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Molecular targets for existing and novel immunosuppressive drugs

Stanislaw M. Stepkowski

Anti-neoplastic cytostatic antiproliferative agents, such as methotrexate, 6-mercaptopurine and cyclophosphamide, were originally used as immunosuppressive drugs. Although these agents induced only modest anti-rejection activity, they caused serious non-specific bone marrow suppression, impairing host resistance and increasing the incidence of infections. Unlike these non-selective agents, cyclosporine A, tacrolimus and sirolimus act more selectively on different stages of the T-lymphocyte (T-cell) and B-lymphocyte (B-cell) activation cycles; however, cyclosporine and tacrolimus are nephrotoxic, whereas sirolimus causes hypertriglyceridaemia. Thus, despite this progress, continued efforts must be made to develop and test new, potentially very selective agents. The agent 15-deoxyspergualin moderately inhibits both mitogen-stimulated T-cell proliferation and the generation of cytotoxic T lymphocytes (CTLs) but does not affect the production of interleukin 2 (IL-2). Another drug, FTY720, has a unique action to prevent rejection, by altering the homing of lymphocytes to the lymphoid compartments. The newest members of the family of antiproliferative agents, namely mycophenolate mofetil, leflunomide and brequinar, are potentially more selective than their predecessors. However, the most promising agents are produced using antisense technology. This approach involves the design of antisense oligodeoxynucleotides; these novel drugs are designed to block allograft rejection by blocking selected messenger RNA (mRNA). This review outlines the mechanisms of action, the limitations of application and the molecular or cellular targets of traditional agents, newly developed drugs and also antisense technology, which is an example of a new application of molecular medicine.

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The first first-generation immunosuppressive drug to be used for organ transplantation, namely 6-mercaptopurine, was originally developed as a cytostatic and antiproliferative agent against neoplastic growth (Ref. 1). Although 6-mercaptopurine prevented allograft rejection in animals, its imidazol derivative, azathioprine, proved to be less toxic and was therefore used clinically for kidney transplantation (Ref. 2). In spite of its lower toxicity, however, azathioprine caused severe depression of the bone marrow and failed to block pre-sensitised T cells. In fact, the widespread incorporation of an active azathioprine metabolite, 6-thioinosinic acid, into both DNA and RNA nucleic acids causes chromosomal breakage in many different types of cells (Ref. 3). Significant progress in transplantation began with the discovery of the second generation of T-cell-selective inhibitors; these included cyclosporine A, tacrolimus and sirolimus. However, cyclosporine and tacrolimus display overlapping nephrotoxicities, whereas sirolimus produces hypertriglyceridaemia. 15-Deoxyspergualin, another T-cell-selective inhibitor, is of limited use for transplantation owing to its low bioavailability and considerable toxicities. The in vivo effects in humans and the mechanism of action of the new agent FTY720 are as yet unknown. Recently, more-selective inhibitors of nucleotide synthesis have been developed, including mycophenolate mofetil, brequinar and leflunomide; these agents constitute the second generation of antiproliferative immunosuppressive drugs. However, although mycophenolate mofetil significantly decreases the incidence of rejections in recipients of kidney allografts, it appears to have no effect on long-term graft survival rates, whereas both brequinar and leflunomide are significantly toxic.

The field of immunosuppressive therapy now faces a new challenge: to develop a third generation of agents that are highly selective for their targets of action, yet free of toxic side-effects. Newly emerging antisense technology could allow the design of gene-targeted immunosuppressive therapies and eliminate concerns over toxicity. This new technology is based on the design of short (16–22 nucleotides) antisense oligonucleotides (oligos) that have a matching sequence to that of the targeted mRNA. The binding of antisense oligos to the targeted mRNA prevents protein production, thereby selectively blocking cell function. Although such an approach offers unlimited possibilities for target selection, chemical improvements must be made to produce oligos that are more resistant to nucleases and to improve their delivery to the cells.

New agents that non-specifically block nucleotide synthesis

Mycophenolate mofetil

Mechanism of action of mycophenolate mofetil

Mycophenolate mofetil (RS-61443; Syntex, San Jose, CA, USA) is the 2-morpholinoethyl ester of mycophenolic acid, which is produced by several species of the fungal genus *Penicillium* (Ref. 4). In the liver, mycophenolate mofetil is converted by ester hydrolysis to its active metabolite, mycophenolic acid (Ref. 5), which non-competitively and reversibly inhibits the activity of inosine monophosphate dehydrogenase (IMPDH) types I and II during DNA synthesis in the S phase of the cell cycle (Fig. 1). Because the salvage pathway of purine synthesis in lymphocytes is less active than de novo synthesis, lymphocytes depend upon the conversion of 5-phosphoribosyl-1-phosphate (PRPP) to inosine monophosphate (IMP). In turn, IMP is converted to guanosine monophosphate (GMP) by IMPDH. In the salvage pathway, guanine is converted to GMP by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRTase). During T-cell activation, the activity of types I and II IMPDH enzymes increases tenfold (Ref. 6). In contrast with mycophenolate mofetil, azathioprine (Imuran; Burroughs-Wellcome, Research Triangle Park, NC, USA) inhibits several enzymes in the de novo synthesis pathway. The reduction of deoxyguanosine triphosphate concentrations by mycophenolic acid was reversed upon the addition of deoxyguanosine ribonucleotide (Ref. 7).

Clinical and experimental results with mycophenolate mofetil

The pharmacokinetic profiles of recipients of cadaveric-kidney transplants who had been treated with escalating doses of mycophenolate mofetil (100–1750 mg, every second day) were examined. Both the maximum concentration of the drug (C_{max}) in the blood at steady-state (1.0–3.2 µg/ml) and the area under the concentration–time curve over a 24-hour period (AUC_{0-24}; 6.4–37.6 µg/hour/ml) increased in a dose-proportionate fashion. The terminal half-life of the drug in the

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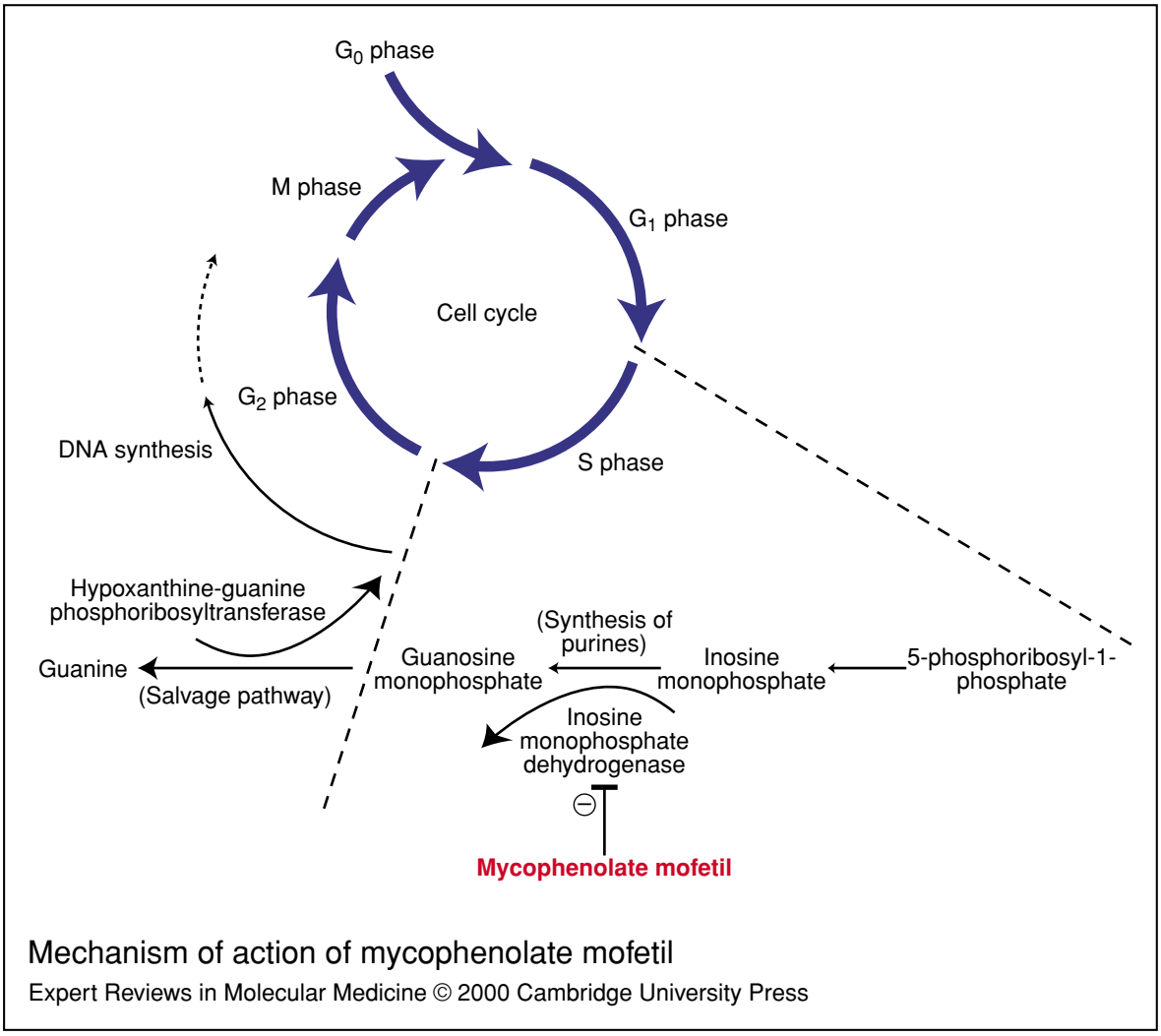

