Is Mycophenolate more than just an Immunosuppressant? – An Overview

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The development of immunosuppressant compounds, such as cyclosporine and tacrolimus was crucial to the success of transplant surgery and for treatment of autoimmune diseases. However, immunosuppressant therapy may increase the concentrations of reactive oxygen species (ROS), inducing oxidative damage such as an increased vascular damage. The major source of ROS in the vascular endothelial cells is NADPH oxidase. The subunit structure and function of this enzyme complex in vascular cells differs from that in phagocytic leukocytes. The enzyme subunits Nox1, Nox2 and Nox4 are only found in vascular cells. The GTP-dependent protein subunit Rac 1 needs to be activated for this enzyme to function. Inhibiting this protein subunit should reduce NADPH oxidase-induced oxidative stress. In the cardiovascular system, oxidative stress is observed as hypertension, hypertrophy, fibrosis, conduction abnormalities and endothelial dysfunction, as well as cardiac allograft vasculopathy in transplant patients. In contrast to cyclosporine and tacrolimus, the immunosuppressant mycophenolate inhibits the Rac 1 subunit thus inhibiting NADPH oxidase in the vasculature. This may reduce oxidative stress, prevent the development of cardiac allograft vasculopathy, decrease the deterioration of vascular function and improve cardiovascular function chronically in transplant patients. This overview discusses whether this antioxidant immunosuppressive property could translate into a more general protective role for mycophenolate in the prevention of cardiovascular disease.

Keywords: Mycophenolate, Immunosuppressants, Oxidative stress, NADPH oxidase, Cardiovascular disease

Introduction

Both acute and chronic changes in vascular tone are mediated by compounds released into the bloodstream by the single layer of endothelial cells that line the blood vessels. These compounds include relaxants, such as nitric oxide (NO) and prostaglandins such as PGI₂, PGD₂ and PGE₂, as well as contractile agents such as endothelin and prostaglandin Ÿ₂α. Compromise of these control mechanisms produces the vascular dysfunction characteristic of cardiovascular diseases, including hypertension, diabetes and atherosclerosis. Vascular dysfunction is prognostic of serious vascular events including stroke and myocardial infarction.

Heart transplant patients typically show a high risk of developing allograft vasculopathy, where the epicardial arteries and microcirculation are damaged. Due to the inflammatory response caused by the immune system, the mononuclear immune cells such as macrophages enter the allograft vessel walls, decreasing vascular endothelial cell function. This occurs soon after heart transplantation and slowly progresses to cardiac allograft vasculopathy.

There is speculation that the immunosuppressive drug regime may initiate and stimulate the progression of cardiac allograft vasculopathy. This overview addresses this speculation by summarizing the role played by immunosuppressants in inducing allograft vasculopathy. Furthermore, we also review some of the recent work on mycophenolate in vivo models and speculate as to whether it could be used as a potential cardioprotective agent by reducing oxidative stress.

Oxidative stress

Free radicals are atoms or molecules having at least one unpaired electron in the outer orbital, making them highly reactive. All aerobic organisms generate free radicals containing oxygen, referred to as reactive oxygen species (ROS) (Table 1). In humans, the endothelium is exposed to agents such as ROS that damage the vasculature. In disease states, stimulation of several enzymes can produce ROS, such as the enzymes of the mitochondrial electron transport chain, xanthine oxidase (XO), cyclooxygenases (COX), lipoxygenases, myeloperoxidases, cytochrome P450 monoxygenase, uncoupled nitric oxide synthase (NOS), heme oxigenases, peroxidases and NADPH oxidases. The importance of these enzymes in producing ROS is well documented in many disease states, including inflammatory disease, diabetes, and transplant rejection. In atheromatous plaques, an increase in ROS is detected in the atheromatous core, and this is associated with an increase in ROS production in the vessel wall. The role of ROS in the pathogenesis of cardiovascular disease is well established, and the link between ROS and vascular dysfunction is becoming clearer.
mechanisms varies in different tissues. Mitochondria are a major source of ROS in the myocardium, while NADPH oxidase is more important in the vascular tissues.

ROS, such as superoxide support physiological functions in various cell-signaling pathways and may also recruit and activate immune cells, such as neutrophils as part of the microvascular inflammatory response to pathogens. Under physiological conditions, ROS concentrations are kept low by endogenous antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and thioredoxin reductase. Non-enzymatic compounds such as ascorbate (vitamin C), tocopherol (vitamin E), glutathione and uric acid also help maintain physiological ROS concentrations. The balance between antioxidant activity and ROS production is crucial in maintaining cardiovascular homeostasis. Oxidative stress is induced when there is a decrease in antioxidant activity compared to the ROS generated in the vasculature.

