Gene Expression Profiles and B-Type Natriuretic Peptide Elevation in Heart Transplantation: More Than a Hemodynamic Marker

Mandeep R. Mehra, Patricia A. Uber, Dirk Walther, Mark Vesely, Jay G. Wohlgemuth, James Prentice, Darren Tayama and Margaret Billingham

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Gene Expression Profiles and B-Type Natriuretic Peptide Elevation in Heart Transplantation

More Than a Hemodynamic Marker

Mandeep R. Mehra, MD; Patricia A. Uber, Pharm D; Dirk Walther, PhD; Mark Vesely, MD; Jay G. Wohlgemuth, MD; James Prentice, MS; Darren Tayama, MD; Margaret Billingham, MD

Background—B-type natriuretic peptide (BNP) is chronically elevated in heart transplantation and reflects diastolic dysfunction, cardiac allograft vasculopathy, and poor late outcome. This investigation studied peripheral gene expression signatures of elevated BNP concentrations in clinically quiescent heart transplant recipients in an effort to elucidate molecular correlates beyond hemodynamic perturbations.

Methods and Results—We performed gene microarray analysis in peripheral blood mononuclear cells of 28 heart transplant recipients with clinical quiescence (absence of dyspnea or fatigue; normal left ventricular ejection fraction [EF >55%]; ISHLT biopsy score 0 or 1A; and normal hemodynamics [RAP <7 mm Hg, PCWP ≤15 mm Hg, and CI ≥2.5 L/min per m^2]). BNP levels were performed using the Triage B-type Natriuretic Peptide test (Biosite Diagnostics Inc, San Diego, Calif) and median BNP concentration was 165 pg/mL. Seventy-eight probes (of 7370) mapped to 54 unique genes were significantly correlated with BNP concentrations ($P<0.001$). Of these, the strongest correlated genes ($P<0.0001$) were in the domains of gelsolin (actin cytoskeleton), matrix metallopeptidases (collagen degradation), platelet function, and immune activity (human leukocyte antigen system, heat shock protein, mast cell, and B-cell lineage).

Conclusions—In the clinically quiescent heart transplant recipient, an elevated BNP concentration is associated with molecular patterns that point to ongoing active cardiac structural remodeling, vascular injury, inflammation, and alloimmune processes. Thus, these findings allude to the notion that BNP elevation is not merely a hemodynamic marker but should be considered reflective of integrated processes that determine the balance between active cardiac allograft injury and repair. **(*Circulation*. 2006;114[suppl I]:I-21–I-26.)**

Key Words: gene expression ■ heart transplantation ■ natriuretic peptides

B-type natriuretic peptide (BNP), a 32-amino acid neurohormone, secreted predominantly from the cardiac ventricle in response to increased wall stress, is chronically elevated in heart transplant recipients.[1] Because an elevation in BNP is associated with cardiopulmonary hemodynamic aberrations, it has been thought to represent diastolic dysfunction or ventriculo-vascular uncoupling of the allograft with its surrounding vasculature.[2,3] More recently, investigations have noted that natriuretic peptides in the chronic phase of transplantation are associated with cardiac rejection[4] and are predictive of cardiac allograft vasculopathy[5,6] and graft loss.[6,7]

The primary purpose of this investigation included investigation of molecular pathways using peripheral gene expression (GE) patterns that correlate with elevated BNP concentrations in otherwise clinically quiescent heart transplant recipients.

Materials and Methods

Study Design

Of 42 consecutive heart transplant recipients initially screened, we enrolled 28 consecutive clinically quiescent patients in this study at least 4 weeks after heart transplantation who met the inclusion criteria. Clinical quiescence was defined as the absence of any symptoms of dyspnea or fatigue; normal left ventricular ejection fraction (EF >55%) by echocardiography; no histological evidence of rejection on endomyocardial biopsy (International Society for Heart and Lung Transplantation [ISHLT] biopsy score 0 or 1A)[8]; and normal hemodynamics (right arterial pressure <7 mm Hg, pulmonary capillary wedge pressure ≤15 mm Hg, and cardiac index ≥2.5 L/min per m^2) measured by invasive right heart catheterization. Furthermore, patients with abnormal renal function as defined by a serum creatinine >1.5 mg/dL were excluded because of the possibility of interference with BNP concentrations.[8] Clinical data, including immunosuppressive drug regimen and measures of allograft function for each patient encounter, were also collected and analyzed. All patients received tacrolimus and mycophenolate

