Abstract

Atherosclerosis is characterized by a persistent, low-grade inflammatory state in which immune cell activation is inseparably linked to plaque formation and destabilization. The T-lymphocyte in particular has emerged as a pivotal player throughout the course of atherogenesis. As a consequence, the concept that immune modulation is a suitable target for cardiovascular prevention is currently an important focus of research.

Mycophenolate mofetil (MMF) has emerged as a non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) that exerts cytostatic effects, particularly on proliferating T-lymphocytes. In addition, MMF has other immune-modulating effects, such as downregulation of the expression of adhesion molecules and attenuation of monocyte and macrophage responses. Given the added benefit that MMF is well tolerated, this immunosuppressive agent constitutes an attractive candidate for the modulation of inflammatory activation in atherogenesis. The present review provides an overview of the potential anti-atherogenic properties of MMF.

Keywords: Atherosclerosis; Mycophenolate mofetil; Leukocytes; Endothelium; Adhesion molecules

1. Introduction

Atherosclerosis is an inflammatory disease in which various cell types have been shown to play an important role, comprising endothelial cells, smooth muscle cells and inflammatory cells such as monocytes/macrophages, T-cells and dendritic cells. A central mediator in atherogenesis is activation of the NFκB pathway by several atherogenic stimuli such as oxidized Low Density Lipoprotein (LDL) and a number of cytokines [e.g. Interleukin (IL)-1, IL-2 and tumor necrosis factor (TNF)-α] [1]. Downstream, NFκB activation mediates upregulation of adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [2], followed by leukocyte rolling and adhesion to an activated endothelial monolayer [3]. Subsequent transmigration of lymphocytes and monocytes into the subendothelial space is concomitantly promoted by various chemokines. Within the subendothelial space, T-lymphocytes can get activated (e.g. by dendritic cells and cytokines) and can polarize into different subsets, of which the pro-inflammatory Th1-cell phenotype predominates in the atherosclerotic plaque. Subsequently, stimulated by local chemokines, monocytes transform into tissue macrophages, with increased expression of scavenger receptors. The presence of modified LDL in the subendothelium is the catalyst for early foam cell formation. These subendothelial inflammatory processes have recently been elegantly reviewed [4].
The introduction of statins represented great progress in cardiovascular protection. Indeed, statin therapy reduces risk of major coronary events by 26–36% [5]. This efficacy however, emphasizes the need for more effective drugs in order to decrease the burden of atherosclerotic disease. Novel strategies include combination therapies targeted to raise HDL on top of aggressive LDL lowering. Therapies with immunomodulatory effects have also been proposed. In the present review we will summarize the data for MMF as potential anti-atherosclerotic candidate.

2. Lymphocytes

2.1. Role of T-lymphocyte in atherogenesis

T-cells play a pivotal role in atherosclerosis. Their atherogenic potential was underlined by several animal studies which showed that downregulation [6], depletion [7] or signal inhibition of T-cells [8–11] results in significant reduction of atherosclerosis ranging from 59% to 73%. In addition, introduction of CD4+ cells in immunodeficient ApoE−/− mice accelerates atherosclerosis [7].

2.2. Effect of MPA on T-lymphocyte proliferation and apoptosis

After intestinal absorption mycophenolate mofetil is hydrolyzed into its active form, mycophenolic acid (MPA). This metabolite is a non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), which is the rate-limiting enzyme in de novo synthesis of guanosine nucleotides [12]. Whereas most cell types have the capacity to synthesize guanosine nucleotides by the IMPDH path as well as by a salvage pathway, lymphocytes are dependent upon de novo synthesis (Fig. 1). To date, two isoforms of IMPDH have been identified. Stimulated T-lymphocytes strongly express type II, which has a 5-fold higher affinity for MPA, compared to IMPDH type I. Hence, IMPDH inhibition with MPA is followed by depletion of the pool of dGTP required for DNA synthesis, predominantly in stimulated T-lymphocytes [12]. As a consequence, MPA inhibits T-cell proliferation by establishing a block at the early to mid-G1 phase of the cell cycle. In addition to this cytostatic effect, MPA also induces apoptosis in activated T-cells. Accordingly, MPA was shown to increase apoptosis from 12% to 82–92% in cultured MOLT-4 cells [13]. In mice injected with the superantigen staphylococcal enterotoxin B (SEB), MMF accelerated elimination of SEB-reactive T-cells by triggering apoptosis of Vβ8+ cells, an effect also seen in humans [14,15].