Mechanism of action of mycophenolate mofetil

Figure 1. Mechanism of action of mycophenolate mofetil. The salvage pathway of purine synthesis in lymphocytes is less active than the de novo synthesis of purines. Inosine monophosphate is converted to guanosine monophosphate by inosine monophosphate dehydrogenase. During T-cell activation, the activity of both types I and II inosine monophosphate dehydrogenase enzymes increases by tenfold. Mycophenolate mofetil is converted in the liver by ester hydrolysis to mycophenolic acid, which in turn non-competitively and reversibly inhibits types I and II inosine monophosphate dehydrogenase activity during DNA synthesis in the S phase of the cell cycle. In the salvage pathway, guanine is converted to guanine monophosphate by the enzyme hypoxanthine-guanine phosphoribosyltransferase **(fig001ssh)**.

blood was 2.3–9.6 hours (Ref. 8). In a phase I study of 48 recipients of cadaveric kidneys, mycophenolate mofetil at doses of 100–3500 mg/day caused dose-dependent gastrointestinal toxicity (including gastritis and mild ileus) without any evidence of bone marrow suppression. In another trial, 21 recipients of renal transplants from cadaveric donors were administered 0.25–3.5 g/day of mycophenolate mofetil in combination with a cyclosporine–prednisone regimen, and experienced limited side-effects [diarrhoea, nausea, elevated liver enzymes and an increased rate of infections with cytomegalovirus (CMV); Ref. 9].

In a phase II trial, mycophenolate mofetil was administered to primary recipients of a kidney transplant from a cadaveric donor at doses in the range of 0.1–3.5 g/day in conjunction with anti-lymphocyte globulin (ALG) and cyclosporine and prednisone. The addition of mycophenolate mofetil at doses higher than 2 g/day reduced, in a dose-dependent fashion, the incidence of rejection episodes (Ref. 8). Similarly, mycophenolate mofetil

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reduced the incidence of acute rejection episodes among paediatric recipients of renal allografts from cadaveric donors (Ref. 10), as well as recipients of heart allografts (Ref. 11). In European phase III trials, 491 recipients of kidney transplants were randomised to receive a placebo (n=166) or 2 g/day mycophenolate mofetil (n=165) or 3 g/day mycophenolate mofetil (n=160), in combination with cyclosporine and prednisone therapy (Ref. 12). The incidence of biopsy-proven rejection episodes was reduced from 46.4% among placebo-treated patients to 17.0% for the 2 g/day mycophenolate mofetil group and to 13.8% for the 3 g/day mycophenolate mofetil group. However, 6 months after the transplant, the incidence of graft loss was 6.7% for the 2 g/day mycophenolate mofetil group and 8.8% for the 3 g/day mycophenolate mofetil group, compared with 10.2% for the placebo group. Similar results were observed in a double-blinded multicentre study performed in the USA, which involved a cohort of 495 kidney transplant patients (Ref. 13). Although mycophenolate mofetil reduced the thickening of the coronary vessels in a rat model, to date, there is no clinical evidence that mycophenolate mofetil affects the progression of graft-vessel disease or chronic rejection (Ref. 14). These clinical studies show that mycophenolate mofetil decreases the incidence and severity of acute rejection episodes, but has little effect on the overall long-term rate of graft survival. Other clinical trials have revealed the superiority of the more selective and non-nephrotoxic mycophenolate mofetil compared with azathioprine when used in combination with cyclosporine or tacrolimus (Refs 15, 16).

Brequinar

Mechanism of action of brequinar

Like other antiproliferative agents, brequinar (DUP785; DuPont-Merck, Wilmington, DE, USA) was originally developed as an anti-tumour agent (Ref. 17). Brequinar is a synthetic difluoroquinolone carboxylic acid derivative [6-fluoro-2-(2'-fluoro-1,1'biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt]. Although brequinar displays anti-tumour effects over a wide range of concentrations, it displays anti-lymphocyte effects over only a relatively narrow range (22–185 nmol/l; Ref. 18). Brequinar inhibits non-competitively the enzyme dihydro-orotate dehydrogenase (DHODH), which converts dihydro-orotate into orotic acid. This conversion is critical for the formation of uridine and cytidine, both of which are required for the synthesis of DNA and RNA (Ref. 19).

Clinical and experimental results with brequinar

Brequinar inhibits the proliferation of lymphocytes in a mixed lymphocyte culture (MLC); it has a 50% inhibitory concentration (IC_{50}) of 0.15 μg/ml (Ref. 20). Brequinar inhibits the rejection responses to a xenograft that is mediated by immunoglobulin M (IgM) antibody in hamster-to-rat heart xenograft models. Although the IgM concentrations increased from 218.3 ± 88.4 mg/l to 1631 ± 359.8 mg/l (n=6, $P<0.01$) in untreated animals, they remained unchanged in brequinar-treated animals (Ref. 21). In rat transplant models, brequinar monotherapy has been shown to prolong the allograft survival of hearts, livers and kidneys. A dose of 12 mg/kg brequinar administered three times a week for 30 days prolonged the survival of heart allografts to 45.5 ± 12.26 days, compared with a survival rate of 7.0 ± 0.69 days for untreated controls. The same brequinar protocol produced long-term survival (>230 days) in 12 out of 26 rat recipients of orthotopic liver (Ref. 20). Furthermore, the oral administration of 4 mg/kg brequinar three times per week prolonged the survival of heterotopic cardiac allografts in non-human primates to 20.0 ± 21.5 days, compared with a survival rate of 8.0 ± 0.5 days for the controls (Ref. 21). Finally, brequinar (3 mg/kg/day for 90 days) prolonged the mean survival time of hamster heart xenotransplants in LEW recipients (an inbred strain of rats). The survival time was increased to 24.5 ± 42.2 days (from 4.0 ± 0.48 days in the control animals); furthermore, four of the hearts continued to beat for more than 90 days (Ref. 21). Thus, in animal models, brequinar has proved to be highly potent for blocking allograft rejection.

Brequinar is water soluble, has a bioavailability of 90%, and reaches peak concentrations in the plasma 2–4 hours after oral administration (Ref. 22). Brequinar is metabolised in the liver by the P450 cytochrome oxidase system, and is excreted primarily in faeces (66%) and, to a lesser extent, in urine (23%). The oral administration of brequinar to rats for 30 days did not affect cyclosporine pharmacokinetics (Ref. 23). Based on results indicating high bioavailability and a long half-life, the oral administration of brequinar was recommended on alternate days. The combination

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of brequinar with cyclosporine and/or sirolimus was synergistic, as shown by the median effect analysis (Refs 24, 25). In primates, the brequinar–cyclosporine combination also prolonged graft survival (Ref. 26).

In a phase I safety trial, 45 cancer patients were administered brequinar by a single daily intravenous infusion for 5 days at a dose range of 36–300 mg/m^2/day; several side-effects including transaminase elevations, thrombocytopaenia, mucositis, phlebitis and dermatitis were recorded (Ref. 27). In a phase II efficacy study of brequinar, doses as high as 1800 mg/m^2 failed to reduce tumour growth in cancer patients (Ref. 28). Although the results of the clinical studies have never been published, the administration of brequinar with a cyclosporine and prednisolone regimen was tested in recipients of cadaveric kidneys (DuPont-Merck, unpublished).

Leflunomide

Mechanism of action of leflunomide

Leflunomide (HWA 486; Hoechst, Basle, Switzerland), an isoxazol derivative [*N*-(4-trifluoro-methylphenyl)-5-methylisoxazol-4-carboxamadide], has been shown to inhibit the symptoms of arthritis (Ref. 29), glomerulonephritis induced by anti-basement membrane antibodies (Ref. 30), systemic lupus erythematosus (Ref. 31) and tubulo-interstitial nephritis (Ref. 32). Leflunomide has also been shown to prolong the survival of kidneys in rats (Ref. 31) and dogs (Ref. 33). Although initial data suggested that leflunomide partially inhibits IL-2 production, the administration of exogenous IL-2 failed to restore T-cell proliferative responses. Thus, it has been hypothesised that leflunomide inhibits T-cell activation by blocking the lck and fyn families of tyrosine kinases. These enzymes are associated with the transduction of such growth factor receptor signals as IL-2, interleukin 3 (IL-3) and tumour necrosis factor alpha (TNF-α) but not interleukin 1 (IL-1; Ref. 34). However, the most recent data show that leflunomide inhibits signal transduction after binding of interleukin 4 (IL-4) to the IL-4 receptor (IL-4R; Ref. 35). Because the addition of uridine and cytidine to T cells that have been treated with leflunomide restored T-core proliferative responses, it is more likely that leflunomide inhibits pyrimidine synthesis (Ref. 36) – possibly like brequinar, by inhibiting DHODH. Following its administration, leflunomide is metabolised quickly to form an active metabolite, A771726, which is only slightly soluble in water. In rodents, A771726 had a half-life of 10–30 hours, which is tenfold longer than its half-life in humans. Because the absorption of leflunomide varies significantly between individuals and it produces severe adverse effects, less-toxic analogues of leflunomide might need to be developed.