From University of Maryland School of Medicine (M.R.M., P.A.U., M.V.), Baltimore, Md; XDx Inc (D.W., J.G.W., J.P., D.T.), South San Francisco, Calif; Stanford University (M.B.), Stanford, Calif.

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Correspondence to Mandeep R. Mehra, Herbert Berger Professor and Head of Cardiology, University of Maryland Medical Center, 22 South Greene Street–S3B06, Baltimore, MD 21201. E-mail: mmehra@medicine.umaryland.edu

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mofetil-based immunosuppression with adjunctive corticosteroids (prednisone). The study was conducted as part of an Institutional Review Board (IRB)-approved protocol. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Blood Sampling

Venous blood samples obtained from enrolled patients were processed on the day of scheduled surveillance biopsies. BNP measurements were performed using whole blood (5 mL) collected into tubes containing potassium EDTA (1 mg/mL blood). The Triage B-type Natriuretic Peptide test (Biosite Diagnostics Inc, San Diego, Calif) was used for this measurement. The Triage BNP test is a fluorescence immunoassay for the quantitative determination of BNP in whole blood and plasma specimens with a turnaround time of 15 minutes and a coefficient of variability of 15%.[1,6] Peripheral blood mononuclear cells (PBMCs) were isolated from 8 mL venous blood using density gradient centrifugation (CPT tubes; Becton-Dickinson). Samples were frozen in lysis buffer (RLT; Qiagen) within 2 hours of phlebotomy. Total RNA was isolated from each sample (RNeasy; Qiagen) and assessed spectrophotometrically (Spectromax). Two micrograms of RNA from each sample were converted to cDNA, which were then used as templates for in vitro transcription with Cy3-dCTP to generate Cy3-labeled RNA.

Endomyocardial Biopsy

Standard techniques were used to obtain biopsy samples, which were graded by local pathologists as well as by 3 independent ("central") pathologists blinded to clinical information. All samples were obtained >4 weeks after transplant, transfusion, or rejection therapy. Absence of rejection was defined as ISHLT 1A or 0 grades, confirmed by a panel of blinded central pathologists.[9]

Gene Expression

Microarray Testing

A custom microarray was designed using sequences from subtracted, suppressed libraries (25 000 sequences) of stimulated and resting human leukocytes (PCR Select; Clontech) and also identified using publicly available databases; 24 000 50-mer oligonucleotide (Sigma) gene probes (8000 probes in triplicate) representing 7370 genes were spotted on a custom microarray (Telechem). Microarrays were imaged on confocal laser scanners (Agilent) and data were extracted (GenePix 3.0; Axon Instruments), corrected for background and normalized. Gene probes were excluded from analysis if: expression values were available for only ≤10 samples (ie, gene was not detectable in a sufficient number of samples); or the standard deviation of expression values across the samples was <0.05 with expression values expressed as log_base10 (ratio[sample/control]) This condition was introduced to base correlation analysis on sufficient variance; or average signal-to-noise ratio associated sample was <5 (empirical threshold). This left 5927 gene probes for analysis. Probes mapping to the same gene transcript were not averaged. A global median normalization was applied to each hybridization sample.

