Ageing and Immunosuppression in Kidney Transplantation

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Modern approaches to tailor-made, individualized immunosuppressive therapy for patients receiving organ transplantation require a rethinking of therapeutic strategies when it comes to older persons receiving kidney transplants, especially from deceased older donors. This review article makes the case for the use of calcineurin-inhibitor–free immunosuppressive induction/maintenance protocols in this "worst-case scenario" and discusses the theoretical and clinical data that support this recommendation.

We will discuss modern theories of ageing, emphasizing the free-radical theory in relation to new insights into the mechanisms of innate immunity. In this context, a new, modified theory of ageing is presented. Increased generation of reactive oxygen species during ageing, via increased leakage of these oxidizing molecules from mitochondria, may contribute to senescence and age-related diseases by direct damage to intracellular DNA, proteins, and lipids. In addition, free-radical–mediated tissue injury, accompanied by induction of damage-associated molecular patterns, may result in activation of both inflammatory and vascular cells of the innate immune system, contributing (via inflammatory processes) to ageing and age-related diseases such as atherosclerosis.

Calcineurin-inhibiting agents have been shown to induce oxidative stress and are thus defined as "proageing" drugs. Their use in older patients may aggravate the preexisting oxidized intracellular state and therefore should be avoided. In contrast, inosine-monophosphate dehydrogenase–inhibiting agents such as mycophenolate mofetil have been shown to even ameliorate oxidative stress and are thus defined as "antiageing" drugs. Therefore, their use for immunosuppression in older patients receiving kidney transplantation is suggested. This recommendation is supported by data from a prospective trial on the application of a calcineurin-inhibitor–free, mycophenolate-mofetil–based induction/maintenance immunosuppressive protocol in older recipients of kidneys from deceased older donors: the 5-year patient and 5-year allograft survival rates are currently 87% and 70%, respectively.

Key words: *Senescence, Oxidative stress, Innate immunity, Immunosuppressive drugs*

The percentage of older persons in the world is growing. The implications of the growing older population are many, including rising total healthcare expenditures, increased needs for long-term care services, and the need for expert and focused healthcare services. Accordingly, older persons are the fastest growing segment of the population with end-stage renal disease, contributing to a steadily growing number of older patients on waiting lists [1]. In light of a desperate organ shortage, there is a worldwide tendency to establish allocation programs—such as the Eurotransplant Senior Program—in which kidneys from deceased older (ie, marginal) donors are preferentially transplanted to those older recipients [2], a policy that is apparently supported by stringent ethical considerations [3]. The resulting ethical/medical problem of this allocation policy (the "worst-case scenario") is obvious: inferior long-term graft outcomes associated with this special kidney transplantation

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program [4,5].

In this article, we examine two questions: Why are renal allograft outcomes inferior when transplanting old organs to older recipients? And does immunosuppressive therapy contribute to this outcome? We address these questions by discussing the theory of ageing in relation to oxidative stress, highlighting new insights into mechanisms of innate immunity in relation to ageing, as well as in relation to posttransplant immune/nonimmune events, and analyzing certain properties of immunosuppressive drugs that may potentially aggravate the process of ageing and thereby negatively influence the posttransplant (late) outcome.

Theories of Ageing

Senescence (ageing) is defined as a decline in performance and fitness with advancing age. Senescence is a nearly universal feature of multicellular organisms, and understanding why it occurs has been a long-standing problem in biology. The process of ageing is a highly complex one, involving multiple mechanisms at different levels and consequently, several theories of ageing exist that try to explain these mechanisms.

The *antagonistic pleiotropy theory* of ageing holds that natural selection has favored genes that confer short-term benefits to the organism at the cost of deterioration in later life. In other words, genes that offer an advantage in youth but are detrimental later on (ie, antagonistic pleiotropy) will be selected if the early advantage outweighs the later disadvantage. The basic idea of this theory is that selection pressure falls over time, as the cumulative risk of accidental death rises. This model partially explains age-related diseases but not ageing [6].

The *disposable soma theory*—sometimes thought of as a special case of antagonistic pleiotropy—argues that there is a tradeoff between resources dedicated to reproduction and those that are reserved for survival. In other words, the theory interprets the antagonistic pleiotropy concept as a life-history strategy in which somatic maintenance is below the level required to prevent ageing, thus enabling higher immediate fertility. Thus, this model explains ageing but not age-related diseases [7].

