

Fibronectin in Immune Responses in Organ Transplant Recipients

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The immune response to an organ allograft involves perpetuation of T cell infiltration and activation. Advances in understanding the mechanisms of T cell activation have placed particular emphasis on the interactions between the T-cell receptor and antigen presenting cells, with little reference to the fact that in vivo activation occurs in the physical context of extracellular matrix proteins (ECM). Indeed, the possibility that ECM proteins may have a determining role in lymphocyte adhesion and tissue localization and function is now becoming more appreciated in view of growing evidence indicating that integrins and other T cell antigens bind ECM components, with some of these components exerting synergistic effects on T-cell activation. This review focuses on the importance of interactions between lymphocytes and fibronectin, a prominent ECM component, for cell migration and function in organ allograft recipients. It explores novel therapeutic approaches based on the assumption that fibronectin represents an active element in the process of T cell activation in the immune cascade triggered by organ transplantation.

Keywords: Fibronectin, Transplantation, Acute Rejection, Chronic Rejection, Tolerance

Lymphocytes recirculate between blood and lymph until the trigger of signals that stimulate their adhesion to vascular endothelium or extracellular matrix (ECM) proteins (de Sousa et al., 1991; Springer, 1994). To emigrate through vascular endothelium, lymphocytes have to acquire strong adhesion interactions to the vessel wall while resisting to continuous sheer forces (Diamond et al., 1994). The emerging relevance of lymphocyte – ECM interactions to cell adhesion, migration, and positioning within specific tissue microenvironments emphasize the role ECM proteins may play in the functioning of the immune system in both physiological and pathological conditions (de Sousa et al., 1991).

Fibronectin (FN) is likely a key ECM protein involved in these events. FN is a large dimeric glycoprotein (440–500 kDa) composed of a series of independently folding modular domains known as FN repeats I, II, III (Hynes, 1986). These repeating units contain regions or domains which interact with a number of other matrix molecules as well as cells, and mediates functions such as cell migration, matrix assembly, differentiation, and proliferation (Yamada et al., 1992; Hynes, 1986). Structural diversity in FN arises by regulated alternative splicing of a single gene transcript in three segments termed EIIIA, EIIIB, and V in rats, and ED-A, ED-B, and IIICS respectively in humans (Tamkun et al., 1984; Kornbli-

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htt *et al.*, 1985). The EIIIA (or ED-A) and EIIIB (or ED-B) domains are either included or excluded as intact type III homology repeats. The V (or IIICS) may be excluded, partially included, or fully included. Alternative splicing of the FN gene allows for the generation of multiple isoforms, with 20 isoforms possible in humans (Schwarzbauer, 1991). The importance of FN in cell migration and differentiation is underscored by the observation that mice made null for this ECM protein die during embryonic development (George *et al.*, 1993; Georges-Labouesse *et al.*, 1996). The role of FN in lymphocyte adhesion, migration, and activation has been illustrated in several studies (Shimizu *et al.*, 1990; Hauzenberger *et al.*, 1994; Ybarrondo *et al.*, 1994; Ostergaard *et al.*, 1995; Hunter *et al.*, 1997). Cellular adhesion to FN is mediated by a superfamily of heterodimeric molecules, the integrins (Hynes, 1987). Lymphocytes have been shown to interact with sequences within the type III repeats of FN primarily via two different receptors of the $\beta 1$ integrin family, the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (Guan, *et al.*, 1990; Hemler *et al.*, 1995). Thus, $\alpha 4\beta 1$ integrin, interacts with the connecting segment-1 (CS-1), which is located within the V region, and with a recently described segment KLDAPT located in FN-III5 (Moyano *et al.*, 1997). In addition, it was reported that the activated $\alpha 4\beta 1$ integrin is also able to interact with the RGD sequence present in the cell adhesion domain (Sanchez-Aparicio *et al.*, 1994). These findings showing the flexibility of the $\alpha 4\beta 1$ integrin in recognizing other domains in the FN molecule, support the important role this ECM protein may play in an array of lymphocyte biological functions. The other main receptor for FN, the $\alpha 5\beta 1$ integrin, known to be present on a subpopulation of resting CD45RA dim memory T cells (Klingemann *et al.*, 1989), interacts via the RGD sequence. Other integrins have also been identified as alternative receptors for FN, such as $\alpha 4\beta 7$ (Postigo *et al.*, 1993), $\alpha 3\beta 1$ (Yamada *et al.*, 1992), and $\alpha v\beta 1$ (Vogel, *et al.*, 1990). The adhesive capacity of integrins expressed on circulating lymphocytes is highly regulated. Thus, adhesion of T cells to FN has been shown to be regulated by the engagement of antigen-specific TCR/CD3 complex, as well as CD2, CD7 and CD28