Microarray Data Analysis

Correlation analysis was performed using Pearson correlation coefficient and rank-based Spearman correlation coefficient. Correlation coefficients (r) and associated *P* values were computed and averaged for Pearson and Spearman methods. Hierarchical clustering was performed using the pair-wise Pearson correlation coefficients computed from the expression values in the 28 samples as a distance measure. Only gene probes significantly correlated with BNP concentrations were subject to clustering. The average linkage method as implemented in the program OC by Geoffrey Barton was used to generate expression-based dendrograms of gene probes.[10]

Estimation of the False Discovery Rate

Gene expression–BNP concentration correlation results in experimental data were compared with randomized BNP concentrations to estimate the proportion of false-positives among the genes identified with *P* below a certain cutoff value. BNP concentrations were randomly reassigned among the 28 samples and correlation analysis was applied and the process repeated 10 times. The number of probes below a given *P* value threshold obtained from the randomization trials was then compared with the number of significant genes in the unscrambled data set at the same *P* value threshold to estimate the proportion of false-positives. As an added precaution for robustness, we implemented the Benjamini and Hochberg frequentist methodology[11] for detection of the false discovery rate.

Results

Patients

The demographic characteristics of the 28 patients enrolled included a mean recipient age of 58±10 years; 68% were men, 96% were white, and it was 16±3 weeks after transplant. The donor characteristics included an average age of 31±6 years; 67% were women and 96% were white. Based on the inclusion criteria of normal hemodynamics, the cohort had a right atrial pressure of 6±1 mm Hg, pulmonary capillary pressure of 11±2 mm Hg and cardiac index of 3.2±0.5 L/min per m^2. The mean serum creatinine was 1.2±0.1 mg/dL (median, 1.0 mg/dL). The average tacrolimus trough concentration was 12.2±4 ng/mL, whereas prednisone daily dose was 18.9±6 mg. Mycophenolate mofetil concentrations were not measured and the average daily dose was 2.4±0.3 grams. These 28 samples on biopsy included 20 with grade 0 and 8 with grade IA pathology as confirmed by the centralized pathologists.