The *free-radical theory*—or still better, the *oxidative damage theory* of ageing—proposes that normal ageing and age-related diseases result from random, deleterious damage to cells/tissues by free oxygen radicals generated during normal aerobic metabolism. The theory suffers, however, from the fact that antioxidant supplements have only a poor effect in preventing ageing [8, 9]. All 3 theories are backed by strong evidence but are flawed.

Recently, a new theory, the *double-agent theory*, has unified the 3 concepts in a more simple way [10]. This Darwinian evolutionary-oriented concept is based on the assumption that humans and animals are structurally designed as a compromise to guarantee optimal survival during the time of reproduction based on strong natural selection pressure that is effective during youth and declines thereafter during continuous ageing. The theory argues that there is a tradeoff between oxidative stress as a critical redox signal that arranges genetic defenses against physiological stress (such as infection) in youth and oxidative stress as a cause of ageing and age-related diseases.

Oxidative Stress and Antioxidative Defense, Ageing, and Age-Related Diseases

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide anions, free hydroxyl radicals, the free radical nitric oxide, and the very toxic peroxynitrite, are continuously generated within cells, either as a result of the mitochondrial electron transfer processes or as a by-product of the catalytic activity of several enzymes [9]. Under normal circumstances, they operate as physiologically important mediators—so-called *second messenger molecules*—and by this, regulate biological signaling processes in cell functions [11]. In this way, and under the control of intracellular antioxidative defense systems, they are responsible for cell homeostasis, normal growth, and metabolism [12].

Intracellular antioxidative defense mechanisms in aerobic organisms, however, do not protect completely against ROS/RNS-mediated damage, thereby enabling ROS and RNS (which are continuously produced during the life span under normal physiological conditions) to potentially damage proteins, lipids, and most importantly, DNA. This cell/tissue damage—and in particular, damage to nuclear, mitochondrial, and telomeric DNA—participates in a network of interacting processes and may contribute to the phenomenon of ageing and age-related diseases via processes such as apoptosis, diminished energy production, and telomere shortening [13-16] (Figure 1). In other words, the

amount of oxidative damage accumulates and increases as an organism ages and is postulated to be a major causal factor of senescence [17].

Theoretical considerations about the underlying nature of this phenomenon include the notion that the continuously ongoing ROS/RNS production during ageing may be the consequence of oxidative stress-genes, evolutionarily selected because they confer benefit in early life (eg, host defense against infection). Such events as phagocyte ROS/RNS-production are beneficial in the short term by preventing death from infection before or during the reproductive years. This oxidative response, useful during youth, however, continues during the human life span leading ultimately to the chronic inflammation characteristic of old age. Ageing and age-related diseases, in this sense, are thus the price human beings pay for redox control of stress-gene expression during youth [9,10].

The rate of ageing generally varies with metabolic rate, and thus, as assumed, varies indirectly with the amount of free radicals generated during metabolism. Indeed, the idea that ageing should be linked to energy expenditure has a long history that can be traced to the late 1800s and the industrial revolution: energy consumption, per se, was responsible for senescence—a concept referred to as the *rate of living model* [18]. There is, however, accumulating evidence in support of the notion that the rate of leakage of free radicals from mitochondria correlates with ageing much more than does metabolic rate. In fact, comparative studies about the relationship between endogenous antioxidants and pro-oxidant factors and maximum longevity of different animal species indicate that the rate of mitochondrial ROS generation (ie, generation of endogenous oxidative damage) determines, at least in part, the rate of aging in animals: a higher rate of ROS generation is associated with a shorter life span [19, 20].

Notably, an increasing leakage of ROS via mitochondrial electron transport associated with age in addition to an exogenously induced increase of ROS production (eg, during reperfusion injury, intracellular metabolism of xenobiotics by the cytochrome p450-enzyme system, and others) may aggravate ongoing ROS-mediated mitochondrial damage. The result is a vicious circle of oxidative damage promoting further ageing.