(Shimizu *et al.*, 1992; Shimizu *et al.*, 1995; Chan *et al.*, 1997). The fact that TCR engagement up-regulates adhesion of T lymphocytes to FN, highlights the importance of this protein in host immune responses. Integrin-dependent binding of lymphocytes to FN is also regulated by a number of chemokines, including the C-C chemokines, such as RANTES, monocyte chemoattractant protein 1 (MCP-1) and macrophage inhibitory protein 1 (MIP-1) (Weber *et al.*, 1996; Carr *et al.*, 1996; Carr *et al.*, 1994). Modifications in FN expression may also have a regulatory role in the integrin-mediated lymphocyte adhesion. Indeed, there is good evidence that ECM protein binding can modulate integrin expression (LaFlamme *et al.*, 1992; Delcommenne *et al.*, 1995). This review focuses on the significance of FN expression in organ allograft recipients, and explores novel therapeutic approaches based on the assumption that FN is an active participant in the immune cascade leading to allograft rejection.

FIBRONECTIN IN ORGAN TRANSPLANT RECIPIENTS

Organ allograft represents a useful *in vivo* experimental system to examine the role of FN in the immune response in which the effector phase is dependent on the migration of alloreactive cells into the foreign tissue (Kupiec-Weglinski *et al.*, 1993; Coito *et al.*, 1994). In our immunohistological and *in situ* hybridization studies of rat cardiac allografts, a markedly increased expression of FN, mostly vascular, in the early post-transplant period preceded cellular infiltration (Coito *et al.*, 1994; Coito *et al.*, 1997). This initial up-regulation of vascular FN is a common step in both allo- and iso- transplants and may reflect a response to injury or ischemia that occur during the interval of about 45 minutes of cardiac engraftment (Coito *et al.*, 1997). The initiation of vascular FN synthesis in such an early time-point may represent an important signal that triggers lymphocyte recruitment at the graft site. At later time-points after allotransplantation, cellular FN is predominantly expressed in the myocardium contrasting with cardiac isografts in