ROS induce vascular damage, both by damaging the vascular endothelial cells and by removing NO by rapidly reacting with superoxide to form peroxynitrite free radicals. Superoxide radical is unique in that it can lead to the formation of many other reactive species, including hydroxyl free radical, hydrogen peroxide, peroxynitrite and perhydroxyl radicals, which are equally or more potent than superoxide itself in inducing oxidative stress by damaging cellular components such as lipids, proteins and DNA molecules. ROS oxidize the amino acids in proteins and also the polynsaturated fatty acids in lipids forming plaque in arteries, thus increasing the risk of atherosclerosis and stroke. Oxidative stress is estimated by quantifying the circulating products, such as malondialdehyde and isoprostanes in the blood.

Cardiac transplantation is associated with oxidant stress, which may contribute to the development of accelerated coronary arteriosclerosis and heart failure. The role of oxidative stress in transplant-associated arteriosclerosis and tissue rejection is supported by animal studies, where antioxidants prolonged survival after cardiac transplantation. In humans, supplementation with antioxidant vitamins C and E retards the early progression of transplant-associated arteriosclerosis and endothelial dysfunction. Microvascular endothelial dysfunction is associated with an enhanced endomyocardial induced NO synthase (iNOS) mRNA expression and higher transcardiac NO production in human cardiac allografts, suggesting peroxynitrite plays a role in the disease process. Thus, oxidative stress by increased ROS formation, combined with the immunosuppressive drug regime may promote the vascular damage observed in cardiac transplant patients.

Table 1—Oxidants that affect cardiovascular homeostasis

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Description</th>
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<tbody>
<tr>
<td>•O₂⁻, superoxide anion</td>
<td>One-electron reduction state of O₂, formed in many autoxidation reactions and by the electron transport chain. Rather unreactive but can release Fe²⁺ from iron-sulphur proteins and ferritin. Undergoes dismutation to form H₂O₂ spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.</td>
</tr>
<tr>
<td>H₂O₂, hydrogen peroxide</td>
<td>Two-electron reduction state, formed by dismutation of •O₂⁻ or by direct reduction of O₂. Lipid soluble and thus able to diffuse across membranes.</td>
</tr>
<tr>
<td>•OH, hydroxyl radical</td>
<td>Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components</td>
</tr>
<tr>
<td>ROOH, organic hydroperoxide</td>
<td>Formed by radical reactions with cellular components such as lipids and nucleobases.</td>
</tr>
<tr>
<td>RO•, alkoxy and ROO•, peroxy radicals</td>
<td>Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.</td>
</tr>
<tr>
<td>HOCl, hypochlorous acid</td>
<td>Formed from H₂O₂ by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.</td>
</tr>
<tr>
<td>OONO⁻, peroxynitrite</td>
<td>Formed in a rapid reaction between •O₂⁻ and NO•. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.</td>
</tr>
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NADPH oxidase

Strong correlations exist between NADPH oxidase activity, atherosclerotic risk factors and endothelial dysfunction\textsuperscript{13,14}. NADPH oxidase is a complex protein made up of 5 subunits including Rho guanosine triphosphatase (Rac1 and Rac2), gp91phox (Nox1 or Nox 4), p22phox, p47phox and p67phox\textsuperscript{1} (Fig. 1). The enzyme becomes highly active during respiratory bursts and forms superoxides by transferring electrons from NADPH inside the cell through the membrane to the oxygen outside. The NADPH oxidase complex expressed in vascular cells differs from that in phagocytic leucocytes\textsuperscript{15}. The isoforms generally expressed in the myocardium are Nox1, Nox2 and Nox4. NADPH oxidase amplifies the ROS formation in the myocardium, as its activity increases during heart failure which in turn induces NOS uncoupling and XO activity\textsuperscript{6}. NADPH oxidase is the major contributor to ROS generation in various cardiovascular disease models and its effect is directly related to the increased protein levels. The expression of NADPH oxidase and its protein subunits such as Rac1 and p67phox are increased during the progression of cardiovascular diseases and heart failure\textsuperscript{16}. NADPH oxidase activation releasing ROS contributes to vascular endothelial dysfunction, apoptosis and inflammation\textsuperscript{16}.

