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Review

The role of inducible nitric oxide synthase in cardiac allograft rejection

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Cardiac transplantation is an effective therapy for end-stage heart failure with one and five year survival rates about 80% and 65% respectively [1]. Despite these good results, cardiac allograft rejection remains a problem which produces impaired ventricular performance and death of cardiac myocytes ultimately causing congestive heart failure, low cardiac output, hypotension and reduced pressor responses to catecholamines. Chronic allograft rejection also results in the development of transplant-associated coronary atherosclerosis, a vasculopathy that produces myocardial ischemia, infarction and sudden death and is the leading cause of death in cardiac transplant recipients after the first year [2]. Although there is a large body of evidence concerning the immunological interactions involved in cardiac allograft rejection, knowledge concerning the cellular and biochemical mechanisms responsible for contractile dysfunction and for death of heart muscle cells remains incomplete. Since an inflammatory reaction in the myocardium is an intrinsic component of the pathological changes observed during cardiac allograft rejection, our laboratory undertook an investigation of the participation of nitric oxide synthases, particularly the inducible nitric oxide synthase (iNOS), in the manifestations of cardiac allograft rejection.

1. NO synthesis

The five electron oxidation of L-arginine to L-citrulline and biologically active nitric oxide (NO) is important to a large variety of physiological and pathological processes [3–4]. NO synthesis is accomplished by the three isoforms of NOS, the neuronal NOS (nNOS, NOS 1) originally identified in brain, inducible NOS (iNOS, NOS 2) originally identified in macrophages and endothelial NOS (eNOS, NOS 3) originally identified in endothelial cells. Constitutive nitric oxide synthases (nNOS and eNOS) require calcium and calmodulin as cofactors and generate low amounts of NO. Small amounts of NO released by endothelial cells in response to hormones or shear stress interact with soluble guanylyl cyclases to increase the formation of cyclic GMP in target cells such as platelets, endothelial cells and vascular smooth muscle cells promoting inhibition of platelet adhesion and aggregation, inhibition of leukocyte adhesion and migration and vasodilation respectively. The NO produced by iNOS acts as a neurotransmitter in brain, in cells of the nonadrenergic, non-cholinergic nervous system and in skeletal muscle [5].

The inducible NO synthases expressed in macrophages, endothelial cells, vascular smooth muscle cells and cardiac myocytes in response to cytokines (such as IL-1β, TNF-α, IFN-γ, IL-6) or bacterial endotoxin do not require calcium and calmodulin as cofactors and generate substantially larger amounts of NO for long periods of time [3–6]. NO produced by iNOS in activated macrophages is cytotoxic and participates in their antimicrobial actions [4,7]. NO produced by vascular smooth muscle cells and cardiac myocytes in response to endotoxin has been implicated in the pathogenesis of hypotension in association with bacterial infections [4]. The continuous large production of NO by iNOS in cardiac myocytes has been shown to influence myocardial contractile responses and to affect heart rate [6].

Other factors important in the synthesis of NO by iNOS include the availability of the substrate, L-arginine, and of the cofactors NADPH and tetrahydrobiopterin (THB) [3–7]. When iNOS is induced by cytokines in macrophages, endothelial cells or cardiac myocytes, cationic amino acid transporter proteins, CAT 1, CAT 2B (both high affinity)
and CAT 2A (low affinity) are coinduced which can increase the intracellular l-arginine concentration. GTP cyclohydrolase, the key enzyme in THB₃ biosynthesis, is also coinduced with iNOS. Other experimental evidence obtained with nNOS and iNOS indicates that NO can exert a negative feedback on NO synthesis [6]. In activated macrophages NO can inhibit iNOS activity by reducing the availability of heme and its insertion into monomers, blocking formation of the dimers required for enzyme activity.