Importantly, the increased oxidizing conditions

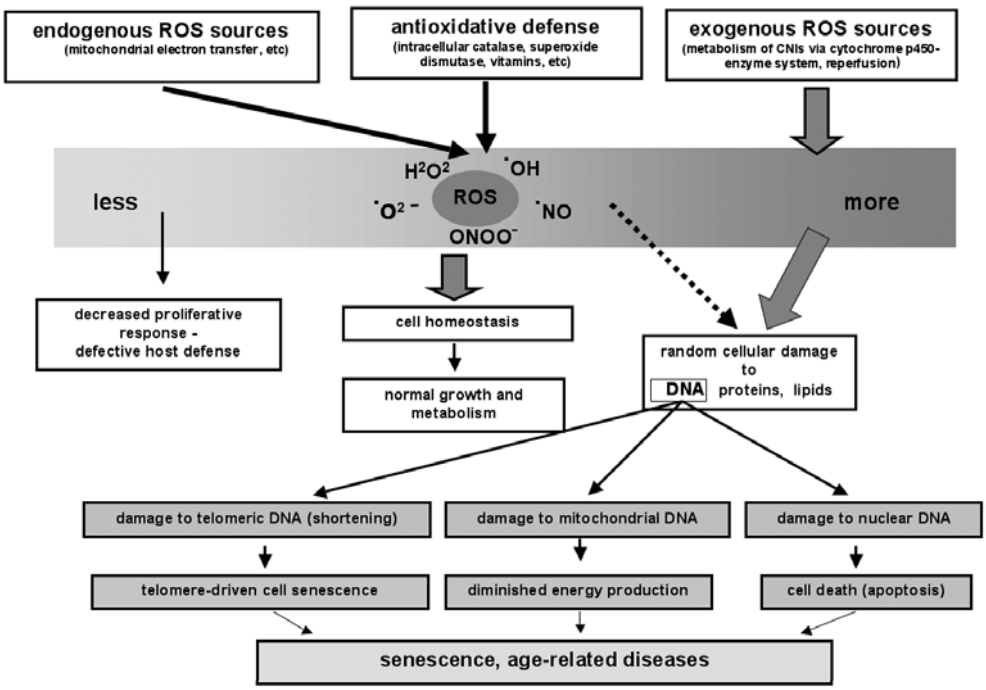

Figure 1. Contribution of oxidative stress to ageing and age-related diseases: Under normal physiological conditions, reactive oxygen species (ROS) are endogenously generated within cells and are responsible for cell homeostasis. However, instead of antioxidative control, ROS are continuously produced during the life span and lead to damage of nuclear, mitochondrial, telomeric DNA, proteins, and lipids (dotted arrow), as does ROS production induced by exogenous events (eg, reperfusion injury, and metabolism of calcineurin-inhibitors [CNIs] via the cytochrome P450-enzyme system). H^2O^2 = hydrogen peroxide; $^{\cdot}O^{2-}$ = free superoxide radicals (superoxide anions); $ONOO^-$ = peroxynitrite; $^{\cdot}NO$ = free nitric oxide radicals; $^{\cdot}OH$ = free hydroxyl radicals.

of old age also may be influenced by a decline of the antioxidative defense system during ageing (decrease of both intracellular antioxidative capacities and major exogenous antioxidants, for example, vitamins E, C, A, and beta-carotene). Preliminary findings in support of this hypothesis are currently available. In fact, the system consists of 3 endogenous defense lines: antioxidant enzymes, detoxifying enzymes, energy-dependent efflux pumps, and in addition, exogenously administered antioxidant nutrients. There is growing evidence suggesting that during ageing, enzymes of the first defense line such as superoxide dismutase, catalase, and glutathione peroxidase are intracellularly depleted. The same appears to be true for vitamins C and E [21-26].

Of considerable interest in this context is a recent report showing that older recipients of renal allografts demonstrate a lower antioxidative capacity as indicated, besides other things, by lower values of the vitamin E analogue, Trolox. The authors suggest that this scenario may be associated with a poorer outcome for kidney transplantations in older patients [27].

In regard to transplantation of old kidneys from deceased donors into older recipients, the consequences of increasing mitochondrial free-radical leakage and declining antioxidative capacity during ageing are deleterious both to the donor organ and to the recipient: The endogenously mediated oxidative injury to the graft is aggravated by exogenously added oxidative stress (eg, during graft reperfusion) resulting in an increased susceptibility of old organs to ROS-mediated injury. Also, the endogenously mediated chronic oxidative injury to the recipient organism is aggravated by exogenously added oxidative stress (eg, by the continuous use of calcineurin-inhibiting drugs [CNIs]), which results in promotion of ageing processes and age-related diseases. In fact, it is generally accepted that oxidative stress plays a critical role not only in ageing but also in the pathogenesis of age-related diseases such as atherosclerosis, autoimmune diseases, and cancer [28-30].