Immunosuppressants

Usually, the immune system distinguishes between self and foreign tissue and will only attack those tissues that are recognized as foreign. Autoimmune disorders result when the immune system attacks self, rather than foreign tissue. When a patient receives a transplant, the immune system will correctly recognize the new organ as foreign tissue and will attack it in a process called tissue rejection\textsuperscript{17}. To prevent rejection, the transplant patient must take drugs that reduce the activity of the immune system, the immunosuppressant compounds\textsuperscript{17}. Clinically, immunosuppressive drugs make organ or tissue transplants more viable and treat autoimmune disorders\textsuperscript{17}.

Glucocorticoids, calcineurin inhibitors and mycophenolate are the most commonly used immunosuppressant drugs in heart transplant patients\textsuperscript{18}. Glucocorticoids suppress the immune system by curbing cytokine production thereby reducing T-cell proliferation and antibody production\textsuperscript{18}. Calcineurin inhibitors suppress T-cell response by deactivating expression of interleukin-2 (IL-2)\textsuperscript{9}. Mycophenolate acts as a non-competitive, selective and reversible inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH), which is a key enzyme in the de novo guanosine nucleotide synthesis, inhibiting B and T lymphocytes that are dependent on this synthesis\textsuperscript{19}.

The calcineurin inhibitor cyclosporine, an 11-amino acid fungal peptide, in the cytoplasm binds to its immunophilin cyclophilin, forming a complex (Fig. 2). The serine/threonine phosphatase activity of calcineurin is blocked by the formation of the cyclosporine–cyclophilin complex. As a result, calcineurin fails to dephosphorylate the cytoplasmic

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Fig. 1—The vascular NADPH oxidase containing Nox subunits as substitutes for the catalytic gp91phox subunit of phagocytic NADPH oxidase [The cytosolic subunits shown will be required for full and sustained activation of the complex in vascular cells. Figure taken from reference 15]

Fig. 2—The mechanism of action of calcineurin inhibitors, cyclosporine and FK506 [Figure taken from reference 20. CsA – cyclosporine, CaN – calcineurin, CpN – cyclophilin, FK506 – tacrolimus, FKB – FK506 binding protein, NF-ATc - nuclear factor of activated T cells]
component of the nuclear factor of activated T cells (NF-ATc), and thereby inhibits the transport of NF-ATc to the nucleus and the binding of NF-ATc to the nuclear component of the nuclear factor inhibiting synthesis of IL-2. Therefore, T cells do not produce the necessary IL-2 for full T-cell activation. The mechanism of action of tacrolimus, otherwise known as FK506, is very similar to that of cyclosporine, inhibiting NF-ATc activation. Tacrolimus (FK506) binds to the FK506-binding protein, forming a FK506-FK506 binding protein complex, which then blocks calcineurin. The NF-ATc is prevented from entering the nucleus, as the FK506–FK506 binding protein–calcineurin complex inhibits the activation of NF-ATc. Although cyclosporine and tacrolimus bind to different target molecules, both drugs inhibit T-cell activation using the same pathways (Fig. 2).

The use of the calcineurin inhibitors, such as cyclosporine and tacrolimus did not reduce the risk of cardiac allograft vasculopathy in transplant patients. Allograft damage leading to vasculopathy can occur through pathways that are not inhibited by calcineurin, such as ROS-mediated oxidative stress. The associated risks of long-term calcineurin inhibitor treatment includes oxidative stress, hypertension, hyperlipidaemia and chronic renal disease; all potential symptoms of cardiac allograft vasculopathy. Cyclosporine, in particular has been increasingly substituted with newer immunosuppressants, since a major concern is nephrotoxicity. It also induces endothelial and smooth muscle cell dysfunction and hypertension by inducing oxidative stress. Cyclosporine decreases superoxide dismutase activity by more than 15% and increases erythrocyte superoxide concentrations by 29%, contributing to vascular dysfunction. It also increases the plasma malondialdehyde concentration, a quantitative measurement of oxidative stress.

Mycophenolate or mycophenolic acid obtained from the fungus Penicillium stoloniferum, inhibits IMPDH that converts inosine monophosphate to guanosine monophosphate. During T-cell activation, the activity of both types I and II IMPDH increases by ten-fold. Mycophenolic acid non-competitively and reversibly inhibits types I and II IMPDH activity during S-phase in DNA synthesis. In the salvage pathway, guanine is converted to guanine monophosphate by the enzyme hypoxanthine-guanine phosphoribosyl-transferase (Fig. 3). By inhibiting IMPDH, T-cell activation is stalled. Mycophenolate diminishes proliferative responses to donor cells in mixed lymphocyte reactions and shows low incidence of cardiac allograft vasculopathy in rat studies. Cardiac transplant patients treated with mycophenolate showed marked reduction in circulating levels of B-lymphocytes and also lowered C-reactive protein concentrations, compared to other immunosuppressants. The immunosuppressive and anti-inflammatory properties of mycophenolate may provide long-term benefits in reducing the risks of cardiac allograft vasculopathy in cardiac transplant patients.