2. iNOS induction in cardiac allograft rejection

During cardiac allograft rejection there is significant release of cytokines by activated T lymphocytes and macrophages which participate in the immune response to foreign HLA and other antigens present in endothelial and other cells of the transplanted heart [8]. Because cytokines, particularly those released by the Th1 subset of lymphocytes, were reported to stimulate iNOS expression in several cell systems, Yang et al. used a rat model to investigate iNOS expression during cardiac allograft rejection [9]. Heterotopic abdominal cardiac transplantation was performed using Lewis strain donor hearts and Wistar–Furth strain recipients. Lewis to Lewis transplants were performed as syngeneic controls. Allograft rejection is complete at days 6–7 with this model. Cardiac allografts examined at day 5 manifested reduced contractility and histological changes of severe rejection, with an inflammatory myocardial infiltrate composed of lymphocytes and macrophages, edema and damage and destruction of cardiac muscle fibers. The mRNA for iNOS and iNOS protein were detected in ventricular homogenates and also in isolated purified myocytes from the rejecting cardiac allografts but not in ventricular tissue or myocytes from the syngeneic control grafts. iNOS enzyme activity and tissue cyclic GMP levels were also increased significantly in the rejecting allografts. Immunostaining with a highly specific antibody indicated that iNOS protein was present in macrophages infiltrating the myocardium and also in cardiac myocytes of the rejecting allografts. Some lymphocytes, macrophages, and microvascular endothelial cells in the allografts also showed positive iNOS immunostaining. The data indicated that myocardial iNOS mRNA, protein and enzyme activity are induced in infiltrating macrophages and cardiac myocytes of rejecting allografts.

Using a similar Lewis to ACI strain heterotopic rat cardiac transplant model Worrall and coworkers confirmed that iNOS mRNA and enzyme activity were increased in rejecting cardiac allografts [10]. In agreement with a previous report serum nitrite/nitrate levels were also significantly increased in the allograft recipients [10–12]. Immunostaining revealed iNOS protein in infiltrating macrophages but not in cardiac myocytes. Whether absent iNOS immunostaining in cardiac myocytes in the Worrall studies [10,11] relates to rat strain differences, milder rejection or methodological problems is unclear. Treatment with aminoguanidine, a drug which inhibits iNOS activity (but which also has antioxidant properties, reduces advanced glycation end product formation and inhibits di-amine oxidase and aldose reductase) improved allograft papillary muscle contractile performance, reduced the extent of pathological changes and prolonged allograft survival from 10 to 15 days [10,11]. EPR signals in myocardial tissue from the rejecting grafts were indicative of the formation of nitrosylferromyoglobin and/or nitrosylferro-hemoglobin and of nonheme iron–dinitrosyl complexes; these signals were also ameliorated by aminoguanidine [10]. Russell et al., using a Lewis-F344 rat cardiac transplantation model, also observed a prolonged increase in expression of iNOS mRNA and immunostaining of myocardial macrophages and of vascular smooth muscle cells in rejecting cardiac allografts [13]. In studies of serial endomyocardial biopsies of patients during the first year following heart transplantation, Lewis and coworkers found iNOS mRNA during the first 180 days [15]. iNOS protein was demonstrated by immunohistochemistry in cardiac myocytes. There was also increased myocardial cGMP. iNOS mRNA expression was not related to the ISHLT histological grade of rejection but was associated significantly with systolic and diastolic dysfunction of the left ventricle. Winlaw et al. in a study of Brown–Norway to Lewis allografts and Lewis–Lewis isografts found that urinary nitrite excretion in untreated allograft rejection was increased 8 fold above basal excretion of isografts and that peak nitrate excretion occurred early in rejection and was attenuated by immunosuppressive therapy [16].