Oxidative Stress-Induced Activation of Innate Immunity, Ageing, and Organ Transplantation

A new door to the interpretation of the free-radical theory of ageing may be opened by recent insights into mechanisms of innate immunity preceding the adaptive immune response, as reviewed elsewhere [31-33]. Thus, the innate immune system evolved to protect the host as a rapid first line of defense against invading pathogens. The family of innate immune signaling receptors, known as the Toll-like receptors (TLRs), has proven (besides other pattern recognition receptors) to be essential in the detection and signaling of infection. Mammalian TLRs comprise a family of germ-line–encoded transmembrane receptors that recognize conserved bacterial, viral, fungal, and protozoal molecular structures called *pathogen-associated molecular patterns* (PAMPs). PAMPS, acting as exogenous ligands of TLRs, activate TLR-bearing cells of innate immunity (dendritic cells, monocytes, macrophages, vascular cells, and others) by interacting with TLRs. This interaction triggers signaling cascades leading (via activation of proinflammatory transcription factors such as NFκB and AP-1) to the activation of stress genes resulting in the production of cytokines, chemokines, and adhesion molecules that establish an acute/chronic inflammatory milieu.

Of importance for a new interpretation of the oxidative damage theory of ageing is the early observation that danger signaling stress proteins—known to be released in a host as a result of any tissue injury—act as putative endogenous ligands of TLRs, and by this interaction, are able to activate cells of the innate immune system as well. Such endogenous ligands were first described in studies with molecular chaperones, the heat shock proteins (ie, stress proteins), including HSP60, HSP70, and gp96 (known to be induced by oxidative stress). Later on, other damage-associated molecules such as fibronectin, heparan sulfate, hyaluronic acid, and fibrinogen, were described and shown to interact with TLRs. Collectively, similar to PAMPs, these molecules may be called danger-associated molecular patterns or damage-associated molecular patterns (DAMPs).

Rising intracellular oxidative stress during ageing may induce a chronically ongoing heat shock response. Consequently, heat shock proteins, acting as DAMPs, interact with TLR-bearing cells of innate immunity, thereby maintaining a state of chronic inflammation in the ageing organism. In this sense, ageing reflects a response of the innate immune system that is continuously activated by oxidative stress (Figure 2).

In addition, according to new notions and assumptions about the nature of atherosclerosis as an age-related disease, the sclerotic lesions reflect an

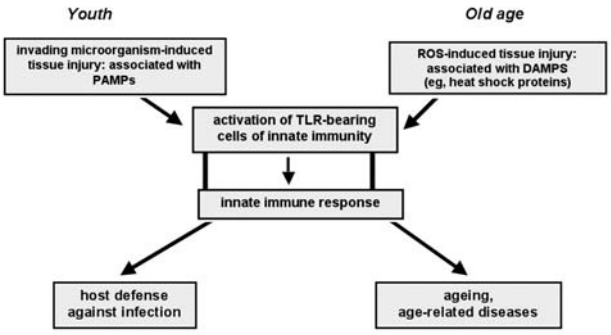

Figure 2. The free-radical theory of ageing in relation to the function of cells of the innate immune system (modified according to the double-agent theory [10]): Under evolutionary pressure, a strong innate immune system has evolved to protect the host as a rapid first line of defense against invading pathogens causing tissue injury. The defense response is provided by TRL-bearing cells of innate immunity that are able to recognize and interact with pathogen-associated molecular patterns (PAMPs). Humans and animals were selected with traits that allow them, during youth, to resist infections and survive, and thus, to concentrate on reproduction. After reproductive decline, activation of the same innate immune system is induced by tissue injury, this time caused by reactive oxygen species generated due to increased mitochondrial leakage during old age. TLR-bearing cells recognize and interact with injury-induced molecules = damage-associated molecular patterns (DAMPs) such as heat shock proteins. The subsequent innate immune response is directed at the organism itself and contributes to ageing and age-related diseases. As these negative effects of innate immunity take place in old age, they exert less selective pressure than their positive benefits in youth.

event of innate immunity and involve an ongoing inflammatory process with autoimmune features. Antigenic heat shock proteins—known to be induced by oxidative stress—play an important role by initiating and inducing events of innate immunity via interaction with TLR-bearing vascular cells [34-36]. According to these assumptions, interaction of HSPs with TLR4-bearing vascular cells such as endothelial cells, smooth muscle cells, and macrophages results in their activation, and via local secretion of cytokines, chemokines, adhesion molecules, and growth factors, they create an inflammatory milieu in the vessel wall, initiating and leading to atherosclerosis.