which myocardial FN expression is absent (Fig. 1 A&B) (Coito et al., 1997). In addition, the simultaneous detection of FN and laminin (LN) in cardiac allografts, by laser scanning confocal microscopy, revealed a preferential accumulation of FN in the interstitial areas where infiltrating mononuclear cells (MNC) localize (Fig. 1 C&D) (Coito et al., 1994). Similarly, sensitized lymphocytes after adoptive transfer to test recipients localize in FN-rich areas of both cardiac transplants (Fig 1E) and lymph nodes (Coito et al., 1994). Moreover, treatment of rats with a neutralizing anti-TNF- α serum significantly prolongs cardiac allograft survival, downregulates the local production of FN and reduces intragraft MNC infiltration (Coito et al., 1995). All these findings support a key role of FN to act as an *in vivo* adhesive factor for lymphocytes to home to specific tissue microenvironments, including an organ transplant. Our combined immunohistological and *in situ* hybridization analyzes have also demonstrated that the prime sources of FN in rejecting cardiac allografts are macrophages in the myocardium and smooth muscle and endothelial cells in the vessels (Coito et al., 1997; Coito et al., 1995). It remains to be determined how different cell types regulate FN expression *in vivo*. While TGF- β 1 is well known to increase FN expression by fibroblasts, its role in modulating FN expression in other cell types remains unclear (Roberts et al., 1988; Ignatz et al., 1987). Our data suggest a potential role for TNF- α in modulating macrophage appearance, and potentially FN expression, during cardiac allograft rejection (Coito et al., 1995). Other data also indicate that, in coronary arteriopathy after transplantation, endothelial and smooth muscle cells produced increased FN under the regulation of IL-1 β and TNF- α (Clausell et al., 1993; Molossi et al., 1995). Moreover, soluble TNF- α receptor reduces the expression of FN and leukocyte infiltration within areas of intimal thickening (Clausell et al., 1994). In cardiac allografts, FN exists in multiple isoforms with a distinct temporal and spatial pattern of expression (Coito et al., 1997). Newly synthesized FN in cardiac grafts includes EIIIA+, EIIIB+ and CS1 variants that are generated by alternative splicing of FN pre-mRNA. The FN variants that include the EIIIA

and EIIIB segments are prominent in FN produced during embryogenesis, and their expression in the adult tissues is minimal unless under certain pathological conditions (Vartio et al., 1987; Carnemolla et al., 1989). For example, adult liver synthesizes the plasma form of FN, and this form excludes the EIIIA and EIIIB domains. However, these EIIIA and EIIIB segments are markedly increased during cutaneous wound healing (Brown et al., 1993; French-Constant et al., 1989), neointimal hyperplasia (Knowlton et al., 1992; Mamuya et al., 1992), and glomerular nephritis (Barnes et al., 1995; Nickenleit et al., 1995). Although, very little is known about the function of the EIIIA and EIIIB domains of FN, by comparing the patterns of EIIIA and EIIIB expression in cardiac allografts and isografts, our data support the hypothesis that these FN variants are involved in lymphocyte adhesion, migration, or differentiation in cardiac allografts. Both EIIIA and EIIIB domains are highly expressed in the myocardium of cardiac allografts associated with increased numbers of infiltrating T cells and appearance of infarcts. In contrast, the FN splicing variants are not detected in the myocardium of cardiac isografts, neither are a similar increase in T cells, nor are infarcts. Recently, it has been shown that EIIIA segment mediates adhesion of fibroblastic cells and promotes the differentiation of lipocytes in the liver to become myofibroblasts (Jarnagin et al., 1994). We think that similar processes may be involved in the lymphocyte biological functions in organ allografts. For example, CD8+ T-lymphocytes-mediated lysis of target cells requires the formation of tight conjugates between these cytotoxic T lymphocytes and the targeted cell. While, the cellular receptors for EIIIA and EIIIB domains are not yet fully characterized, the CS-1 domain recognized by the α 4 β 1 integrin, is known to mediate lymphocyte adhesion (Guan et al., 1990; Wayner et al., 1989). The CS-1 subunit of FN is the first alternatively splicing domain found to be increased in cardiac allografts, it is up-regulated as early as at 3 hours post-transplant, and it is preferentially expressed after day 4 (Coito et al., 1997). The presence of specific FN variants may provide a spatial address at which appropriated co-stimulatory signals could initiate or augment criti-