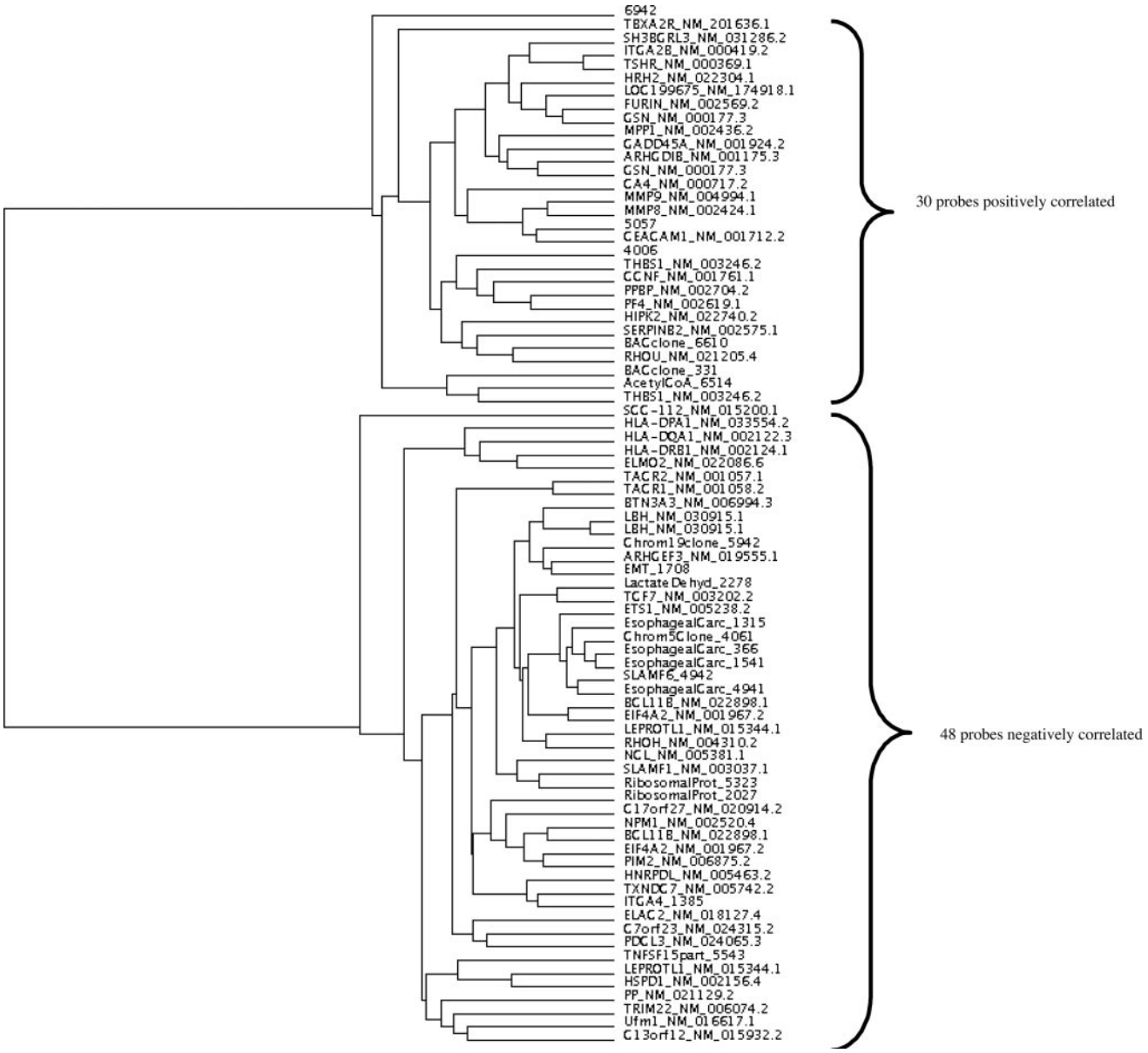
BNP Concentration

For the patient cohort, median BNP concentration was 165 pg/mL (mean 255±32 [SEM] pg/mL). Women displayed higher BNP concentrations than men (medians 191 versus 156 pg/mL, $P=0.012$). There were no correlations of BNP concentration with renal function or hemodynamics in this specially selected cohort.

BNP and Gene Expression Profiles

Of the 5927 probes used, 3850 mapped to 2863 unique annotated genes and 2077 did not map to any presently characterized human transcript sequences in the RefSeq database. A total of 78 gene probes were significantly correlated with BNP concentrations at $P<0.001$. At this level of significance, the false discovery rate (FDR) correcting for multiple testing was estimated at 10%. An average of only 8 probes were found to be <0.001 of the *P* value threshold in the randomization trials. The FDR by the method of Benjamini and Hochberg was estimated at <7.6%.

These 78 probes mapped to 54 uniquely characterized genes (existing gene symbol) and 19 probes were associated sequences not mapping to currently annotated genes; 48 probes were significantly negatively correlated with BNP concentrations (ie, increased gene expression was associated with decreased BNP concentrations), whereas 30 probes were positively correlated with BNP. These 78 gene probes are depicted in the dendrogram with their annotations in the Figure.



Hierarchical clustering dendogram (all 28 samples, genes with $P<0.001$). Labels indicate gene symbols and associated Refseq IDs. "Naked" IDs refer to array probes that do not map to known expressed sequences in the human genome. IDs not preceded by "NM," but with annotation were mapped to the non-redundant National Center for Biotechnology Information database (nr) and the top hit used for annotation.

Specific Gene Correlates of BNP

We reviewed all uniquely characterized genes (n =54) and categorized them into domains of cellular remodeling (those encoding proteins involved with cellular structure), vascular injury and repair (those encoding proteins involved with platelet and endothelial function), and alloimmune inflammatory interactions (those genes encoding proteins involved with cellular and humoral immune processes). Additional genes involved in stem cell mobilization pathways, antiviral activity, and apoptosis were also described. These genes and their ascribed functions (n=25) are detailed in the Table. We did not display those significant genes with a nonspecific function. Several genes (n =29) encoding cell signaling proteins and G proteins fell into this category. Examples of these genes include *PP* (inorganic pyrophosphatase), *HNRPDL* (heterogeneous nuclear ribonucleoprotein D-like), *PDCL3* (phosducin-like 3), *ARHGEF3* (Rho guanine nucleotide exchange factor [GEF] 3), NCL (nucleolin), *ELMO2* (engulfment and cell motility 2), *HIPK2* (homeodomain interacting protein kinase 2), *MPP1* (membrane protein, palmitoylated 1), and *CCNF* (cyclin F).