Clinical and experimental results with leflunomide

Leflunomide is currently undergoing clinical evaluation in a phase I/II clinical trial to test its efficacy for preventing the rejection of kidney allografts.

Limitations of agents that non-specifically block nucleotide synthesis

Both the old and the new antiproliferative agents are limited in that they have non-specific effects on various types of proliferating cells: these drugs not only prevent the expansion of lymphocytes, but also impair the physiological function of the bone marrow and the gastrointestinal tract. Therefore, none of these immunosuppressive agents is optimal for the prevention of allograft rejection, although the more-selective action of mycophenolate mofetil has allowed its use as a supplement to a standard cyclosporine and prednisone immunosuppression regimen. Of these three new antiproliferative agents – mycophenolate mofetil, brequinar and leflunomide – mycophenolate mofetil proved to be better than azathioprine when used in combination with cyclosporine and prednisolone. However, all of the antiproliferative agents depress bone marrow function, leading to an increased incidence of infections.

Agents that specifically block T-cell function

Cyclosporine

Mechanism of action of cyclosporine

Cyclosporine (Novartis, Basle, Switzerland), a cyclic undecapeptide that is derived from extracts of the fungus *Tolypocladium inflatum*, exhibits potent immunosuppressive activity. As the first T-cell-selective drug, cyclosporine reinvigorated the field of transplantation, which had long become stagnant owing to the complications of bone marrow suppression (mylosuppression) that accompany azathioprine-based therapy (Refs 37, 38). Cyclosporine binds to its immunophilin, cyclophilin, to inhibit the phosphatase calcineurin

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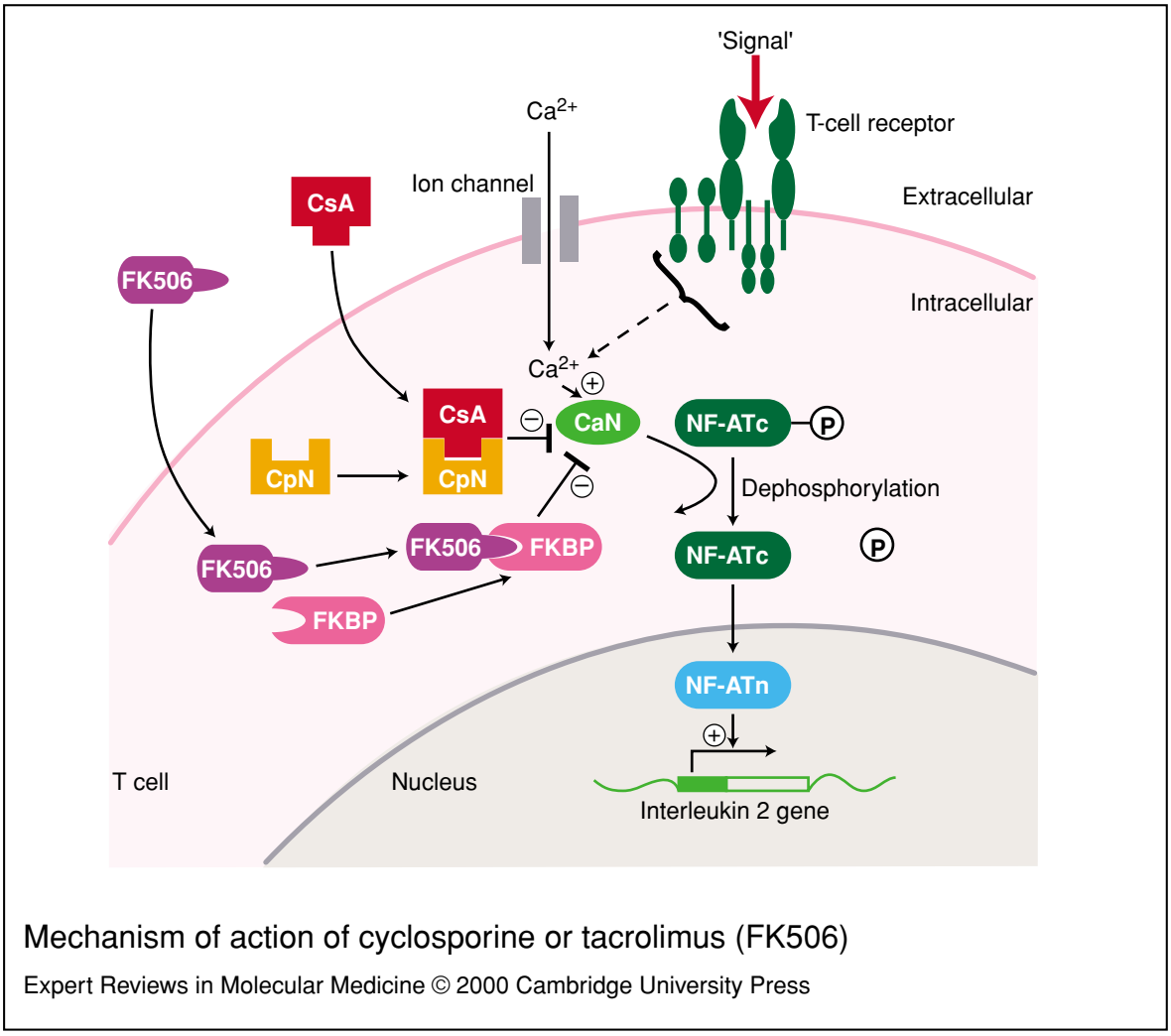

Mechanism of action of cyclosporine or tacrolimus (FK506)

Figure 2. Mechanism of action of cyclosporine or tacrolimus (FK506). In the cytoplasm, cyclosporine (CsA) binds to its immunophilin, cyclophylin (CpN), forming a complex between cyclosporine and CpN. The cyclosporine–CpN complex binds and blocks the function of the enzyme calcineurin (CaN), which has a serine/threonine phosphatase activity. As a result, CaN fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc), and thereby the transport of NF-ATc to the nucleus and the binding of NF-ATc to the nuclear component of the nuclear factor of activated T cells (NF-ATn). The NF-ATc–NF-ATn complex binds to the promoter of the interleukin 2 (IL-2) gene and initiates IL-2 production. Consequently, T cells do not produce IL-2, which is necessary for full T-cell activation. Tacrolimus (FK506) binds to FK506-binding protein (FKBP), forming a FK506–FKBP complex, which binds to and blocks CaN. The FK506–FKBP–CaN complex inhibits the activation of NF-ATc, thus preventing its entrance into the nucleus. Although the pre-drugs cyclosporine and FK506 bind to different target molecules, both drugs inhibit T-cell activation in the same fashion **(fig002ssh)**.

and thereby prevent the generation of the potent nuclear factor of activated T cells (NF-AT), as shown in Figure 2. This step is essential for the up-regulation of the mRNA of various cytokines (Ref. 39), particularly IL-2, which is necessary for the proliferation and maturation of T cells, and interferon gamma (IFN-γ) which, in turn, is critical for the activation of macrophages.

Clinical and experimental results with cyclosporine

The superior selectivity and efficacy of cyclosporine compared with those of azathioprine were shown by an increase in 1-year survival rates of renal allografts from cadaveric donors to 80% for cyclosporine-based regimens from only 51% for azathioprine-based regimens

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(Ref. 40). Furthermore, in contradistinction to the azathioprine–prednisolone regimen, cyclosporine–prednisolone therapy facilitated the successful clinical transplantation of hearts (Ref. 41), livers (Ref. 42), lungs (Ref. 43) and combined heart and lung (Ref. 41) allografts. Cyclosporine-based therapy significantly reduced patient morbidity and overcame at least some of the negative effects of the risk factors that are associated with azathioprine therapy. The risk factors included pre-transplant blood transfusions, the possibility of re-transplantation, and the requirement for matching of alleles of the human leukocyte antigen (HLA) system (Ref. 38).

The major limitations of cyclosporine are its various toxicities; its nephrotoxicity, especially, is a serious one, because several cell types, including those of the brain, heart and kidney, require calcineurin to function. Despite extensive testing, none of the cyclosporine analogues has proved to be free of nephrotoxic effects. The nephrotoxicity of cyclosporine is associated with mucoid deposits in arterioles, focal fibrosis potentiated by vasoconstriction, a disturbed balance of ionic calcium, an altered balance of prostacyclin to thromboxane and pro-rennin activation (Ref. 44). Regrettably, cyclosporine concentrations that are only minimally effective produce nephrotoxic effects, especially in kidneys that have pre-existing damage. To date, no effective therapy has been found to prevent or even mitigate cyclosporine-induced nephrotoxic injury.

The other major limitation of cyclosporine is its inability to prevent chronic transplant nephropathy. First, cyclosporine is less effective in blocking the function of B cells than that of T cells, and hence poorly inhibits antibody production (Ref. 45). Anti-donor antibodies are involved in chronic nephropathy. Second, although cyclosporine inhibits IL-2 and IFN-γ production by T-helper 1 (Th1) cells, it is less effective in blocking the synthesis of IL-4, interleukin 5 (IL-5) and interleukin 7 (IL-7) by T-helper 2 (Th2) cells (Ref. 46). Third, cyclosporine directly up-regulates the transforming growth factor beta (TGF-β) that is responsible for fibrogenic activity in kidney transplants (Ref. 47). Finally, donor age, pre-transplant ischaemia-reperfusion injury and intra-individual variability in cyclosporine concentrations in recipients of kidney transplants are all risk factors that might lead to chronic rejection in cyclosporine-treated recipients.