cal T cell signaling events. Supporting this assumption, are our recent findings in rat recipients rendered tolerant to MHC-incompatible cardiac allografts by exogenous immunosuppressive therapy. In long-term tolerant recipients, FN is found up-regulated in the vessels in the absence of other vascular adhesion molecules such as VCAM-1 and ICAM-1, highlighting a putative role for FN in the recruitment of leukocytes at the graft site (Coito *et al.*, 1998). Moreover, despite the high number of MNC infiltrating well-functioning grafts, intramyocardial infiltrating macrophages failed to express FN. This contrasted with long-term control recipients undergoing chronic rejection, in which FN expression was readily detectable in the myocardium (Coito *et al.*, 1998). The observation that MNC do accumulate in the myocardium in the absence of cellular FN in tolerant hosts, indicates other role for this ECM protein that is unrelated to cell migration or tissue positioning. Furthermore, by recreating rejection in tolerant hosts, we have pointed out a novel role for FN in the upregulation of IL-2 and IFN- γ expression, indicating that FN by itself may play an active role in cellular activation (Coito *et al.*, unpublished). Our results are consistent with the concept that FN exerts synergistic effects on T cell activation by acting as a co-stimulator for both CD4+ and CD8+ T cells through TCR (Ostergaard *et al.*, 1995; Matsuyama *et al.*, 1989) and cytokine release. For example, the density of immobilized CD3 or TCR mAb required to induce degranulation and tyrosine phosphorylation of cellular proteins by CD8+ T cells is about 10-fold lower in the presence of FN (Ostergaard *et al.*, 1995). Several studies have also shown that adhesion to FN activates tyrosine phosphorylation of several T cell proteins (Ostergaard *et al.*, 1995; Nojima *et al.*, 1992; Ticchioni *et al.*, 1995). The production of IL-2, IFN- γ , and TNF are also stimulated *in vitro* by interactions between CD4+ cells and FN (Yamada *et al.*, 1991; McCarthy *et al.*, 1997; Brunmark *et al.*, 1997; Hershkovich *et al.*, 1993). Moreover, it has been reported that the binding of CD4+ cells to ECM proteins, most likely through conformational changes resulting in better presentation to their receptor, may in turn enhance cytokine activity (Crawford *et al.*, 1998; Lortat-Jacob *et al.*, 1991; Lortat-Jacob *et*

al., 1991). Taken together, these findings support the idea that FN is an active participant in the immune cascade leading to graft rejection.

FIBRONECTIN – MONONUCLEAR CELL INTERACTIONS: POTENTIAL TARGETS FOR THERAPEUTIC INTERVENTION IN TRANSPLANT RECIPIENTS

That MNC-FN disturbed *in vivo* adhesive interactions play immunopathological role, and may represent important targets for novel therapeutic interventions, has been documented in both autoimmune disease and transplantation animal models. Multiple-organ MNC infiltration occurs in TGF- β 1 knockout mice, followed by cachexia, and death (Hines *et al.*, 1994). In addition, cells from TGF- β 1 deficient mice exhibit increased adhesion to FN matrices *in vitro*. Such an increased adhesion in culture has been inhibited following incubation of cells with synthetic FN peptides that interact with integrins (RGD/CS-1) and/or cell surface proteoglycans (C/H-I/II/III/V). Interestingly, daily systemic injections of FN peptide preparations virtually blocked leukocyte tissue sequestration, and the development of autoimmune-like lesions, and retarded the lethal wasting syndrome characteristic for this model. The efficiency of FN peptides has been also documented in the rat model of erosive polyarthritis (Wahl *et al.*, 1994). Not only were FN peptides inhibitory to acute and chronic synovial pathology, but were also found to prevent and reverse local recruitment, and the evolution of arthritis.

Our own data are consistent with the model in which *in vivo* interactions between the α 4 β 1 integrin receptor and the cell-associated CS1 motif of FN are critical in the acute allograft rejection cascade (Coito *et al.*, 1998). Indeed, treatment of rat recipients of cardiac allografts with a 7 day course of bioactive CS-1 peptides: (i) abrogated acute rejection, and doubled the transplant survival time, (ii) diminished vascular expression of adhesion molecules such as VCAM-1 and ICAM-1, (iii) reduced intragraft infiltration by CD4+ and CD8+ cells, and (iv) decreased allo-Ag activation at the graft site, as evidenced by decreased