3. iNOS in transplant vasculopathy

Allograft associated vasculopathy is considered to be a form of chronic rejection [2]. Transplant associated coronary artery disease tends to be diffuse, to extend to small arteries and arterioles and to involve a proliferative intimal response of smooth muscle cells and to a lesser extent of macrophages. Russell et al. and Akyurek et al. reported that eNOS was present in endothelial cells and that iNOS expression was upregulated in macrophages and smooth muscle cells in the intimal lesions found in rat models [14,17]. Smooth muscle immunostaining was particularly apparent at later time points in lesions from the rat. Ravalli et al. used in-situ hybridization and immunostaining to examine iNOS expression in human transplant coronary artery disease [18]. Both iNOS mRNA and protein were detected in neo-intimal macrophages, neo-intimal ‘foam’ cells and neo-intimal smooth muscle cells. Immunostaining for nitrotyrosine was also found in the same cell types. These findings are similar to those found in human atherosclerotic lesions and in mouse models of the disease [19,20]. Whether iNOS expression is pathogenetic in pro-
moting the vascular disease (e.g. by increasing oxidant stress and the expression of oxidant sensitive genes) or is protective against the development of vascular disease is unclear. In the study of Ajji et al. the administration of L-arginine supplements to LDL receptor knockout mice fed a high cholesterol diet reduced xanthoma formation and the extent of atherosclerotic aortic lesions by 40% [20]. This beneficial effect was abrogated when a NOS inhibitor was coadministered with the L-arginine supplement suggesting that NO produced by eNOS and iNOS was responsible for lesion reduction. NO is known to inhibit platelet and white cell adhesion and transmigration of white blood cells across the endothelium and to block smooth muscle cell proliferation [3,4,6]. Another possibility is that NO contributes to vascular remodeling by its known effects to promote apoptosis of macrophages and vascular smooth muscle cells [21,22]. A recent preliminary report which indicated that transplant associated vasculopathy was more extensive in allografts in iNOS deficient mice is also consistent with a protective effect of iNOS in this disorder [23].

4. Relationships of NO, cytokines and cellular immune responses in cardiac allograft rejection

Cellular immune responses constitute major components of acute and chronic allograft rejection [24]. Activated T lymphocytes, both CD4+ and CD8+, along with macrophages and endothelial cells expressing class II MHC antigens and multiple cytokines released by macrophages and other cells (including IL-1, IL-2, IFN-γ, TNF-α, IL-6 and IL-10) are present in the myocardium and vessels undergoing allograft rejection [8,25]. iNOS expression occurs early in the rejection process as reflected by immunostaining of myocardial tissues and also by urinary excretion of nitrite/nitrate [9–11,16]. The administration of immunosuppressive therapy such as dexamethasone, cyclosporine A and FK506 alone or in combination resulted in an amelioration of the pathological changes in the myocardium and also a reduction of the levels of iNOS mRNA, and (in another report) a delay in the onset and peak rise in urinary excretion of nitrite/nitrate [9–11,16,26]. As mentioned previously, the administration of aminoguanidine, a somewhat selective iNOS inhibitor, also resulted in an improved survival and a delay in the histological appearance of severe rejection [10,11]. These data suggest that iNOS in macrophages and cardiac myocytes plays a role in the pathogenesis of rejection but this is not exclusive or necessarily the most important role.

The specific signal which induces iNOS expression during allograft rejection has not been defined. Nevertheless, it is probable that cytokines produced by activated macrophages play an important role [8,25]. Interferon-γ, TNF-α and IL-1β have been reported to induce iNOS mRNA, protein and enzyme activity in macrophages, vascular smooth muscle cells and isolated rat cardiac myocytes in vitro [3,4,6]. It is also probable that interaction between the CD40 ligand expressed on CD4+ T-lymphocytes and its target molecule CD40 also plays a role. Interaction by cell contact between the CD40 ligand on T-cells and CD40 on macrophages and has been reported to induce iNOS in vitro [27]. In recent studies of human coronary arteries using immunohistochemistry, Szabolcs et al. found that in both transplant coronary artery disease and atherosclerosis CD40 ligand + T-lymphocytes were present in the intimal lesions along with abundant positive immunostaining for CD40 on endothelial cells, macrophages, ‘foam’ cells and smooth muscle cells [28]. Larson et al. using a mouse model of heterotopic cardiac transplantation reported that treatment with an antibody to CD40 ligand (anti-gp39) at the time of transplantation markedly prolonged graft survival [29]. Allografts from treated recipients showed decreased expression of mRNA for iNOS but unaltered expression of transcripts for T-cell cytokines or the costimulatory molecules B7-1, B7-2 [29]. In a subsequent publication this group demonstrated that simultaneous therapy which blocked CD40 ligand-CD40 interactions with an antibody which inhibited CD28-B7 interactions aborted T-cell clonal expansion in vitro and in vivo and resulted in long term acceptance of cardiac and skin allografts and inhibition of the development of coronary vascular disease in the transplanted hearts [30].