Tacrolimus (FK506)

Mechanism of action of tacrolimus

Tacrolimus (FK506; Fujisawa, Osaka, Japan), the macrolide product of the bacterium *Streptomyces tsukubaensis*, inhibits IL-2 production by T cells in a fashion that is similar to that of cyclosporine (Ref. 48). After tacrolimus forms a complex with FK-binding proteins (FKBPs), the complex inhibits the phosphatase activity of calcineurin (Fig. 2), thereby blocking the activity of the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc; Ref. 49).

Clinical and experimental results with tacrolimus

In a European multicentre clinical trial involving 545 liver transplant recipients, the tacrolimus-treated group displayed an acute rejection episode rate of 43.4%, compared with 53.6% for the cyclosporine-treated group (Ref. 50). In this study, both renal toxicity and neurological complications were more common among those patients who had been treated with tacrolimus. Similar results were observed in a trial carried out in the USA, which involved 478 recipients of liver transplants (Ref. 51). The so-called rescue treatment, which used tacrolimus for the treatment of acute refractory allograft rejection that was attributed to cyclosporine, resulted in serum creatinine concentrations being returned to baseline (normal) levels; improvement was also confirmed by the histological analysis of renal biopsies (Ref. 52). Similar rescues after refractory rejection episodes have been reported for liver, cardiac and combined pancreas–kidney transplants (Refs 53, 54, 55). Tacrolimus-induced toxicities include hyperglycaemia (in 31.4% of all examined patients), nephrotoxicity (27.1%), chest pain, chest discomfort, palpitations, abnormal electrocardiogram (18.6%), abdominal distension (18.6%) and tremors (10%; Ref. 56).

Sirolimus

Mechanism of action of sirolimus

Sirolimus (rapamycin or rapamune; Wyeth-Ayerst, Princeton, NJ, USA), the macrocyclic compound that is produced by the bacterium *Streptomyces hygroscopicus* (Ref. 57), was originally developed as an anti-candidal and anti-tumour agent (Refs 58, 59). Although both tacrolimus and sirolimus bind to FKBP 25, the molecular effect of sirolimus is distinct from the calcineurin-based effect of either tacrolimus or cyclosporine.

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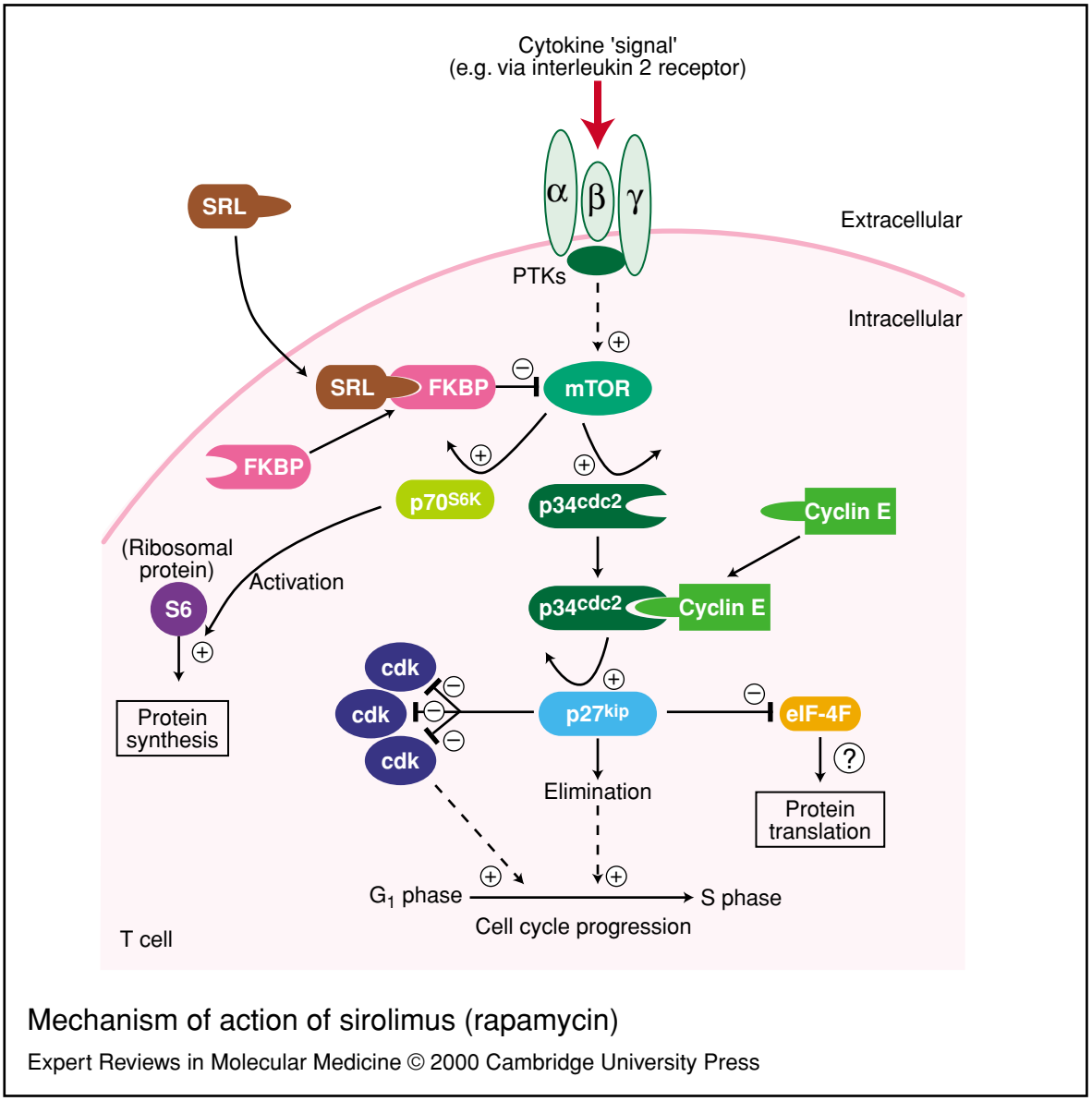

Mechanism of action of sirolimus (rapamycin)

Figure 3. Mechanism of action of sirolimus (rapamycin). Although the pre-drug sirolimus (SRL) binds to FK506-binding protein (FKBP, which is the same molecule that is bound by FK506), the complex that is formed between SRL and FKBP binds to the mammalian target of rapamycin (mTOR). The SRL–FKBP–mTOR complex inhibits biochemical pathways that are required for cell progression through the late G_1 phase or entry into the S phase of the cell cycle. Thus, unlike cyclosporine (CsA) and FK506 (which block the production of cytokines), SRL blocks cytokine signal transduction. SRL is thought to target: (1) the 70-kD S6 protein kinase $p70^{S6K}$; (2) the eukaryotic initiation factor eIF-4F; (3) the G_1-controlling cyclin-dependent kinase (cdk) proteins, such as the D2 cycline cdk2, the D2 cycline cdk6 or the E cycline cdk2 and (4) the kinase inhibitory protein Kip1 ($p27^{kip}$), which blocks cell progression to the S phase. Abbreviations used: $p34^{cdc2}$ = a kinase; PTKs = protein tyrosine kinases **(fig003ssh)**.

Sirolimus inhibits a variety of cytokine-mediated signal transduction pathways (Ref. 60). In particular, sirolimus blocks the activity of the mammalian target of rapamycin (mTOR), a 282-kD phosphatidylinositol 3-kinase (Fig. 3; Ref. 61). The mTOR is necessary for the activation and subsequent protein synthesis of a 70-kD S6 kinase ($p70^{S6k}$), and an enzyme that is critical to

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the activation of the ribosomal protein S6 (Ref. 62). The inhibition of mTOR prevents the activity of $p34^{cdc2}$ kinase, which forms a heterodimeric complex with cyclin E, thus preventing the elimination of both $p27^{kip}$, which is a negative regulatory factor for cyclin-dependent kinases (cdks), and the eukaryotic initiation factor (eIF-4F) binding protein, which is necessary for protein translation. As a result of these activities, sirolimus inhibits the progression of T cells from the G_1 phase to the S phase of the cell cycle (Ref. 63).

Clinical and experimental results with sirolimus

All three immunosuppressants – cyclosporine, tacrolimus and sirolimus – are metabolised by the cytochrome P450 3A/4 system (Ref. 64). In animal models, treatment with sirolimus alone prolonged the survival of organ allografts. Continuous (intravenous) infusion of sirolimus (0.08–0.8 mg/kg for 14 days) extended the survival of rat heart or rat kidney allografts in a dose-dependent fashion (Ref. 65). Similarly, oral gavage with 1.0–8.0 mg/kg sirolimus extended the survival of rat heart allografts (Ref. 64).

One of the most interesting properties of sirolimus is its synergistic interaction with cyclosporine. In in vitro assays, the sirolimus–cyclosporine combination produced an inhibitory effect that was between tenfold and 1000-fold greater than that produced by either agent alone (Ref. 66). Similarly, in vivo, the strong synergism of sirolimus and cyclosporine to prolong the survival of rat cardiac allografts was shown using the median effect analysis (Refs 64, 65). The beneficial interactions of cyclosporine and sirolimus have also been documented for kidney allografts in dogs (Ref. 67).