2.3. Effect of MPA on lymphocyte recruitment

Depletion of GTP by MPA inhibits the transfer of mannose and fucose to lymphocyte glycoproteins [12]. These proteins comprise lymphocytic adhesion molecules, responsible for attachment to endothelial cells. Thus, treatment of human T-lymphocytes with MPA resulted in

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**Pathways of Purine Biosynthesis**

- **DeNovo Pathway**
  - Glycoprotein
  - Synthesis → Guanosine TP
  - Ribose-5P + ATP → PRPP Synthetase
  - 5-phosphoribosyl-1-pyrophosphate (PRPP) → IMP Dehydrogenase (IMPD)
  - Inosine MP → Adenosine MP
- **Salvage Pathway**
  - Guanine → HGPATase → Guanosine MP
  - PRPP (Lesch-Nyhan) → IMP Dehydrogenase (IMPD) → Mycophenolic Acid
  - Ribonucleotide Reductase → Deoxyguanosine TP → DNA

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Fig. 1. Pathways of purine biosynthesis, showing the central position of inosine monophosphate (IMP). Mycophenolic acid inhibits IMP dehydrogenase, thereby depleting GMP, GTP and dGTP. Two rate-limiting enzymes in lymphocytes are activated by guanosine ribonucleotides and dGTP, but inhibited by AMP, ADP and by dATP, respectively. © [2005] Edward Arnold (Publishers) Ltd. [Lupus 2005; 14 (Suppl 1): s2–s8] (Ref. [67]).
decreased attachment of lymphocytes to human umbilical vein endothelial cells (HUVECs). Notably, incubation of both cell lines with MPA resulted in an even larger decrease, underscoring an independent effect on MPA on endothelial cells (see Endothelium of MPA on endothelial cells) [16]. Of note, also leukocytes positive for LFA-1α, the counter-receptor for VCAM-1, were significantly reduced in kidney allografts of MMF treated Lewis rats [17]. Similarly, LFA-1 positive leukocytes in the perivascular space of Fisher rats undergoing heart transplantation were also significantly reduced in the group treated with MMF [18]. Recently, in vitro and in vivo experiments in Lewis rats have confirmed MMF mediated reduction in LFA-1 expression by lymphocytes [19]. MPA also interferes with the mannosylation of VLA-4 [16], the counter-receptor of VCAM-1. In vitro studies demonstrated strong inhibition of both CD4+ and CD8+ T-cell adhesion and penetration by MMF and suggest this occurs mainly as a result of a reduced binding capacity of adhesion molecules such as VLA-4 and LFA-1 [20].

Combined, these findings substantiate that MMF treatment has the capacity to reduce infiltration of circulating lymphocytes to sites of inflammation including the atherosclerotic plaque, as has been confirmed in several animal studies [21,23].

3. Monocytes/Macrophages

3.1. Role of monocytes/macrophages in atherogenesis

Endothelial activation with subsequent upregulation of adhesion molecules by chemokines results in transmigration to the subendothelium in the initial phase of atherogenesis upon continuing risk factor pressure. This is followed by expression of scavenger receptors and internalization of modified LDL, ultimately giving rise to foam cells. Several studies in transgenic mice have established that atheroma formation and progression is heavily dependent on involvement of monocytes and macrophages. Blocking monocyte adherence to the vascular wall [24,25], inhibition of transmigration driven by chemokines [26] or blocking the subsequent expression of scavenger receptors [27,28], all result in significant reduction of atherosclerotic lesion development.

3.2. Effect of MPA on monocytes/macrophages

MPA lowers GTP levels in monocytes [12] and, in doing so, can directly attenuate monocyte and macrophage responses that contribute to plaque progression. Indeed, MPA treatment of monocytes decreased mannosylation of glycoproteins and their attachment to endothelial cells [29]. Recently, these findings have been confirmed, showing an up to 30% reduction of monocyte binding to HUVECs after MMF treatment. Monocyte binding to immobilized E-selectin was also reduced, whereas upregulation of ICAM-1 and MHC-II expression by monocytes in response to LPS was attenuated significantly during MMF [30]. Similar to the pro-apoptotic effects in lymphocytes, MMF can also induce apoptosis in monocytes (THP-1 and U937 cells) [13].