In conclusion, there is now accumulating evidence to support the notion that rising oxidative stress, via activation of TLR-bearing cells of innate immunity, may not only contribute to ageing processes but also may contribute to age-related diseases such as atherosclerosis, which in turn, may aggravate the ageing process. In other words, PAMPs-induced mechanisms of innate immunity against infection, and ROS/DAMPs-induced mechanisms of innate immunity against any tissue injury, as well as mechanisms that cause ageing and age-related diseases, represent common mechanisms (Figure 3). Indeed, there are already initial reports in support of this concept. In a liver reperfusion model, it has been demonstrated that ROS-induced injury is mediated via the Toll-like receptor 4 [37]; in a mouse model of defective innate immunity, it has been shown that a risk factor in atherogenesis, cholesterol, operates via activation of innate immunity [38]; and in a rat model, experimental evidence has been provided suggesting that vascular oxidative injury is increased in rat renal transplants with older age [39].

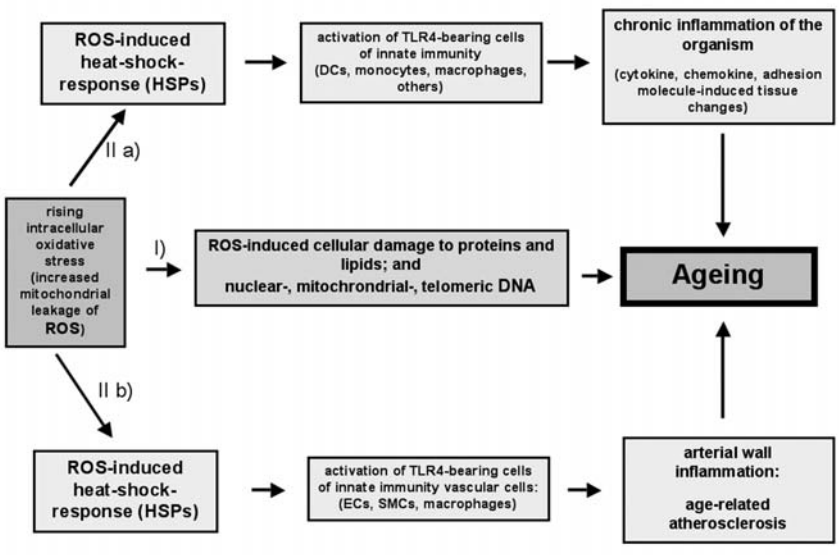

Figure 3. Increased oxidative stress (ie, increased leakage of ROS from mitochondria) may contribute to ageing processes via 3 pathways: direct intracellular damage of DNA, proteins, and lipids; activation of TLR4-bearing inflammatory cells of innate immunity resulting in chronic inflammation of body tissue; and activation of TLR4-bearing vascular cells of innate immunity contributing to development of age-related atherosclerosis.

Immunosuppressive Drugs and Their Different Influences on ROS-Mediated Ageing Processes and Age-Related Diseases

Concerning the scenario outlined here, older recipients of kidneys from deceased older donors may suffer in two ways. The contribution of oxidative stress to the development of ageing and age-related atherosclerosis may reflect a sort of double jeopardy to the transplant outcome. Concerning the donor organ, oxidative stress may represent a risk factor for chronic renal allograft failure. And with respect to the recipient, it may reflect a risk factor for cardiovascular diseases. The question is: Does immunosuppressive therapy currently used in kidney transplanted patients influence these events, or more precisely, do calcineurin-inhibiting drugs, associated with nephrotoxicity and atherogenicity, contribute to these inferior outcomes? In fact, one must state that there are different effects of immunosuppressants on the generation of ROS. In particular, there is no doubt that calcineurin-inhibitors contribute to the generation of ROS (ie, the very toxic peroxynitrite) and, in contrast, inosine 5´-monophosphate dehydrogenase (IMPDH) inhibitors may even inhibit their generation.