Actin cytoskeleton genes (gelsolin) and matrix metallopeptidase genes (MMP8 and MMP9) were strongly upregulated in the presence of elevated BNP concentrations and these genes denote collagen turnover and remodeling at a myocyte, interstitial and vascular level. Gelsolin was increased 25.2-fold (r value 0.74), MMP9 increased 10.5-fold (r value 0.68), and MMP8 increased 6.1-fold (r value 0.67). Genes involved in vascular injury were strongly represented and these included the domains representing platelet function (platelet

Uniquely Characterized Genes (n=25) Correlated With BNP

Action	Gene Name	Gene Symbol	Functional Description of Encoded Protein	Expression
Remodeling	Gelsolin	GSN	Actin-binding protein involved in remodeling the actin cytoskeleton	Overexpression
	Matrix	MMP9	Breakdown of extracellular matrix in normal physiological and disease processes	
	Metallopeptidases	MMP8	Degrades type IV and V collagens (9) or type I, II, and III collagens (8)	Overexpression
Vascular injury and repair	Endoplasmic reticulum protein 5	ERP5	Regulates binding of fibrinogen, cell surface exposure of P-selectin, and co-association of beta 3 integrin in stimulated platelets.	Underexpression
	Thrombospondin 1	THBS1	Plays a role in platelet aggregation, angiogenesis, and tumorigenesis	Overexpression
	Integrin, Alpha 2b (platelet glycoprotein IIb, antigen CD41B)	ITGA2B	Encodes integrin alpha chain 2b; adhesion, participate in cell surface-mediated signaling	Overexpression
	Platelet factor-4 (chemokine [C-X-C motif] ligand 4)	PF4	Platelet-dependent thrombosis; inhibit T cell proliferation as well as IFN-gamma and IL-2 release; inhibition of angiogenesis and hematopoiesis; promotion of neutrophil	Overexpression
	Plasminogen activator inhibitor-2	PAI-2	Regulator of monocyte proliferation and differentiation; polymorphism of the PAI-2 gene is associated with an increased risk of myocardial infarction	Overexpression
	Thromboxane A2 Receptor	TBXA2R	Thromboxane A2-mediated platelet secretion and aggregation are important in thrombosis and the stable TXA2 analogue, U46619, induces 2 waves of platelet secretion	Overexpression
	Pro-platelet basic protein (chemokine [C-X-C motif] ligand 7)	PPBP	Platelet-derived growth factor that is a potent chemoattractant and activator of neutrophils	Overexpression
Inflammation and alloimmune activation	B-cell lymphoma 11B (zinc finger protein)	BCL11B	Translocation may be associated with B-cell malignancies; appears to play a key role in T-cell differentiation	Underexpression
	Erythroblastosis virus E26 oncogene homolog 1	ETS1	Genes that are negatively regulated by ETS1 and upregulated by SP100 have antimigratory or antiangiogenic properties; interleukins 2 and 15 regulate Ets1 expression via ERK1/2 and MNK1 in human natural killer cells	Underexpression
	Butyrophilin, subfamily 3, member A3	BTN3A3	Involved in the extended major histocompatibility complex	Underexpression
	Mast cell-expressed membrane protein 1	MCEMP1	Mast cell transmembrane protein	Underexpression
	Signaling lymphocytic activation molecule family member 1	SLAMF1	Activation of peripheral blood cells with agonistic anti-CD3 antibody and exogenous IL-2, as used for generation of cytokine-induced killer cells, results in significant SLAM activation 5 days after T-cell stimulation	Underexpression
	Heat shock 60-kDa protein-1 (chaperonin)	HSPD1	This gene encodes a member of the chaperonin family; the encoded mitochondrial protein may function as a signaling molecule in the innate immune system	Underexpression
	Major histocompatibility complex, class II, DP alpha 1; DR beta 1; DQ alpha 1	HLA-DPA1 HLA-DRB1 HLA-DQA1	Plays a central role in the immune system by presenting peptides derived from extracellular proteins	Underexpression
	Histamine receptor H2	HRH2	Messenger molecule released from mast cells; functionally linked to cellular processes	Underexpression
	Ras homolog gene family, member U	RHOU	Encodes a member of the Rho family of GTPases; mediates regulation of cell morphology, cytoskeletal organization, and cell proliferation	Underexpression
Miscellaneous	Tripartite motif-containing 22	TRIM22	This protein localizes to the cytoplasm and its expression is induced by interferon; function of this protein may be to mediate interferon's antiviral effects	Overexpression
	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	NPM1	Involved with upregulation of genes involved in the maintenance of a stem-cell phenotype; may derive from a multipotent hematopoietic progenitor	Overexpression
	Growth arrest and DNA damage-inducible, alpha	GADD45A	Member of a group of genes whose transcript levels are increased after stressful growth arrest conditions and treatment with DNA-damaging agents	Underexpression
	Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	D4-GDI of Rho family GTPase may be regulated during apoptosis through the caspase-3 mediated cleavage of the GDI protein	Underexpression