These in vitro studies suggest that MMF can inhibit monocyte and macrophage responses; this was confirmed recently in vivo when MMF treatment in streptozotocin-treated diabetic rats prevented glomerular macrophage infiltration [31]. Of note, direct inhibitory effects of MMF on macrophages are reinforced by the effects of MMF on subendothelial T-cells. The T-cell–mononuclear cell interaction contributes to the activation of inflammatory responses within the subendothelium [4]. Abrogation of this interaction was accompanied by a 40% reduction in atheroma formation. In humans it remains to be established to what extent subendothelial T-cell–mononuclear interactions contribute to the low-grade inflammatory state in atherogenesis.

4. Endothelium

4.1. Adhesion molecules

In the early phases of atherogenesis there is increased expression of various leukocyte adhesion molecules in the endothelium. MPA reduces expression of these adhesion molecules in lymphocytes, as well as on the endothelium. This effect of MMF was shown recently in several in vitro studies. Activation of the nuclear factor NFκB by oxidized LDL and inflammatory cytokines results in transcriptional upregulation of the genes for E-selectin, ICAM-1 and VCAM-1. Concomitant treatment with MPA decreases NFκB activation in TNF-α or PMA treated endothelial cells [32].

In HUVECS, MPA significantly reduces TNF-α mediated expression of VCAM-1 and E-selectin, compared to TNF-α treatment alone. Similarly, IL-1β mediated upregulation is significantly reduced by concomitant incubation with MPA [33]. Moreover, MMF treatment inhibits adhesion and transendothelial infiltration rates of T-lymphocytes as determined by reflection interference contrast microscopy [34].

4.2. Vasodilation

Endothelial dysfunction has been widely acknowledged as the earliest stage in atherogenesis. A hallmark of endothelial dysfunction is impaired bioavailability of endothelium-derived nitric oxide (NO), which is synthesized from the amino acid L-arginine by endothelial nitric oxide synthase (endothelial NOS; NOS3). In contrast to the picomolar concentrations of endothelium-derived NO, inducible NOS (NOS2) is involved in inflammatory pathways producing NO in the nanomolar range. Both NOS isoforms require tetrahydrobiopterin (BH4) as a crucial
cofactor for conversion of L-arginine to NO. However, whereas BH₄ is tightly bound to NOS3, the inducible form requires continuous de novo production of the cofactor [35]. NOS2 is generally considered to exert pro-atherogenic actions by contributing to the formation of peroxynitrite with ensuing nitrosylation of proteins as well as by downregulation of NOS3 [36].

MPA reduces intracellular levels of BH₄ by reducing intracellular GTP levels [37]. Accordingly, MPA treatment of rodent endothelial cells inhibits iNOS activity whereas basal NO production, mediated by NOS3, remained unaffected [38]. Of note, MMF also affects other vasoactive mediators. In fact, in endothelial cells, MPA treatment results in significantly decreased mRNA expression of endothelin-1 [39] and increased PGÌ₂ release [40]. Therefore, it might be postulated that MPA exerts beneficial effects on the vaso dilatory function of the endothelium.

5. Smooth muscle cells

Smooth muscle cells (SMC) play an important role in atherogenesis. Upon activation, SMC migrate towards the sub intimal space, followed by proliferation and secretion of matrix proteins [41]. Proliferation of SMC isolated from rats was significantly reduced by MMF as quantified by ³H-TdR uptake [42]. Shimizu et al. also showed that MPA treatment can dose-dependently inhibit proliferation of vascular SMC induced by ET-1, whereas it can prevent transplant arteriosclerosis by direct inhibition of vascular smooth muscle cell proliferation [43]. These in vitro findings were confirmed in a rat aortic allograft model where MMF treatment reduced the appearance of SMC in the intima and significantly reduced the replication rate of SMC in the media [42]. In human SMC, similar antiproliferative effects of MMF have been demonstrated [44]. These data imply that MMF treatment may be associated with decreased SMC proliferation and matrix secretion, which in turn has been associated with increased plaque stability.