Calcineurin-inhibiting drugs: Concerning drug-induced atherogenicity, there are numerous reports in the literature from experiments clearly showing an atherogenic effect of cyclosporine (CsA), as indicated by induction of endothelial dysfunction, endothelial cell injury, and arterial arteritis. In addition, there are several reports on the phenomenon of CsA-induced generation of ROS (in particular, of free hydroxyl radicals in kidneys of CsA-treated rats) and of heat shock proteins induced by ROS, respectively [40-42]. Of special importance is the in vitro finding that CsA is able to induce the very toxic peroxynitrite in murine endothelial cells. In fact, peroxynitrite formation and protein nitration are considered a mediator and marker, respectively, of ROS/RNS-induced vascular damage, as observed, for example, in atherosclerotic lesions [43]. The literature about this issue regarding tacrolimus is sparse. Obviously, the experiments done in this field have mainly focused on CsA. Nevertheless, a recently published paper describes an important role for hydrogen peroxide in the FK506-mediated nephrotoxicity [44].

Mechanisms of CsA-induced ROS include the generation of superoxide anions as well as free radical nitric oxide. Free superoxide radicals appear to be generated during the metabolism of CsA within the cytochrome P450 enzyme system [45]. However, this observation has not been confirmed, and there seem to be some unknown sources of ROS-generation not yet detected [46]. Nitric oxide is generated during the reaction of L-arginine to citrulline, which is catalyzed by nitric oxide synthetase (ie, NOS). CsA has been shown to upregulate endothelial NOS; superoxide anions and nitric oxide, then, form the very toxic peroxynitrite [43] (Figure 4).

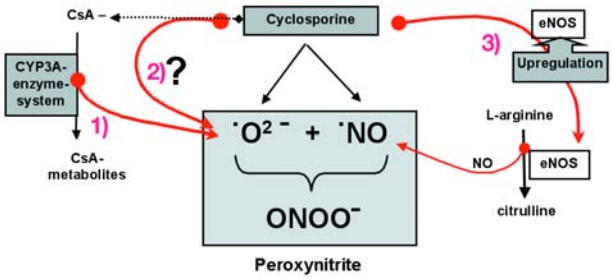

Figure 4. Contribution of cyclosporine (CsA) to the generation of reactive oxygen species. Possible pathways: 1) generation of superoxide anion radicals ($^{\cdot}O^{2-}$) via CsA metabolism by the cytochrome P450-enzyme system; 2) generation of superoxide anions via a still unknown pathway; and 3) generation of nitric oxide radicals ($^{\cdot}NO$) via upregulation of endothelial nitric oxide synthetase (eNOS) catalyzing nitric oxide formation. Superoxide anions and nitric oxide form the very toxic peroxynitrite ($ONOO^{-}$).

IMPDH-inhibiting drugs: There is a contrary effect of IMPDH-inhibiting drugs. Instead of upregulation of oxidative stress, they may even ameliorate it. Mycophenolic acid (MPA) is able to inhibit the action of inducible NOS, and by this, inhibit NO-generation, which forms peroxynitrite in the presence of superoxide anions. The underlying mechanism appears to be that NOS needs a cofactor to reach full activity, which is BH4 (a tetrahydrobiopterin). This protein needs GTP arising from IMP. MPA inhibits generation of GTP, which leads to inhibition of BH4 formation, associated with inactivity of NOS [47,48] (Figure 5).

Immunosenescence and Immunosuppressive Therapy

As reviewed elsewhere, immunosuppression in older recipients must be performed moderately and very cautiously in view of immunosenescence. Overimmunosuppression must be avoided [49]. In fact, many studies performed in mice, rats, dogs,