factor-4, thromboxane-2, PAI-1), adhesion cell molecules, and integrins (platelet glycoprotein 2B-3A). Platelet factor-4 expression was increased 17.4-fold (r value 0.72). Alloimmune activity related genes were both positively (Histamine Receptor H2 involved in mast cell regulation) and negatively (B cell genes that play a role in T cell differentiation; human leukocyte antigen system genes) correlated with BNP concentrations.

Discussion

This investigation has correlated, for the first time to our knowledge, gene expression pathways associated with increased expression of BNP in the clinically quiescent phase of heart transplantation. Contrary to common perception that BNP is a neurohormone principally responsive only to hemodynamic perturbations, we have confirmed that an elevation of this peptide during normal hemodynamics and in the absence of histological allograft rejection, reflects upregulation of molecular pathways representing ongoing inflammation, alloimmune activation, cardiac structural remodeling, and vascular perturbations. These observations lend credence to the notion that BNP elevations in the quiescent phase after heart transplantation are reflective of ongoing allograft injury and remodeling at activity levels not discernable with clinical techniques. This may explain observations from studies that have found this biomarker to possess prognostic predictive power for the development of cardiac allograft vasculopathy and allograft failure.[5–7]

The strong association of the gelsolin gene and matrix metallopeptidase genes with an elevated BNP point to the important regulatory role of this natriuretic peptide in cardiac structural remodeling. The gelsolin gene family encodes a number of actin-binding proteins that are thought to function in the cytoplasm by severing, capping, nucleating, or bundling actin filaments.[12] Others have demonstrated that the binding step of collagen phagocytosis is facilitated by Ca(2+)-dependent, gelsolin-mediated severing of actin filaments and that phosphatidylinositol-4,5-bisphosphate regulation of gelsolin promotes the actin assembly required for internalization of collagen fibrils.[13] Matrix metallopeptidases (MMPs) and their inhibitors regulate the cardiac extracellular matrix by controlling fibrillar collagen. Specifically, MMP8 and MMP9 have been shown to be selectively increased in transplanted hearts as early as 2 weeks after transplantation and correlate with an increase in connective tissue in the allograft.[14] MMP9 activity has been found to reflect increased T cell alloreactivity,[15] whereas other studies have pointed to a vascular role for this proteolytic enzyme as an effector molecule of oxidant-mediated coronary vasomotor dysfunction.[16] It has also been described that systemic activation of MMP2 and MMP9 in donors with intracerebral hemorrhage and subsequent heightened expression of these peptidases in the allograft are associated with the development of allograft vasculopathy.[17] Thus, BNP elevation serves as a surrogate for gelsolin and MMP activity, which represent ongoing extracellular matrix and vascular remodeling.