In a double-blinded phase I study, either sirolimus or the placebo was administered for 14 days (twice-daily doses) to stable recipients of renal transplants who were being treated with a cyclosporine–prednisolone regimen (Ref. 68). In this study, 30 patients were divided into three dose groups, and given a low dose (1–3 mg/m^2/day), a medium dose (5–6 mg/m^2/day) or a high dose (7–13 mg/m^2/day). These patients were then compared with ten patients who had received the placebo. The major toxic effect produced by sirolimus was hypertriglyceridaemia. In addition, sirolimus caused a dose-dependent decrease in platelet counts and, to a lesser extent, in white blood cell counts. Importantly, the addition of sirolimus treatment did not affect kidney function, liver function or blood pressure. Subsequently, increasing doses of sirolimus (0.5–7.0 mg/m^2) were administered in combination with cyclosporine and prednisone to a group of 30 patients. This combined treatment reduced the incidence of acute rejection within the first 6 months to 6.7% (2/30), compared with 36.9% for a group of 65 patients who had been treated with cyclosporine and prednisone alone (P=0.02; Kahan et al., unpublished, University of Texas Medical School, Houston, Texas, USA). These results were confirmed in a phase I/II study of 40 recipients of living-related transplants who received escalating doses of sirolimus (0.5–7.0 mg/m^2/day) in addition to cyclosporine–prednisolone therapy. The addition of sirolimus reduced the overall incidence of rejection episodes to 7.5%, from 32%, in patients who were treated with cyclosporine and prednisone alone (Ref. 69). Thus, clinical results confirmed the potent synergistic interaction between cyclosporine and sirolimus for recipients of kidney transplants. Finally, a multicentre trial involving 149 renal transplant patients who had been treated with cyclosporine and prednisone confirmed that the addition of sirolimus (at 1, 3 or 5 mg/m^2/day) reduced the incidence of acute rejection episodes (Ref. 70). Although within 6 months the cyclosporine–prednisone group showed a 40% incidence of rejection episodes, patients treated adjunctively with 1 or 3 mg/m^2/day of sirolimus experienced only a 10% incidence of rejection. The studies also showed that 1, 3 or 5 mg/m^2 of sirolimus did not reduce the number of rejection episodes in African-American patients who were treated with reduced cyclosporine doses; this finding was in contrast with what was found when this regimen was used to treat patients of non-African-American origin. However, the most recent clinical trial revealed that the adjustment of the cyclosporine–sirolimus regimen in African-American patients (using higher doses than those given to non-African-Americans) improved the 1-year survival rate of kidney allografts. The survival rate was increased to 97% from only 70% for those recipients who were treated with the cyclosporine–prednisolone regimen (Ref. 71).

Treatment with sirolimus reduces thickening of the intima of the heart vessels and prevents the development of experimental chronic heart allograft vasculopathy (Ref. 72). Furthermore, the intimal thickening and proliferation of

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endothelial cells that is observed in the carotid artery after mechanical balloon injury in rats was reduced by 85% after sirolimus therapy (Ref. 73). This inhibitory effect is attributed to the blockade by sirolimus of the signal transduction that is initiated when sirolimus binds to specific receptors of the epidermal-derived growth factor (EDGF-R) and fibroblast-derived growth factor (FDGF-R) as well as to receptors for other cytokines that induce endothelial cell proliferation. In another study, sirolimus treatment inhibited an ongoing process of allograft rejection (Ref. 74). Sirolimus treatment, when initiated on day 3 or 4 (but not on day 5) after transplantation, reversed the rejection of heart allografts in recipients who were treated with sub-therapeutic doses of cyclosporine (Ref. 74). Finally, sirolimus prevented the accelerated rejection of heart allografts in pre-sensitised rat recipients, an effect that correlated with significantly lower concentrations of donor-specific IgG antibody compared with those in pre-sensitised but untreated hosts (Ref. 75). These experimental results suggest that sirolimus might block the vascular changes that are associated with the chronic rejection of grafts in long-term surviving heart and kidney allografts.

15-Deoxyspergualin

Mechanism of action of 15-deoxyspergualin

Spergualin (1-amino-19-guanitido-11,15-dihydroxy-4,9,12-triazathioprinenonadecane-10,13-dione; Nippon Kayaku Co., Tokyo, Japan) is the fermentation product of the bacterium *Bacillus laterosporus*. It is a water-soluble peptide with a molecular weight of 496 daltons (Ref. 76), and is synthetically dehydroxylated to produce 15-deoxyspergualin. Although 15-deoxyspergualin displays both antibacterial and anti-tumour properties (Refs 77, 78), it also inhibits allograft rejection (Ref. 79). 15-Deoxyspergualin only moderately inhibits both the mitogen-stimulated proliferation of T cells and the generation of cytotoxic T cells (Refs 80, 81), without affecting IL-2 production. Because 15-deoxyspergualin blocks T-cell function in vitro when added 3–4 days after the stimulation of T cells (Ref. 80), it is believed to act at a late stage of the cell cycle of T cells. However, this inhibitory effect can be reversed by the exogenous supplementation of IFN-γ, but not of IL-2 (Ref. 82).

Although the molecular mode of action of 15-deoxyspergualin is unknown, the compound binds specifically to two heat-shock proteins (Hsp), namely Hsp70 and Hsp90, which represent two members of a class of immunophilins (Ref. 83). An analogue of 15-deoxyspergualin, methyldeoxyspergualin, has been shown to induce apoptosis in rapidly dividing cells (Ref. 84). In addition, 15-deoxyspergualin inhibited the monocyte and macrophage functions of: (1) liposomal enzyme release, (2) superoxide production, (3) major histocompatibility antigen (MHC) class II up-regulation and (4) IL-1 production (Ref. 85). 15-Deoxyspergualin also reduced the production of antibodies in immunotoxin-treated mice (Ref. 86), and in in vitro studies reduced the expression of IgM on the surface of B cells after the administration of lipopolysaccharide or IFN-γ to a mouse cell line of pre-B cells (Ref. 87).

Clinical and experimental results with 15-deoxyspergualin

15-Deoxyspergualin is a highly polar molecule that has poor (3–6%) oral bioavailability (Ref. 88). After intravenous infusion (80–2160 mg/m^2/day), 15-deoxyspergualin and one of its six metabolites, desaminopropyl 15-deoxyspergualin, reach a steady-state concentration within 2 hours and do not accumulate over 5 days (Ref. 89). The AUC (area under the curve) of 15-deoxyspergualin correlates well with the administered dose (Refs 89, 90). The treatment of baboons with 4 mg/kg/day of 15-deoxyspergualin improved the survival of heart, but not renal, allografts (Ref. 91). Furthermore, 15-deoxyspergualin slightly delayed the rejection of heart allografts in rat recipients that had been pre-sensitised with skin allografts (Ref. 92). Experiments in dogs showed that 15-deoxyspergualin treatment reversed acute rejection episodes (Ref. 93), but caused significant gastrointestinal disturbances.

Results from an initial clinical report suggested that 15-deoxyspergualin (40–220 mg/m^2) was highly effective in reversing rejection episodes in 27 of 34 (79.4%) recipients of renal transplants when used alone (Ref. 94), and even more effective when administered in combination with methylprednisolone (87.5%; Ref. 95). In another study, 15-deoxyspergualin (3–5 mg/kg) was administered in daily 3-hour intravenous infusions for 7 days, and reversed 76% of the rejection episodes in 260 patients within the first 6 months after transplantation. Based on Banff criteria, 15-deoxyspergualin was more

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effective in reversing grade I (20/22; 88%) than grade II (3/8; 37.5%) rejection episodes (Ref. 96). In addition, 15-deoxyspergualin reversed 70% of steroid-resistant rejection episodes. Patients who were treated with 15-deoxyspergualin for allograft rejection experienced adverse effects, including numbness of the face, lips and limbs (in 14% of patients), gastrointestinal toxicity (in 9%) and bone marrow suppression (in 54%). Because of its low bioavailability, 15-deoxyspergualin must be administered intravenously but for only a short time, which limits its widespread application in transplantation.

FTY720

Mechanism of action of FTY720

FTY720 (Novartis, Basle, Switzerland) is a semi-synthetic derivative of myriocin (ISP-1), a compound that was isolated from the culture broth of the fungus *Isaria sinclairii* (Ref. 97). FTY720 features a phenyl ring inserted into the side chain of ISP-1 (2-octadecyl-2-amino-1,3-propanediol), but displays significantly reduced toxic effects compared with those of ISP-1 (Ref. 98). Although the parent compound, ISP-1, is up to tenfold more potent than cyclosporine at inhibiting the proliferation of lymphocytes in allogenic MLCs, ISP-1 was ineffective at blocking the proliferation of human leukaemia cells. In vivo, ISP-1 was more effective than cyclosporine at inhibiting T-cell-dependent antibody production (tenfold) and alloantigen-specific cytotoxic T cell production (100-fold; Ref. 98). In contrast, even high concentrations of FTY720 (1000 nM) failed to inhibit the proliferation of human MLCs (Ref. 99) or the production of IL-2 or IL-3 (Ref. 100).

