Figure 9.1.**

*Figure 9.1. Western blot of cell culture supernatants for MMP-1 from experiment 6. The first (darkest) channel is the MMP-1 positive control. The next 6 channels are with increasing concentrations of sirolimus in the presence of IL-1. The next 6 channels are with increasing concentrations of sirolimus in the absence of IL-1.*
<table>
<thead>
<tr>
<th></th>
<th>MMP-1 band density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27,000</td>
</tr>
<tr>
<td>Sirolimus 0.01ng/ml</td>
<td>25,904</td>
</tr>
<tr>
<td>Sirolimus + IL-1</td>
<td></td>
</tr>
<tr>
<td>Sirolimus 0.1ng/ml</td>
<td>32,812</td>
</tr>
<tr>
<td>Sirolimus 1ng/ml</td>
<td>40,426</td>
</tr>
<tr>
<td>Sirolimus 10ng/ml</td>
<td>41,282</td>
</tr>
<tr>
<td>Sirolimus 100ng/ml</td>
<td>34,526</td>
</tr>
<tr>
<td>Control</td>
<td>10,975</td>
</tr>
<tr>
<td>Sirolimus 0.01ng/ml</td>
<td>10,882</td>
</tr>
<tr>
<td>Sirolimus only</td>
<td></td>
</tr>
<tr>
<td>Sirolimus 0.1ng/ml</td>
<td>10,233</td>
</tr>
<tr>
<td>Sirolimus 1ng/ml</td>
<td>8,306</td>
</tr>
<tr>
<td>Sirolimus 10ng/ml</td>
<td>5,483</td>
</tr>
<tr>
<td>Sirolimus 100ng/ml</td>
<td>3,542</td>
</tr>
</tbody>
</table>

Table 9.7. This shows the density of the bands seen in Figure 9.1.

The western blot densitometries correlate with the RNA analysis from experiment 6 ($\rho = 0.886, p = 0.019$). They show an increase in MMP-1 production with increasing concentrations of sirolimus in the presence of IL-1. This demonstrates that in this experiment, the concentrations of MMP-1 RNA did reflect the presence of the MMP-1 protein. Because of this good correlation Western blots were not performed on all of the sets of supernatants.
Summary

- Cell culture initially demonstrated that sirolimus altered collagen 1 and MMP-1 gene expression in the MRC5 cell line.
- These results were found to be unreliable in subsequent experiments and no explanation was found for their variability.

Discussion

In these cell culture experiments an immortalised cell line has been used to study the effect of sirolimus on the expression of 2 genes, collagen 1 and MMP-1. The initial experiments appeared to show that sirolimus might inhibit the expression of the collagen 1 gene and it might potentiate expression of the MMP-1 gene. Although the effect looked quite large, it appeared to be lost in subsequent experiments. In some of the experiments (4, 5 and 6) there is a systematic concentration effect of both IL-1 and sirolimus with larger amounts of MMP-1 being produced at higher concentrations. The effect is not apparent in experiments 7 and 8 despite the same cells being used under the same conditions.

We were unable to explain the variability of the results in the cell culture system. Aliquots of sirolimus and growth factors were made at an early stage so that they would not undergo repeated freeze-thaw cycles and other culture conditions were kept the same from one experiment to the next. Cells were not allowed to become overcrowded in the flasks or culture plates and when experiments were repeated there were no apparent differences in the conditions used. We therefore presumed that there must be an effect on the cells after repeated passaging causing aging of the cell line and that the cells lost their ability to be stimulated in the way in which they had been in earlier experiments.
These are therefore interesting preliminary experiments that could be taken further, perhaps with some adjustment to the methods. If the same cell line were to be used, cells could be removed from the freezer at intervals and used at the same number of passages after thawing for different experiments. This should reduce the chance of the cells losing their phenotype or losing the ability to be stimulated in a reproducible way. Alternatively another cell line could be used which has previously been documented to demonstrate reproducible effects over repeated passages. The work could also be done on primary cells used at early passages before they become senescent. A fuller range of matrix metalloproteases could be assayed together with their inhibitors to get a clearer picture of the effect that sirolimus is having on the components of fibrosis control. It would be particularly important to measure tissue inhibitor of metalloprotease 1 (TIMP-1) because this is known to bind to MMP-1 and prevent it acting as a collagenase, but we have not measured it in these samples because the results would be difficult to interpret due to the variability seen in the results described above.

If the cell line culture could be made to work in a reproducible way, it would be interesting to investigate other stimuli such as heparin, TNF or other cytokines and the effect which sirolimus may have on them.