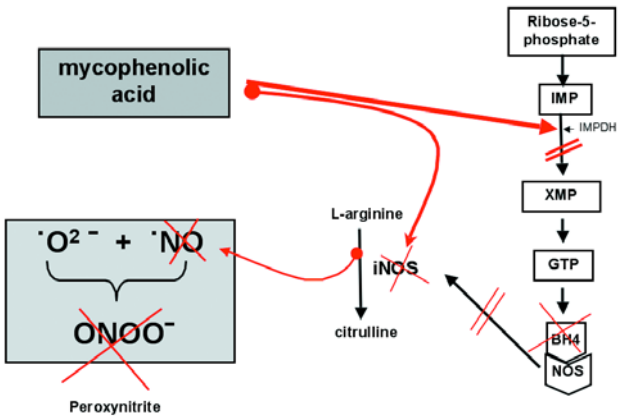

Figure 5. Amelioration of oxidative stress by inosine 5´-monophosphate dehydrogenase (IMPDH)-inhibitors. Possible pathway: Mycophenolic acid inhibits the formation of BH4 (tetrahydrobiopterin), a cofactor of inducible nitric oxide synthetase (iNOS) arising from guanosintriphosphate (GTP), which arises from inosinmonophosphate (IMP). iNOS loses its full activity, leading to inhibition of nitric oxide ($^{\cdot}NO$) formation and subsequent peroxynitrite ($ONOO^-$) formation.

monkeys, and man have established that age-associated immune decline is characterized by a decrease in both innate responses and cellular/humoral adaptive responses [50,51]. Consequently, a decline in acute rejection has been observed in older recipients of allografts [52]. Thus, the principle of tailor-made, individualized immunosuppression must be applied to this population of patients.

Clinical Trial: CNI-Free, Mycophenolate Mofetil-based Induction/Maintenance Immunosuppression in Older Recipients of Kidneys from Deceased Older Donors

At the end of the 1990s, our Munich institution performed a clinical pilot trial in older recipients of renal allografts from deceased older donors by applying a CNI-free mycophenolate mofetil (MMF)-based immunosuppressive induction/maintenance protocol, which was designed, at least in part, using much of the theoretical background described here. Induction therapy was provided in terms of a combination protocol consisting of MMF, steroids, and a short course (4-7 days) of rabbit ATG. In addition, we applied a transient antioxidative cocktail consisting of vitamins C and E and acetyl-cysteine intraoperatively until day 4 postoperatively. Calcineurin inhibitors were introduced (transiently or permanently) only in patients experiencing acute rejection episodes. The long-term results look promising: the 5-year patient and 5-year allograft survival rates are currently 87% and 70%, respectively. The 5-year renal graft survival is 88% when censored for patient death and nonimmune graft loss [53,54]. These encouraging results call for the design and performance of a multicenter randomized control trial. However, there is no money for funding such a trial at the present time! In addition, such a control trial is not necessary in view of the impressive long-term data obtained in this worst-case scenario as well as in regard to the well-fitting theoretical scientific background of the study presented in this article. Meanwhile, more than 100 consecutive patients have been treated with this protocol, and we recommend continuing with this protocol without following the constraints of "trialomania" [55].

In addition, the successful Munich concept of MMF-based long-term treatment is supported by recent reports of improved long-term survival in patients and their grafts under an MMF-containing immunosuppressive combination regimen following kidney, heart, and liver transplantation [56-60].

Conclusion

Any redesign of immunosuppressive protocols in older recipients of kidneys, in particular, of kidneys from deceased older donors, should include reflections about both the special nature of immune responses during age and modern theories of ageing. Accordingly, the state of immunosenescence usually encountered in kidney transplanted older patients allows application of nonaggressive protocols, at least in the course of maintenance treatment.

Among the concepts of ageing, the oxidative damage theory of ageing, which may also explain age-related diseases (atherosclerosis), continues to gain credence and thus, calls for therapeutic approaches in clinical application of antiageing (ie, nonoxidizing) instead of proageing (ie, oxidizing) immunosuppressive agents. In fact, there is growing experimental evidence suggesting that the clinical use of calcineurin-inhibiting agents is associated with oxidative stress, which may aggravate the already existing increased mitochondrial leakage of ROS during ageing. On the other hand, IMPDH-inhibiting drugs may even diminish this age-related increased intracellular oxidized state. Notably, there is preliminary evidence from clinical trials that MMF-based induction/maintenance protocols, without or even with the simultaneous use of CNIs, are associated with improved patient

and allograft survival. Consequently, application of CNI-free protocols in older patients receiving older organs currently appears to be the treatment of choice. Therefore, future trials using TOR-inhibiting drugs would be of interest with regard to their capability to potentially counteract the two most frequent disasters in immunosuppressed older patients: malignancies and cardiovascular death.

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