5. iNOS and ventricular contractile function

Impaired ventricular contractile performance and cardiac myocyte death are the hallmarks of cardiac allograft rejection. Much evidence suggests that NO produced by iNOS contributes to allograft contractile dysfunction. NO acts upon soluble guanylyl cyclase to form cyclic GMP which is known to decrease cardiac myocyte L-type calcium channel current and the contractile responses of cardiac myofilaments to calcium [6,31]. Increased myocardial cGMP has been found in guinea pigs treated with endotoxin and in rat and human cardiac allografts [9,15,32]. In studies by Brady et al. the amplitude of contraction of isolated guinea pig cardiac myocytes was reduced in cells from animals treated with endotoxin to induce iNOS and in myocytes from normal guinea pigs that were treated with media containing NO or the NO donor drug sodium nitroprusside [32,33]. In studies of isolated rat cardiac myocytes by Balligand, the induction of iNOS in myocytes by cytokines was not associated with a depression of basal contractile function but with a reduction in the contractile responses to β adrenergic agonists that could be reversed by administration of NOS inhibitors [34,35]. Worrall et al. reported that iNOS derived NO in early rejection of cardiac allografts (prior to widespread cell death) was associated with impaired contractility of isolated papillary muscles at baseline and during βaden-
ergic, adenylate cyclase and calcium stimulation[10,11,36]. There were also associated membrane dysfunctions[36]. The impaired contractile responses were ameliorated by aminoguanidine or therapy with corticosteroids which inhibited iNOS expression. As mentioned previously, in studies of patients following cardiac transplantation Lewis et al. found that iNOS mRNA was inversely related to cardiac function, i.e. ejection fraction[15]. Paulus et al. found that in human allografts, iNOS mRNA (determined by RT-PCR in endomyocardial biopsies) was an independent variable relating a reduced peak contractile response to dobutamine to simultaneous shortening of systole, i.e. there was earlier onset of left ventricular relaxation and reduction of left ventricular end-systolic pressure[37].

The mechanisms by which NO produced by iNOS influences ventricular performance remain largely unexplored but are potentially multiple[6]. In addition to increasing cGMP, NO can produce auto-ADP ribosylation of glycolytic enzymes, inhibition of ribonucleotide reductase, and activation of poly ADP ribose synthetase which can lead to depletion of cellular energy stores. NO can also inhibit enzymes involved in the mitochondrial respiratory chain, reducing oxygen consumption and can inhibit the citric acid cycle enzyme aconitase. NO and its derivatives such as nitrososnium ion (NO +) can react with SH groups to form S nitrosoproteins which can in turn support additional transnitrosation reactions with other SH containing proteins influencing their activity. Peroxynitrite, the product of the interaction of NO and superoxide, is a strong oxidant which can cause autoperoxidation of lipids and proteins and decompose to form toxic hydroxyl radicals[38–40]. Peroxynitrite can also produce nitration of tyrosines in cell proteins. Nitration of contractile proteins such as actin may impede the contractile function of cardiac myofilaments[40]. As will be discussed subsequently NO in large doses may also contribute to death of cardiac myocytes.

### 6. iNOS and cardiac myocyte death

Death of cardiac myocytes is the hallmark of cardiac allograft rejection. Because iNOS expression was apparent in macrophages and cardiac myocytes in rejecting allografts and because the generation of NO by iNOS in macrophages is important in the immune defenses against invading viruses, bacteria and other microorganisms, Pinsky et al. performed in vitro experiments to investigate potential toxic effects of NO in the heart[41]. In the first experiments, J774 macrophages were stimulated with cytokines to induce iNOS. Stimulated and control macrophages were cocultured with isolated rat cardiac myocytes using transwell filter inserts to prevent physical contact between the two cell types. There was significantly greater myocyte death (assessed by creatine kinase release and by trypan blue dye exclusion) in cocultures with stimulated macrophages expressing iNOS. The death rate returned to control levels when cocultures with activated macrophages were treated with l-NMMA which inhibits NO enzyme activity. The data indicated that NO produced by iNOS in activated macrophages can be lethal to adjacent cardiac myocytes. In additional experiments they showed that rat cardiac myocytes treated with TNF-α, IL-1β and IFN-γ expressed iNOS mRNA, protein and enzyme activity and also exhibited a higher death rate than control myocytes[41]. The increased death of cardiac myocytes was inhibited by the administration of l-NMMA to block NO formation and by coincubation of the stimulated myocytes with TGF-β which reduced iNOS expression. The data indicate that proinflammatory cytokines can induce iNOS expression in adult cardiac myocytes and that NO so produced can be autotoxic to the cardiac myocytes. Taken together the experiments suggested that NO produced by macrophages infiltrating the myocardium or by cardiac myocytes in a cytokine rich milieu is potentially cytotoxic to heart muscle cells. Such cardiotoxicity may be manifest not only in allograft rejection but in other pathological states such as myocardial infarction or cardiomyopathy in which macrophages and cytokines are abundant and in which there is myocardial expression of iNOS.