6. Dendritic cells

Dendritic cells (DC) are antigen presenting cells that can activate naïve T-cells, thereby initiating proliferation and differentiation of T-cells to Th1 cells. For this activation, maturation of DC as well as upregulation of co-stimulatory molecules (e.g. CD40, CD86) and Major Histocompatibility Complex molecules are required. Atherogenic stimuli induce DC maturation (e.g. OxLDL and nicotine) and DC recruitment into the vascular wall (e.g. OxLDL, TNF-α and hypoxia) [45]. Although the exact role of DC in atherogenesis remains to be determined, their potential contribution was underlined by the detection of DC in the plaque co-localized with T-cells. The frequent DC–T-cell contacts in the rupture-prone regions suggest that DC activate T-cells thereby promoting plaque progression and destabilization [46]. In vitro experiments showed that MMF decreased the ability of murine DC to stimulate allogenic T-cells in MLR assays. Accordingly, MMF reduced expression of CD40 and CD86 dose-dependently. In addition, MMF reduced IL-12 production by LPS triggered DC. The ability to produce IL-12 is acquired by matured DC and is necessary for the development of the pro-atherogenic Th1 cells [47]. These findings were confirmed in human monocyte-derived DC. MMF reduced the number of immature MDDC in culture, dose-dependently by inducing apoptosis and inhibited their stimulatory activity on allogenic lymphocytes. This corre-
lated with downregulation of co-stimulatory and adhesion molecules such as CD40, CD54, CD80 and CD86. MDC differentiated in the presence of MMF showed significantly reduced maturation upon stimulation with LPS, as judged by lower expression of CD83 and co-stimulatory molecules, lower production of TNF-α, IL-10, IL-12 and IL-18 as well as lower stimulation of alloreactive T-cells including naive CD4⁺CD45RA⁺ T-cells [48].

7. Atherosclerosis in animal studies

Several animal experiments have been performed in order to evaluate the impact of MMF on atherogenesis. Greenstein et al. used New Zealand White rabbits that were fed a high-cholesterol diet for 4 weeks as a model for atherosclerosis and treated them with MMF (80 mg/kg). At the end of 4 weeks, they observed a trend towards a reduction of foam cell formation in the MMF treated animals [49]. In a second, larger study, these observations were repeated with a follow up period of twelve weeks. In the MMF treated group, the neointimal plaque area was significantly decreased by 46% and the number of macrophages in this group was reduced to a comparable level of the control group [50]. Also, a significant reduction of atherosclerotic plaques covering the thoracic and abdominal aorta was found by other researchers in the MMF treated rabbits with a reduction in cholesterol content of the aorta (mg/g) in this group [51]. Hence, data from animal models, albeit limited in number, unanimously support the anti-atherogenic potential of MMF.

8. Conclusion

As can be deduced from the presented overview, MMF exerts a plethora of anti-inflammatory effects that could be hypothesized to attenuate pivotal processes in atherosclerosis (see Fig. 2). Its principal mode of action is IMPDH inhibition which results in decreased lymphocyte proliferation rates. In view of the crucial role of T-lymphocytes in the subendothelial amplification loop between lymphocytes and macrophages, this MMF effect might be expected to induce a decrease of the inflammatory responses within the subendothelial space. The latter contention is supported by other anti-atherogenic effects of MMF at the level of monocytes, endothelial cells, dendritic cells and NOS2 inhibition (Table 1). In addition, MMF was reported to decrease platelet aggregation [52] and conversion from Cyclosporin to MMF prescription in 15 renal transplant recipients lowered plasma vWF [53]. Additional studies are required to verify whether MMF can truly inhibit the coagulation cascade.

Based on the beneficial effects on key pathways involved in atherogenesis as well as on the preliminary data obtained in animal models, MMF is an attractive candidate for testing in clinical trials in humans. The selectivity of MMF offers a clear advantage over the non-selective azathioprine, which inhibits several enzymes of purine synthesis. In addition, both AZA as well as cyclophosphamide are potentially mutagenic agents. MMF per se has no effect on serum lipids, blood pressure and does not increase homocysteine levels [54]. In addition to these advantages over other drugs MMF is generally well tolerated [55]. Whereas gastrointestinal toxicity may occur these are mainly limited to high dosages used in transplantation medicine while lower dosages have very few side effects. Obviously, when considering prolonged immunosuppression the risk of additional side effects such as bone marrow suppression and opportunistic infections should be taken into account. It is not known whether the risk of these complications, observed in transplantation medicine and limited to high dosages, is similar when MMF is used at lower dosages.

Currently, there is significant in vitro and in vivo data suggesting that MMF might have significant effects on modulating atherosclerosis in humans in vivo. Clearly, pilot studies are needed to formally test this hypothesis.

Table 1

<table>
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<tr>
<th>Mode of action</th>
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<tr>
<td>Induction of apoptosis</td>
<td>[13–15]</td>
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<tr>
<td>Downregulation of adhesion molecules</td>
<td>[16,19,20]</td>
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<tr>
<td>Downregulation of adhesion molecules</td>
<td>[29,30]</td>
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<tr>
<td>Inhibition of maturation</td>
<td>[47,48,66]</td>
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<tr>
<td>Inhibition of T-cell activation</td>
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