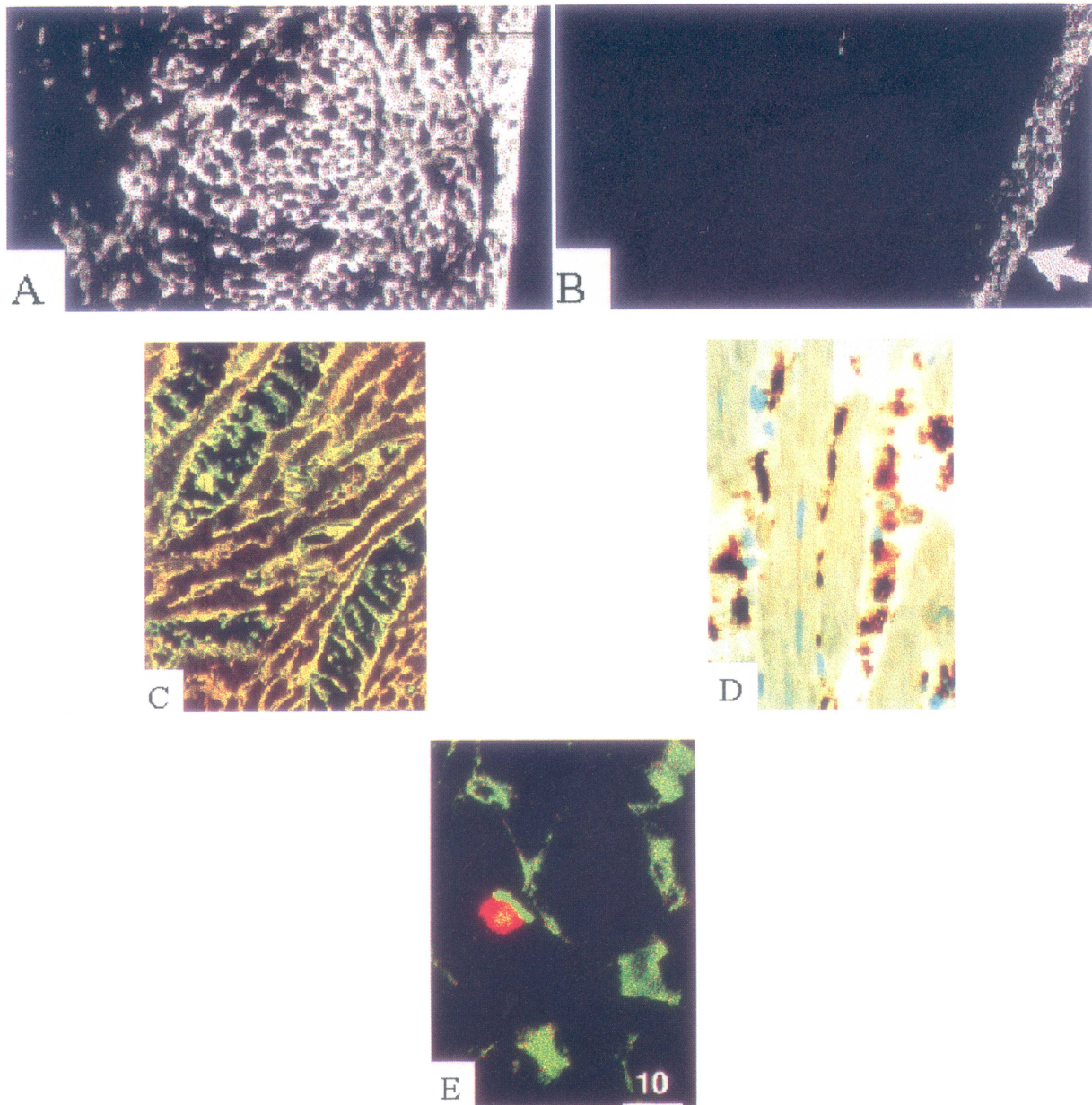


FIGURE 1 FN and infiltrating MNC in cardiac transplants. Immunofluorescence localization of cellular FN is shown in cardiac allograft (A) and isograft (B) at day 6 post-transplant. In cardiac allografts, cellular FN (white) is detected in elevated amounts in both myocardium and epicardium; in cardiac isografts cellular FN is restricted to the epicardium (arrow) but absent from the myocardium. Simultaneous detection of FN and laminin (LN) in rejecting cardiac allograft by confocal microscopy (C) shows a preferential accumulation of FN in the interstitial areas where infiltrating MNC localize (D). The yellow around the myocytes represents mixed deposition of FN and LN; the green in the interstitial areas represents exclusively FN deposits. Panel D shows monocyte/macrophages stained in brown by immunoperoxidase. Panel E illustrates the immunohistologic localization in a cardiac allograft of specifically sensitized lymph node lymphocytes labeled in vitro with Di "T" in red after adoptive transfer. As documented by confocal microscopy, labeled red cells are detected in association with FN deposits (stained in green) at the graft site. Original magnification $\times 109$ (A, B); $\times 400$ (C); $\times 230$ (D); and $\times 1200$ (E) (see Color Plate XIV at the back of this issue)

infiltration by CD25+ cells, and diminished expression of Th1 and Th2 type cytokines. These immunosuppressive effects could be reversed and acute rejection recreated after adjunctive treatment with recombinant IL-2, suggesting that CS1 peptides may induce a temporary state of cytokine-responsive T cell anergy, *in vivo*.

Most of the adhesion functions mediated by $\alpha 4\beta 1$ integrin are attributed to interactions with its two known ligands, the CS1 motif in the FN molecule, and VCAM-1 expressed on endothelial cell surfaces. Although it is possible the CS1 peptide may interfere with $\alpha 4\beta 1$ – VCAM-1 interactions, $\alpha 4\beta 1$ has been shown to have distinct ligand/binding sites for VCAM-1 and FN (Elices *et al.