### 7. Necrosis and apoptosis

Although there is considerable information concerning immune mechanisms of cardiac myocyte death, knowledge concerning mechanisms of myocyte death during cardiac allograft rejection is incomplete[24]. During advanced episodes of rejection there can be necrosis of heart muscle cells. This is characterized by swelling of the cells, disruption of internal and external cell membranes and eventual lysis[42]. Necrosis is often associated with a marked inflammatory response. Recently, it has become increasingly apparent that apoptosis of cardiac myocytes also occurs in a variety of important cardiovascular diseases. Apoptosis is a morphologically and biochemically distinct form of programmed cell death[42,43]. During apoptosis there is condensation of nuclear chromatin and cell shrinkage with preservation of intracellular organelles. Subsequently there is blebbing of nuclear and cytoplasmic membranes and fragmentation of the dying cell into membrane bound ‘apoptotic’ bodies which undergo phagocytosis. The process is relatively rapid lasting 2–12 hours. The sequence of events during apoptosis requires energy and follows an internally determined program involving the activation of endonucleases which degrade chromosomal DNA into oligosomal fragments that are multiples of 180–200 base pairs in lengths (which form DNA ‘ladders’ after electrophoresis on agarose gels)[42,43]. The program also involves the expression of multiple genes which promote or hinder the death pathway (e.g. Bcl-2 or Bax) and the expression of genes for proteases (e.g. CPP-32) involved in cell destruction[44,45].
8. Apoptosis and iNOS expression in cardiac allograft rejection

Albina and coworkers have reported that NO produced by iNOS in cytokine-treated macrophages can trigger apoptosis of the macrophages and also of certain tumor cells cocultured with the macrophages [22,46]. Drugs that release NO have also promoted macrophage apoptosis. Accordingly, Szabolcs et al. used the Lewis to Wistar–Furth heterotopic heart transplantation model to investigate whether apoptosis occurs during cardiac allograft rejection and whether the time course of apoptosis was related to the induction of iNOS [47]. They found that significant apoptosis of macrophages and of cardiac myocytes occurred during cardiac allograft rejection. Apoptosis was identified by in situ labeling of DNA fragments in cell nuclei by two methods and also by the presence of DNA ladders on agarose gels. Apoptotic cardiac myocytes were distinguished by their characteristic striations, immunoreactivity for muscle actin and from the appearance of intact shrunken myocytes with intact membranes and hyperchromatic or fragmented nuclei (Fig. 1a). In the rejecting allografts the number of apoptotic nuclei increased sharply during days 3–5 after transplantation and at day 5 there was a significant increase in apoptotic nuclei (macrophages, endothelial cells and cardiac myocytes) in comparison to syngeneic controls Fig. 1a. Apoptosis of myocytes was present in the rejecting allografts at a rate that was 30 fold higher than that observed in the non-rejecting controls (Fig. 2a). Apoptosis of myocytes was present in both areas with macrophage-rich inflammatory infiltrates and in areas with no inflammation. Positive immunostaining for iNOS was present in macrophages, mononuclear cells and cardiac myocytes in the rejecting allografts Fig. 2b. Immunostaining for nitrotyrosine, a marker of peroxynitrite, was found in the rejecting biopsies in regions where iNOS protein expression was also present (Fig. 2c). The data indicate that apoptosis is a major form of cardiac myocyte death during human cardiac allograft rejection and that it occurs in association with the expression of iNOS and nitration of proteins indicative of the presence of peroxynitrite.