Vascular injury is sentinel to the development of cardiac allograft vasculopathy and this entity is the strongest limitation to long-term survival in heart transplantation.[18] The genesis of cardiac allograft vasculopathy is determined by both alloimmune and nonimmunological factors but inflammation is central in its development.[19] Thrombosis and inflammation are intertwined in the development of vascular disease. Platelet factor 4 (PF-4), a member of the CXC subfamily of chemokines, is secreted in high amounts by activated platelets. PF-4 specifically binds to human polymorphonuclear granulocytes, but requires tumor necrosis factor alpha-α as a costimulus for the induction of effector functions in vascular cells.[20] It has been demonstrated that long-term survivors of heart transplantation are characterized by activation of platelets, which contain increased levels of soluble CD-40 and release enhanced cytokines when stimulated.[21] Animal studies have shown that lack of plasminogen activator inhibitor-1 expression in donor tissue greatly exaggerates the extent of vascular intimal proliferation after allogeneic transplantation.[22] Similarly, integrins are cell surface adhesion receptors that mediate cell–cell and cell–extracellular matrix interaction with several ligands, including fibrinogen, fibronectin, thrombospondin, and prothrombin. Myocardial ischemic injury after cardiac transplantation is associated with upregulation of integrins, tissue factor, and activation of the MMP induction system, which may contribute to the subsequent development of allograft remodeling and vasculopathy.[23] Thrombospondin-1, a matrix glycoprotein, inhibits angiogenesis and facilitates smooth muscle cell proliferation. Increased levels of thrombospondin-1 in human cardiac allografts may alter vascular responses to angiogenic growth factors by inhibiting angiogenesis and promoting smooth muscle cell proliferation characteristic of cardiac allograft vasculopathy.[24] Our investigation suggests that BNP elevation is indicative of upregulation of pathways in thrombosis, inflammation, and vascular remodeling in heart transplantation. Importantly, the strong expression of genes regulating platelet function points to the need for anti-platelet therapeutic studies in heart transplantation.

Although we enrolled a selective subset of patients without overt rejection as evidenced by normal allograft function and absence of histological rejection, our investigation correlated genes in the mast cell function domain with BNP elevation. Human mast cells, by elaborating various cytokines, chemokines, and proinflammatory mediators play a complex role in inflammatory disorders and have been linked with cardiac rejection.[25] However, in post-transplant hearts during ongoing acute rejection, the mast cells are phenotypically different from nonrejecting hearts and isografts.[25] Others have shown that mast cells are not necessarily associated with rejection but underlie enhanced inflammation, neovascularization, and fibrosis during cardiac allograft arteriosclerosis.[26] Thus, in our study, it is more likely that the correlation of mast cell genes reflects ongoing inflammation rather than a signature for allograft rejection.

Finally, we acknowledge several limitations of this work. First, the patient number is small; however, we chose to develop a highly selected cohort of clinically quiescent patients with normal renal function within the first year of transplant and in this context represents a reasonable sample size. Second, we correlated peripheral blood gene expression patterns with BNP concentrations and did not evaluate

intragraft events. Therefore, it should be recognized that overexpression or underexpression of a specific gene in peripheral blood should not necessarily be construed to describe the direction of that pathway within the allograft. Third, we used a custom leukocyte microarray and it is entirely possible that we might have missed some significant pathways if a whole genome approach had been undertaken. However, the fact that a number of genes that correlated with BNP concentrations all mapped to the same domain of molecular pathways increases the likelihood of the robustness of our findings. We concede that independent validation studies should help confirm these findings.

Conclusion

In the clinically quiescent heart transplant recipient, an elevated BNP concentration is associated with molecular patterns that point to ongoing to active cardiac structural remodeling, vascular injury, inflammation, and alloimmune processes. Thus, these findings allude to the notion that BNP elevation is not merely a hemodynamic marker but should be considered reflective of integrated processes that determine the balance between active cardiac allograft injury and repair.

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Disclosures

M.R.M. is a consultant for XDx, Inc, and D.W., J.G.W., J.P., and D.T. are or were employees of XDx Inc.

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