A possible explanation for this difference could be that ISP-1, but not FTY720, inhibits the activity of serine palmitoyl transferase in IL-2-dependent CTLL-2 cells (Ref. 101). One hypothesis, based on an in vitro experiment in which lymphocytes that were suspended for 3 hours in a high concentration of FTY720 formed apoptotic bodies (Ref. 102), suggests that FTY720 induces the apoptosis of activated alloantigen-specific T cells. A second hypothesis, based on the observation that a single injection of FTY720 at 10 mg/kg to rats caused profound lymphocytopaenia within 3 hours, suggests that FTY720 alters the 'traffic' of lymphocytes (Fig. 4). These results might be explained by a possible change in the expression or function of α4/β7 integrin, an adhesion molecule that is responsible for the homing of lymphocytes into the lymphoid tissues. This hypothesis is supported by the fact that FTY720 did not affect the number of circulating polymorphonuclear leukocytes or monocytes. At the same time, the number of lymphocytes (T cells and B cells) increased in Peyer's patches, as well as in mesenteric and peripheral lymph nodes (Ref. 103). Such sequestration of lymphocytes might be caused by the up-regulated expression of α4/β7 integrins on lymphocytes that bind Gly cellular adhesion molecule 1 (CAM-1) and Mad CAM-1; both of these adhesion molecules are expressed on the cell surface of high endothelial venules in lymph nodes and in Peyer's patches.

Clinical and experimental results with FTY720

Although in rat models the intraperitoneal injection of ISP-1 (0.03–0.3 mg/kg) prolonged the survival of rat skin allografts in a dose-dependent fashion, this treatment was severely toxic. In contrast, the intraperitoneal administration of FTY720 (0.1–10 mg/kg) to rats prolonged the survival of skin allografts without any side-effects. Furthermore, the treatment of rats with 0.5 mg/kg of FTY720 for 14 days prolonged the survival of heart or liver allografts. In dogs, a 2-day course of FTY720 (5 mg/kg) initiated either the day before or on the day of transplant extended the median survival time of kidney grafts (Ref. 102). When used in combination therapy in a rat model, FTY720 (0.1 mg/kg) potentiated the immunosuppressive effects of cyclosporine, prolonging the survival of rat skin allografts (Ref. 104). The most recent results, analysed using the median effect equation, indicated that FTY720 in combination with either cyclosporine or sirolimus displayed potent synergism in the rat heart allograft model (Ref. 99). In addition, the synergistic interaction between FTY720 and either cyclosporine or sirolimus was shown by the inhibition of the proliferation of OKT3-stimulated human peripheral blood lymphocytes. Similarly, FTY720 potentiated the immunosuppressive effect of cyclosporine to prolong the survival of kidney allografts in cynomolgus monkeys (Ref. 105). Based on these in vitro and in vivo studies, a clinical trial is under way to study the effects of the FTY720 and cyclosporine combination in recipients of a primary kidney transplant.

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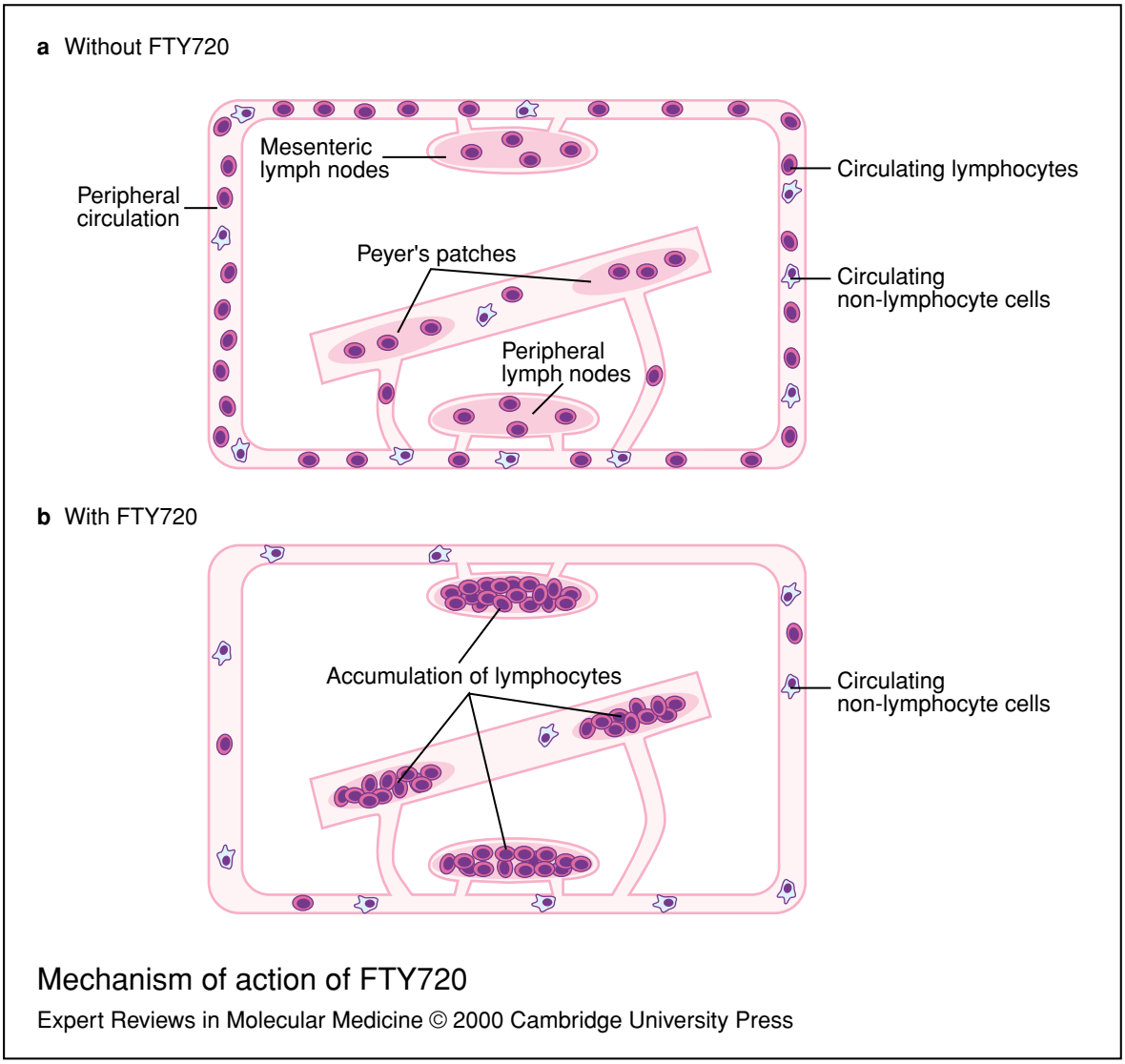

Figure 4. Mechanism of action of FTY720. In rats, a single injected dose of FTY720 causes profound lymphocytopaenia within 3 hours, suggesting that FTY720 alters the 'traffic' of lymphocytes (compare panels 'a' and 'b'). In particular, FTY720 may change the expression or function of α4/β7 integrin, an adhesion molecule that is responsible for the homing of lymphocytes into the lymphoid tissues. In contrast, FTY720 does not affect the number of polymorphonuclear leukocytes or monocytes that are circulating in the blood. FTY720 increases the number of lymphocytes (i.e. T cells and B cells) that are present in Peyer's patches and in mesenteric and peripheral lymph nodes. Sequestration of lymphocytes might be caused by the up-regulated expression of α4/β7 integrins on lymphocytes that bind Gly cellular adhesion molecule 1 (CAM-1) and Mad CAM-1, both of which are adhesion molecules that are expressed on the cell surface of high endothelial venulas (HEV) both in lymph nodes and in Peyer's patches **(fig004ssh)**.

Limitations of agents that specifically block T-cell function

Both cyclosporine and tacrolimus are nephrotoxic and fail to block chronic allograft rejection. Although sirolimus interacts synergistically with cyclosporine, it elevates cyclosporine concentrations, thereby increasing the risk of nephrotoxic effects. In addition, sirolimus causes hypertriglyceridaemia. The use of 15-deoxyspergualin is limited by its toxicity and low bioavailability; the effects of FTY720 in humans are as yet unknown.

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Agents that selectively block gene function: antisense technology

Mechanism of action of antisense technology

Antisense technology proffers one of the most effective methods of drug design for selectively inhibiting the expression of a specific mRNA (Ref. 106). In theory, the metabolic stages of a specific mRNA should be blocked after an antisense oligonucleotide (oligo) with a matching sequence binds to a particular region of the specific mRNA. However, in fact, an antisense oligo can access and bind to only a few nucleotide sequences that are positioned outside the complex three-dimensional structure of an mRNA molecule. Yet despite these limitations, antisense technology offers a clear advantage over all of the previously described drugs. The advantages include: (1) a possibility to target any mRNA, (2) higher affinity and specificity in comparison with those of classical drugs and (3) improved drug design because both the antisense oligo and the specific mRNA share the same chemistry. Consequently, it is possible to target those molecules that are important in the rejection process or those that are used exclusively by T cells or B cells, thereby avoiding several non-specific toxic effects. For example, an intercellular adhesion molecule 1 (ICAM-1) antisense oligo was designed to bind to the 3' end of the mRNA coding for ICAM-1, which is localised in the cytoplasm of cells (Fig. 5). After binding, the duplex of the ICAM-1 mRNA with the ICAM-1 antisense oligo is digested by the cell's ribonuclease H enzyme, thereby destroying the ICAM-1 mRNA and preventing the synthesis of the ICAM-1 protein in ribosomes.