*, 1990), suggesting that the CS1 peptide that binds to VLA-4 does not likely inhibit the binding by VCAM-1. Moreover, although VCAM-1 and CS1-FN may share spatially overlapping binding sites on $\alpha 4\beta 1$, the concentration of FN peptides that interfere with the $\alpha 4\beta 1$ – VCAM-1 binding are several-fold higher than those required for the $\alpha 4\beta 1$ – CS1 blockade (Makarem *et al.*, 1994). Hence, it seems quite unlikely that the effects of relatively low dose CS1 peptide regimen upon the kinetics of, and local cell recruitment associated with acute graft rejection, could be attributed to the blockade of $\alpha 4\beta 1$ – VCAM-1 interactions. Supporting this notion are findings from mouse cardiac transplant models of persistent T cell infiltration in allografts despite anti-VCAM-1 mAb therapy, and transient T cell infiltrate in isografts, which are devoid of VCAM-1 expression (Orosz *et al.*, 1993). These strongly suggest a role for VCAM-1-independent collateral system(s) that mediate leukocyte sequestration during the course of acute graft-deteriorating rejection. It has been shown that the blockade of $\alpha 4\beta 1$ -dependent leukocyte – endothelial adhesive interactions by treatment with anti-VLA-4 mAb (TA-2) was insufficient to delay acute rejection of pancreatic islets in rat recipients (Brown *et al.*, 1993). In contrast to the blockade of $\alpha 4\beta 1$ – FN interactions by CS1 peptides, the protective effects afforded by targeting the $\alpha 4\beta 1$ integrin by mAb alone are rather short-lived, and insignificant upon allo-Ag-driven mononuclear and endothelial cell activation leading to graft rejection in

transplant recipients. However, in a more recent study, administration of CS1 peptides to mice recipients of islet allografts not only abrogated graft rejection, but also prevented graft infiltration, suggesting a role for $\alpha 4$ /FN interactions in leukocyte homing to the graft site (Stegall *et al.*, 1999).