9. iNOS expression in myocardial infarction and cardiomyopathy

As discussed previously, Lewis et al. found that the expression of iNOS mRNA in endomyocardial biopsies of human heart transplant recipients was inversely related to left ventricular performance, i.e. ejection fraction [15]. Since apoptosis produces the gradual loss of cardiac myocytes, it is possible that apoptotic myocyte loss triggered by NO may contribute to the decline in ventricular function which is observed in many cardiac allografts over time. iNOS has also been demonstrated in ventricular tissues from animals with reperfusion injury or myocardial infarction and from patients with idiopathic dilated cardiomyopathy [52–56]. In addition, recent studies have demonstrated that apoptosis of cardiac myocytes occurred in iNOS-rich regions at the margins of acute myocardial

Fig. 1. Rejection in rat heterotopic cardiac allograft labelled for apoptosis (blue nuclei) and cardiac myocytes (brown cytoplasm) (a), iNOS (brown) (b) and nitrotyrosine (brown) (c). a: Cardiac myocytes (brown) adjacent to macrophage-rich inflammatory infiltrates show the highest rate of apoptotic nuclei (blue) (arrow). Many apoptotic nuclei are already phagocytosed by macrophages, which prevents determination of their cellular origin. b: Numerous macrophages show strong reactivity for iNOS (brown). Adjacent cardiac myocytes also show weak iNOS labelling. c: Nitrotyrosine which is formed after exposure of cellular proteins to peroxynitrite is present in areas of myocyte damage and inflammation.
infarctions in rabbits [52]. Other studies have independently documented iNOS enzyme activity. iNOS mRNA and protein in cardiac myocytes in myocardial tissues from patients with idiopathic dilated cardiomyopathy many of whom have elevated circulating levels of cytokines and a macrophage-rich myocardial inflammatory infiltrate [53,54]. Additional recent reports have documented that apoptosis of cardiac myocytes occurs in ventricular tissue from patients with idiopathic dilated cardiomyopathy who died of advanced congestive heart failure [57,58]. Conceivably apoptosis of heart muscle cells triggered by iNOS may contribute to the progression of ventricular failure in these and other inflammatory diseases of the myocardium.

10. Possible therapeutic implications in allograft rejection and other cardiac disorders

Apoptosis of cardiac myocytes in association with iNOS expression in allograft rejection may have several therapeutic implications. First, it raises the possibility that drugs which selectively inhibit iNOS enzyme activity or which interfere with NO production by macrophages or cardiocytes might be beneficial in reducing myocyte loss during allograft rejection or other diseases such as dilated cardiomyopathy. In a rat model, amino-guanidine, a semi-selective inhibitor of NO production by iNOS but which also has other effects, prolonged survival and reduced the pathological changes in the grafts [10,11]. In preliminary studies of our rat cardiac transplantation model, Yang et al. administered to the allograft recipients CNI-1493, a tetra-valent guanylhydrazone compound which blocks L-arginine transport into macrophages inhibiting iNOS synthesis of NO and which also blocks the release of TNF-α and other cytokines from macrophages [59–61]. In comparison to vehicle treated allografts, administration of CNI-1493 was associated with prolongation of survival, significant reduction of apoptosis, myocyte loss and macrophage infiltration of the myocardium [59]. Second, the finding of apoptosis of cardiac myocytes in association with iNOS raises the possibility that one could use molecular techniques such as transfection of Bcl-2 to alter the apoptotic gene program favorably to preserve myocyte viability [62]. Finally, it suggests that one might reduce cardiac myocyte death by developing inhibitors or inactivators of the proteases (caspases) such as CPP32 which are involved in the final stages of apoptotic destruction of the cardiac myocytes [63,64].

Acknowledgements

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References


Fig. 2. Human cardiac allograft rejection labelled for apoptosis (blue nuclei) and cardiac myocytes (brown cytoplasm) (a), iNOS (brown) (b) and nitrotyrosine (brown) (c). a: Similar to the experimental results in rat apoptosis of cardiac myocytes (arrow) occurs predominantly adjacent to macrophage-rich inflammatory infiltrates in human allograft rejection. b: Macrophages (arrow) and adjacent cardiac myocytes show strong reactivity for iNOS (brown). c: Damaged cardiac myocytes (arrow) show immunoreactivity for nitrotyrosine (brown) representing exposure to peroxynitrite.