Because natural phosphodiester oligos (PD-oligos) are rapidly hydrolysed by many nucleases following intravenous injection, various chemical modifications have been tested to improve the in vivo resistance of oligos (Ref. 107). One of these modifications, the phosphorothioate oligo (PS-oligo), contains a sulphur group instead of non-bridging oxygen atoms in the phosphate backbone, and proved resistant to both endonucleases and exonucleases. In addition, PS-oligos are more stable than PD-oligos, and because of their negative charge they can attach to positively charged elements on cell membranes (Ref. 108). During the past 5 years, most of the experimental work and all of the clinical trials have used antisense PS-oligos (Ref. 106). Many chemical modifications to such oligos have been developed and tested both in vitro and in vivo; for example, PS-oligos have had 2'-methoxyethyl (ME) groups attached at either end or both ends, or they have been mixed with either PD-oligos or other PS-oligos. These modifications have improved the affinity of the oligos to the target mRNA between fivefold and 20-fold; they have also improved the half-life of oligos in vivo and their resistance to nucleases (Ref. 109). However, oral formulations of PS-ME-oligos must be developed for long-term administration (Ref. 110). Although in rats PS-oligos displayed excellent bioavailability after intravenous administration, as well as good bioavailability following intraperitoneal or subcutaneous injections (both 30%), oral bioavailability was negligible (2%; Ref. 111). Extensive studies have been conducted using an enhancer to increase the bioavailability of PS-ME-oligos. New chemistries will be tested for the next generation of oligos in an effort to increase their localisation in different organs.

Clinical and experimental results with antisense technology for transplantation

Pharmacological studies with antisense technology in transplantation

Unlike natural PD-oligos, PS-oligos are degraded slowly, and have a half-life of 12–24 hours (Refs 106, 112). Following intravenous injection, PS-oligos bind with low affinity (200–400 μM) to serum α2-microglobulins and albumins (Ref. 113). Following intravenous injection, the distribution of PS-oligos from the blood to tissues was extremely rapid, with a half-life of only 1 hour and a terminal elimination of 40–60 hours. Within 4 hours of intravenous infusion, the PS-oligos were located intracellularly in almost all tissues, including in the liver, kidney, bone marrow, skeletal muscle and skin, but not in the brain. The rate of elimination of intact PS-oligos from tissues varies; elimination from the liver is rapid (within 62 hours) and much slower from the kidneys (within 156 hours; Ref. 112). Four hours after a single intravenous injection to rats, PS-oligos had accumulated predominantly in the liver (23% of the oligo), kidney renal cortex (14%), kidney medulla (3%), bone marrow (14%) and skin (13%). Although after 24 hours only 15% of the intact PS-oligos remained in the liver, 40% of intact PS-oligos remained in the kidneys. However, PS-oligos are slowly metabolised by exonucleases and endonucleases into shorter PS-oligos, which are ultimately degraded completely during the

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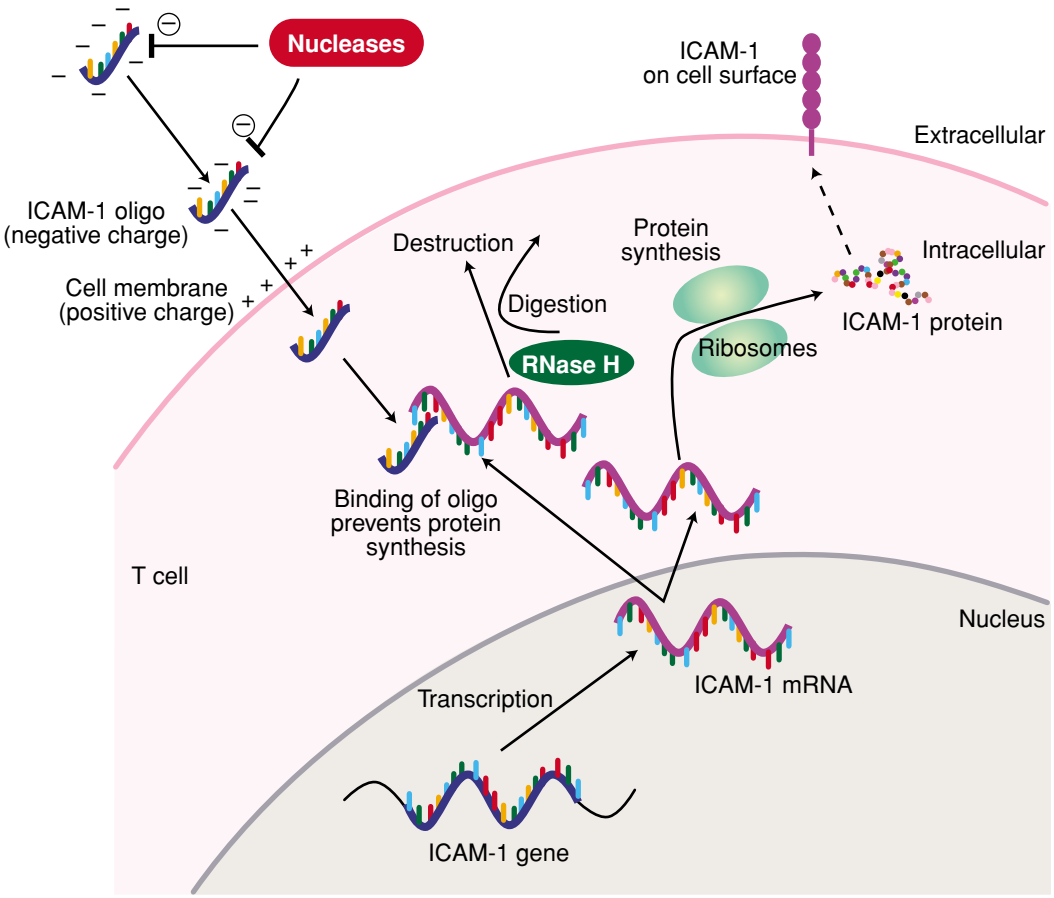

Mechanism of action of intercellular adhesion molecule 1 (ICAM-1) antisense oligonucleotides

Figure 5. Mechanism of action of intercellular adhesion molecule 1 (ICAM-1) antisense oligonucleotides. Before entering cells, the ICAM-1 antisense oligonucleotides may be damaged by nucleases. Different chemical modifications (e.g. the attachment of phosphorothiate groups) prevent damage by nucleases. The negative charges on the antisense oligonucleotides facilitate their binding to the positively charged cell membranes. In the cytoplasm, ICAM-1 antisense oligonucleotides bind to the specific sequence on ICAM-1 messenger RNA (mRNA) molecules. The formation of oligonucleotide–mRNA duplexes activates the ribonuclease H (RNase H) enzyme, which cuts ICAM-1 mRNA, preventing the synthesis of ICAM-1 protein **(fig005ssh)**.

metabolic process. PS-oligos and PS-ME-oligos showed similar tissue distributions, but the PS-ME-oligos had a significantly longer half-life in tissues (Ref. 114).

In mice, as much as 500 mg/kg of PS-oligo or PS-ME-oligo was needed to produce acute toxic effects, as defined by 50% of the lethal dose (LD_{50}). A PS-oligo dose of 20 mg/kg delivered for 4 weeks caused chronic changes, including splenomegaly, mild hyperplasia and organ infiltration by mononuclear cells (Ref. 109). Toxicology profiles of PS-oligos in monkeys revealed sporadic reduction in blood pressure associated with bradycardia (Ref. 115). In particular, in monkeys, the PS-oligos caused the concentration-dependent activation of the complement component 5 (C'5), leading to the activation of the alternative C' pathway and

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prolongation of thromboplastin time (Refs 115, 116). Slow intravenous administration of PS-oligos prevented these side-effects, which were shown to be related to the number of phosphorothioate linkages (Ref. 116). Toxicological profiles in humans following the intravenous injection of 2.0 mg/kg of PS-oligos on alternate days revealed only an insignificant increase in thromboplastin time, and no signs of hypotension or immune stimulation (Ref. 113). The most recent phase I clinical trial, in which PS-oligos were delivered every second day by intravenous infusion, showed no prohibitive toxic side-effects in long-term recipients of kidney transplants (Ref. 117).

In vivo effects of antisense technology

Many studies have shown that antisense PS-oligos might inhibit protein expression by various mechanisms, namely degradation of the targeted RNA, promotion of translational arrest and/or inhibition of RNA processing (Ref. 106). Many experimental systems have shown that specific PS-oligos inhibited the expression of their targeted mRNA in a sequence-specific fashion (Refs 106, 118). For example, ex vivo gene therapy with an antisense PS-oligo to ICAM-1 (delivered by hyperbaric transfection) reduced both the expression of the ICAM-1 protein and chronic heart vascular disease (Ref. 119). The inhibition of the mRNA for the gene-expression modulator 91 (GEM91) by an antisense PS-oligo blocked the replication of human immunodeficiency virus 1 (HIV-1) in a dose-dependent and sequence-specific fashion (Ref. 120). The GEM91 antisense oligo prevented the cytopathic effect of HIV-1 in primary $CD4^+$ T cells (a subset of T cells that is targeted by HIV). In mice, antisense PS-oligos that were designed to block the translation initiation codon of the hepatitis C virus (HCV) inhibited the expression of HCV protein (Ref. 121). Similarly, in mice, antisense PS-oligos that were specific for C-raf inhibited the expression of the C-raf protein and its mRNA in tumour cells and prevented the development of tumours (Ref. 122). In humans, an antisense oligo to ICAM-1 (IP-2302) was tested for its ability to prevent symptoms caused by the up-regulated ICAM-1 protein in inflamed mucosa in patients who have Crohn's disease (Ref. 123). After a 26-day course of IP-2302 (delivered intravenously every second day), 47% of the patients (7 of 15) were in remission, in comparison with 20% (1 of 5) of the placebo-treated control patients. These experiments demonstrate that the antisense oligo might selectively block mRNA in vivo and block the development of specific symptoms.