We have also tested the efficacy of CS-1 peptides in presensitized rat recipients in which therapy with rapamycin (RPM) abrogates rejection at 24h, but it does not prevent the ultimate cardiac allograft loss at 40–50 days (Korom *et al.*, 1998). These long-term grafts show progressive development of arteriosclerosis, a hallmark of chronic rejection (Wasowska *et al.*, 1996). Indeed, an adjunctive course of CS-1 peptides in the early post-transplant phase resulted in the disappearance of the inflammatory and smooth muscle cells from the arterial intimal of long-term recipients. Unlike medium or large arteries in rats undergoing monotherapy, those following adjunctive CS-1 peptides had normal internal elastic laminae, and were free of neointimal thickening, confirming that CS-1 peptides successfully prevented the development of arterial chronic injury. Moreover, treatment with CS1 peptides reduced gene transcript and product levels for T cell- and macrophage- derived cytokines and chemoattractants, which are known to contribute to the development of progressive chronic-type allograft failure. A similar finding was observed in a cholesterol-fed rabbit model in which treatment with CS-1 peptides specifically blocked vascular changes, and markedly diminished accelerated coronary arteriopathy (Molossi *et al.*, 1995). Therapy with FN peptides effectively depressed intragraft transendothelial infiltration by macrophages and T cells, documenting the functional role of FN in the trafficking of inflammatory cells in progression of cardiac arteriopathy. However, unlike in our rat study, CS1 peptides did not affect the grade of cardiac rejection in the rabbit model, as judged by the extensive myocardial cell infiltration, myocyte necrosis, hemorrhage, and fibrosis. As integrin – FN interactions are critical for T cell activation, adhesion, and local retention to perpetuate chronic inflammatory responses, antagonism of cellular activation, and recruitment by FN peptides provides an important mechanism for modulating the

multi-step adhesion process, and attenuating aberrant inflammatory responses (Rodriguez et al., 1992). Given the efficacy of FN peptides to block neointimal thickening in coronary vessels, this novel therapy may interfere with the migration of smooth muscle cells from the media into the intima, with resultant reduction of neointimal hyperplasia, consistent with the expression of $\alpha 4\beta 1$ integrin by smooth muscle cells (Molossi et al., 1995). In addition to regulating cell growth and differentiation, FN may also function as a lymphocyte survival factor in cardiac allografts. Indeed, there is a growing body of evidence that anchorage-dependent cells when prevented from attaching to ECM proteins may undergo apoptosis (Meredith et al., 1993; Boudreau et al., 1995; Sethi et al., 1999).

The CS1-mediated prevention of chronic rejection may result from a far more reaching effect on long-term in vivo interactions between MNC, endothelial vascular lining and the ECM scaffolding. Treatment with RPM abrogates rejection in presensitized hosts by depressing humoral and T cell cytotoxic and proliferative responses (Wieder et al., 1993; Schmidbauer et al., 1994). At this point, host-vs-graft interactions are limited to the cellular contact with ECM and the endothelial lining. Adjunctive administration of CS1 peptides during this early post-transplant phase may impair the engagement of T cells with FN as part of the transplant structural framework. At the same time, CS1 peptides may modify intragraft vasculature by reducing its adhesiveness for host leukocytes. During the ensuing maintenance phase, the alterations in intragraft MNC homing and positioning are mimicked by the prevailing macrophage response. The perpetual cycle of late macrophage-associated proinflammatory mediators, smooth muscle proliferation, neointimal formation, and subsequent vasculature occlusion fails to be initiated.

Collectively, these data advance the hypothesis that local synthesis of FN is an ongoing feature of, and adhesive FN – MNC associations are critical for the development of acute and chronic rejection in transplant recipients. Further elucidation of FN – MNC interactions may ultimately offer potential novel sites for intervention in the control of transplant rejection,

and may lead to the development of refined strategies based upon new concepts of host immunomodulation.

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