It is difficult to draw any conclusions from these experiments because of the variability of the results. Combining the results of some experiments, sirolimus could cause inhibition of collagen 1 gene expression and increased secretion of MMP-1. Looking at other experiments it may have no effect on either of these genes. In view of the unreliability of the results, therefore, no further experiments were performed with this cell line.
Chapter 10. Final discussion

Liver transplantation is an effective treatment for end stage chronic liver disease and most liver transplant recipients receive a calcineurin inhibitor in the long term for immunosuppression post transplantation. These studies have been designed to investigate the effect of a different immunosuppressive drug, sirolimus, on the transplanted liver and investigate some of the effects of the drug which have previously been observed. They were based on observations made at Addenbrooke’s hospital during the first trial of sirolimus after liver transplantation and were thought to be feasible over a short period because of the rapid rate at which fibrosis has been seen to progress in transplant recipients with hepatitis C. The object of the studies was aimed particularly at investigating any anti-fibrotic effect which sirolimus may exert on the liver and the mechanisms by which it may do this. I have also gathered 5 year follow up data on the original cohort of patients treated with sirolimus and studied the effect of sirolimus on renal function, EGF, vEGF, serum markers of fibrosis and neutrophil activation. Because the clinical study was a pilot study, the number of patients involved is small, making the statistical significance of any findings less likely. The relatively short follow-up period of most patients (1 year) also limits the degree of certainty with which the clinical findings can be extrapolated to the longer term although the follow up data available at 2 years in the 6 patients for whom it was available looks very good.

I have demonstrated a beneficial effect on renal function in patients treated with sirolimus compared to those taking a calcineurin inhibitor over a period of 3 years. Most liver transplant recipients develop some degree of renal impairment over time, which is caused by the calcineurin inhibitor used for immunosuppression. In patients
in whom this appears to be becoming a problem as assessed by a rising creatinine level, switching to sirolimus is likely to provide a long term benefit to renal function.

The effect of sirolimus on the progression of graft fibrosis caused by recurrent hepatitis C virus infection is also significant. Despite the small number of patients and short follow up a significant reduction in fibrosis progression when compared to historical controls is seen. In time, the effect may become even more pronounced as evidenced by the effect seen in the patients for whom biopsy data at 2 years is available. Further analysis will need to be undertaken on the future biopsy data of these patients over the coming years, perhaps after 3 and then 5 years of follow up. Although the dose dependent effect demonstrated in the in vitro studies in Southampton suggests that patients may get more benefit from higher doses of sirolimus there seems to be a good effect seen using lower doses and higher levels may be at the expense of increased toxicity.

Sirolimus appears to be a safe drug to use after transplantation in these patients and the 2 week overlap period when taking sirolimus in addition to a calcineurin inhibitor has not caused any problems (such as opportunistic infection). The patient who died while taking sirolimus was not helped by maximal therapy with other drugs and he was in a patient group with a very poor expected outcome. On the basis of this study, therefore, we would recommend that patients switching to sirolimus from a calcineurin inhibitor can do so safely with a short period of overlap in which the 2 drugs are given simultaneously.

We have now performed a power calculation for a randomised controlled trial based on the fibrosis stage in the liver biopsies taken 1 year apart as compared to the historical cohort. If the drug were to have the same effect in patients switched and the controls behaved in the same way as the historical cohort, 72 patients would need to
be included into the trial (36 randomised to each arm) to give 80% power of detecting a difference between the 2 arms, at the 5% significance level. It is likely that a smaller number of patients could be enrolled if a biopsy taken at 2 years were to be used although a meaningful power calculation is not possible from 6 patients.

It is clear from the measurement of the serum markers of fibrosis, hyaluronic acid, MMP-2 and TIMP-1, that these will not replace liver biopsy as the current standard for assessing graft fibrosis in transplant recipients. Although the numbers are small, the correlation is so poor that it does not appear to be worth investigating further and future assessment of graft fibrosis will continue to be by liver biopsy unless other markers prove to be reliable in the transplant population. Clearly they will have to be examined specifically in these patients in addition to any other settings in which they might be used.

Several laboratory based experiments have been undertaken to further investigate the actions of sirolimus. The first of these was examining the effect on neutrophil activation in response to a stimulus. The magnitude of the oxidative burst was reduced by 25%, suggesting that this is a possible mechanism by which sirolimus could cause increased susceptibility to infection. The other laboratory studies were less successful. The investigation of collagen deposition and resorption in a cell line yielded inconsistent results and was discontinued because of this. The growth of slices of liver tissue in Southampton did not yield any useful results because of refinement of the technique to the point where the control slices of liver tissue did not develop fibrosis, meaning that there was no fibrosis for sirolimus to inhibit in the model.

In summary, these studies have demonstrated that sirolimus can be used safely after liver transplantation with a short overlap period with a calcineurin inhibitor. Sirolimus also has a beneficial effect on graft fibrosis over 1 – 2 years and renal function in the
long term. Further analysis will be undertaken in the future to investigate the longer term effect on graft fibrosis in these patients. We also have sufficient data to design a randomised trial in which other UK centres will be invited to participate. If the same effect were to be seen in subsequent patients switched to sirolimus, this would need to include a total of 72 patients, half of whom would switch to sirolimus.
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