Application of oligos in animal transplant models

Results from our own laboratory show that a mouse antisense PS-oligo to ICAM-1 (IP-3082), which targets the 3' end of ICAM-1 mRNA, reduced the expression of ICAM-1 protein and its mRNA in the endothelial cell line (Ref. 124). IP-3082, when delivered intravenously for 7 days, also prolonged the survival of heart allografts in a dose-dependent fashion. Furthermore, the combined administration of IP-3082 and a monoclonal antibody that was specific for leukocyte-function-associated antigen 1 (LFA-1) induced tolerance to heart allografts. Similarly, a combination therapy of anti-ICAM-1 monoclonal antibody and anti-LFA-1 monoclonal antibody delivered intraperitoneally for 7 days induced tolerance to the transplantation of heart allografts (Ref. 125). The same protocol also prevented autoimmune diabetes (Ref. 126), apparently by impairing IL-2 production because of the predominance of Th2 cells over Th1 cells (Ref. 125).

Recent studies examined the effects of the blockade of ICAM-1 and LFA-1 on the survival of pancreatic islet allografts using the PS-oligo IP-3082, anti-ICAM-1 monoclonal antibody and/or anti-LFA monoclonal antibody (Ref. 127). The rationale for this is that the expression of ICAM-1 and its ligand LFA-1 after pancreatic islet transplantation can contribute to both non-specific and alloantigen-specific graft destruction. Freshly isolated pancreatic islets from the C57BL/10 ($H2^b$) mouse strain were either transplanted under the renal subcapsular space or embolised into the liver after portal vein injection into the C3H (MHC type $H2^k$) mouse strain. The survival of pancreatic islets was extended by 7 days of intraperitoneal therapy with 5.0 mg/kg of the IP-3082 PS-oligo, or 100 µg of the anti-ICAM-1 monoclonal antibody or 50 µg/day of the anti-LFA-1 monoclonal antibody (Ref. 127). Although a combination of IP-3082 and anti-ICAM-1 monoclonal antibody therapy produced results similar to those obtained using either agent alone, the combined IP-3082 and anti-LFA monoclonal antibody therapy extended survival to 78.3 ± 16.5 days (n=6; P=0.001), with one recipient mouse surviving more than 100 days. Similar results were obtained using a combination of anti-ICAM-1 monoclonal

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antibody and anti-LFA-1 monoclonal antibody (64.2 ± 31.3 days; n=5; P=0.001), with one mouse surviving in the long-term. The same therapeutic protocols produced almost identical results after portal vein injection of pancreatic islets. Thus, although the targeting of a single ligand prolongs the survival of pancreatic islets, the blockade of two ligands is much more beneficial. However, neither the combination of IP-3082 and anti-LFA-1 monoclonal antibody nor that of anti-ICAM-1 monoclonal antibody and anti-LFA-1 monoclonal antibody induced the transplantation tolerance that was previously observed for heart allografts. Interestingly, the pre-treatment of donors the day before transplantation with IP-3082 improved the survival of islet cells in IP-3082-treated recipients. The beneficial effects of ICAM-1 and LFA-1 blockade were confirmed both by the glucose-tolerance test and by immunochemical staining of transplanted islets.

In in vitro studies, an antisense oligo to rat ICAM-1 (IP-9125), which targets the 3'-untreated region of rat ICAM-1 mRNA, inhibited by 85% the expression of ICAM-1 protein in rat L2 cells in a dose-dependent and sequence-specific manner (Ref. 124). In a rat model, an intravenous infusion of the PS-oligo IP-9125 using an osmotic pump (2.5, 5.0 or 10.0 mg/kg) for 7 or 14 days significantly prolonged the survival of heart or kidney allografts; in contrast, identical doses of control oligos were ineffective. In situ experiments showed that 5.0 mg/kg of IP-9125 markedly reduced both the production of ICAM-1 protein and the infiltration of kidney allografts with $CD8^+$ T cells. Although perfusion with scrambled oligo also showed reduced expression of ICAM-1 protein and infiltration with $CD8^+$ T cells, kidney allografts displayed histological changes within necrotic tissue, confirming acute rejection. Thus, this ICAM-1 antisense oligo inhibited ICAM-1 protein expression and allograft rejection in a rat model.

In another rat model, IP-9125 oligo and cyclosporine acted in a synergistic fashion to prolong the survival of rat heart or kidney allografts (Ref. 124). Cyclosporine monotherapy for 7 days by oral gavage (2.0 mg/kg) extended the survival of heart allografts to 13.2 ± 1.6 days in these animals, compared with 8.5 ± 1.1 days in untreated controls. The combination of 10.0 mg/kg of the IP-9125 oligo and cyclosporine therapy produced graft survival of 21.2 ± 4.7 days. Although a 14-day oral gavage with 1.0 mg/kg of cyclosporine alone extended survival to 20.2 ± 4.0 days, the combination of 10.0 mg/kg of IP-9125 and cyclosporine extended recipient survival to 88.5 ± 7.5 days. The median effect analysis of IP-9125 and cyclosporine demonstrated a synergistic interaction.

The injection of donors intravenously with 10 mg/kg of IP-9125 (on days –2, –1 and 0 or on days –4, –3, –2, –1 and 0) prolonged the survival of kidney allografts compared with that of the controls (Ref. 124). Furthermore, ex vivo perfusion of kidney allografts with 5, 10 or 20 mg of IP-9125 (suspended in 2 ml of saline) also extended the survival of kidney allografts. The perfusion of kidneys with rhodamine-labelled IP-9125 oligo showed localisation after grafting; 4.25% of the oligo that had been administered resided in the kidney immediately after perfusion, 2.9% after 4 hours and 1.1% after 24 hours. Thus, the PS-oligo IP-9125 blocks the rejection of organ allografts when used: (1) for the post-operative treatment of a recipient, (2) for the pre-transplant treatment of a donor or (3) for the perfusion of grafts.

Further development of antisense technology

Ischaemic-reperfusion injury represents a transient period of warm and cold ischaemia followed by graft reperfusion (Ref. 128). As a result, grafts display an increased expression of multiple adhesion molecules on the cellular surface, thus increasing the adhesiveness of blood-borne recipient leukocytes to endothelial cells in the graft (Ref. 89). In particular, the up-regulation of ICAM-1 on endothelial cells facilitates the attachment of neutrophils by the leukocyte β2 integrins that initiate graft damage (Ref. 129). The perfusion of kidneys with monoclonal antibodies directed against β2 integrins or ICAM-1 (Ref. 130), used either alone or in combination, diminished tissue damage and improved the initial function of the kidneys. Despite this advantage, the perfusion of organs with monoclonal antibody before grafting might produce non-specific binding of circulating leukocytes and/or induce potent immune responses to the foreign antibodies (anti-xenoantibodies; Ref. 131). Graft perfusion with the ICAM-1 antisense oligo was shown to: (1) reduce the expression of ICAM-1 protein, (2) reduce the infiltration of neutrophils in kidneys and (3) significantly improve kidney function (Ref. 132). However, the oligo was

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formulated in lipofectin, which might be harmful to both the kidneys and to the recipient (Refs 132, 133). Our most recent results showed that the perfusion of kidneys with ICAM-1 antisense oligo (IP-9125) formulated in Euro-Collins solution prevented ischaemic-reperfusion injury (Ref. 134). Similarly, the prevention of ischaemic-reperfusion injury can improve the function of liver, heart and small-bowel transplants. Although the reduction of the expression of inducible nitric oxide (iNOS) by iNOS-specific antisense PS-oligo protected rat kidneys from ischaemia (Ref. 135), the application of NO precursors (e.g. L-arginine) protected the liver or small bowel against ischaemic-reperfusion injury (Ref. 136). The blockade of apoptosis, achieved by designing oligos for this purpose, might also be important for preventing ischaemic-reperfusion injury, especially in liver and small-bowel transplants (Ref. 137).

Conclusions

Over the past decade, the treatment of patients with immunosuppressive agents has extended the survival rates of both the transplanted organs and the patients themselves. Furthermore, the toxic side-effects of the immunosuppressive drugs have been reduced. However, the problems of chronic rejection and the nephrotoxic effects that are induced by current immunosuppressive drugs still need to be overcome. It is hoped that a new approach, known as antisense technology, will lead to the development of agents that can selectively target molecules that are directly involved in the process of allograft rejection, particularly in the activation of T cells and B cells. Further research in this promising field is likely to concentrate on improving the resistance of antisense oligos to nucleases and improving the delivery of the antisense oligos to the cells.

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Features associated with this article

Figures

Figure 1. Mechanism of action of mycophenolate mofetil (fig001ssh).
Figure 2. Mechanism of action of cyclosporine or tacrolimus (FK506) (fig002ssh).
Figure 3. Mechanism of action of sirolimus (rapamycin) (fig003ssh).
Figure 4. Mechanism of action of FTY720 (fig004ssh).
Figure 5. Mechanism of action of intercellular adhesion molecule 1 (ICAM-1) antisense oligonucleotides (fig005ssh).

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