Oxidative status of immunosuppressants
Numerous in vivo and in vitro experiments suggest cyclosporine induces superoxide and thromboxane (TX) production and lipid peroxidation. This is associated with decreased expression of endogenous antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidases. Both short-term and long-term administration of tacrolimus significantly increases blood pressure in rats. Increased kidney malondialdehyde concentrations, a clear sign of oxidative stress, occur with long-term treatment with tacrolimus. Anti-oxidant treatment reverses the production of ROS and thromboxane, as well as reversing lipid peroxidation and the chronic nephrotoxic effects induced by cyclosporine in rats. Cyclosporine-induced intracellular peroxynitrite and 3-nitrotyrosine formation in cultured bovine aortic endothelial cells (BAEC) is also inhibited by a glutathione donor and N-acetylcysteine, indicating the
importance of a suppressed antioxidant system in the responses to cyclosporine. Increased superoxide production by NADPH oxidase in the endothelial cells is one of the main reasons for vascular dysfunction in cardiac allograft vasculopathy. Both cyclosporine and tacrolimus increase the endothelial NADPH oxidase activity. The vascular damage caused by these compounds is attenuated by a selective NADPH oxidase blocker gp91ds-tat, suggesting the involvement of endothelial NADPH oxidase-mediated oxidative stress.

In contrast, mycophenolate inhibits NADPH oxidase-mediated superoxide formation. Mycophenolate neither acts as a superoxide scavenger nor does it alter the transcriptional activity of NADPH oxidase. Rac1, a small GTP-binding protein subunit important in NADPH oxidase activation, is inhibited by mycophenolate. IMPDH inhibition by mycophenolate causes depletion of guanine nucleotides, reducing intracellular GTP concentrations in lymphocytes by inhibiting DNA synthesis, proliferation and immunosuppression. The reduced intracellular GTP concentrations inactivate the small GTP-binding Rac1, in turn, inhibiting endothelial NADPH oxidase activity. In neutrophils, Rac2, another small GTP-binding protein, is also inactivated in the same way. Rac1 is an essential component of endothelial NADPH oxidase activation; several other vascular NADPH oxidase isoforms are also dependent on Rac1 for activation. The inhibition of Rac1 and Rac2 subunits is responsible for Nox2 inhibition. Mycophenolate also reduces hydrogen peroxide formation in endothelial cells, after stimulation with a phorbol ester, a potent protein kinase C activator that stimulates Nox2 by phosphorylating p47phox. This inhibitory effect of mycophenolate on Nox subunits in endothelial cells may explain its reported beneficial vascular activity in allografts.

Thus, mycophenolate shows marked advantages over the calcineurin inhibitors cyclosporine and tacrolimus. These inhibitors induce excessive ROS generation, hypertension, hyperlipidaemia and endothelial dysfunction, leading to cardiac allograft vasculopathy in transplant patients. On the other hand, mycophenolate is not nephrotoxic, does not affect blood lipid concentrations, blood pressure or vascular reactivity and the chances of developing cardiac allograft vasculopathy are very low. In addition, mycophenolate also acts as a potent antioxidant compound by reducing the NADPH oxidase-mediated production of superoxide. This is contrary to the calcineurin inhibitors, which induce oxidative stress causing vascular damage in transplant patients. Mycophenolate also inhibits endothelin-1 formation, another activator of NADPH oxidase, as well as reducing the expression of adhesion molecules, and enhancing prostaglandin I2 release. These findings that mycophenolate is an inhibitor of NADPH oxidase may extend its use to cardiovascular disease, since few selective oxidase inhibitors are currently available.

Conclusion
The ability of immunosuppressant compounds such as the calcineurin inhibitors, cyclosporine and tacrolimus to induce allograft vasculopathy seems to be correlated with NADPH oxidase/Nox-mediated superoxide production in the endothelial cells. This suggests that the explanation for the low reported incidence of cardiac allograft vasculopathy in mycophenolate-treated patients is the effective inhibition of NADPH oxidase by mycophenolate. Further, some studies have explored this beneficial anti-oxidant/anti-inflammatory property of mycophenolate in the treatment of various cardiovascular disease states in animal models, but none have tested this concept in humans. More elaborate studies will be required to determine whether this proposed mode of action for the protective role of mycophenolate as an anti-oxidant/anti-inflammatory compound can be translated into a more general protective role in cardiovascular disease pathology.

References
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