Platelet-Derived CD154: Ultrastructural Localization and Clinical Correlation in Organ Transplantation

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Platelet-derived CD154: ultrastructural localization and clinical correlation in organ transplantation

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Abstract

CD154 is an immunostimulatory ligand for CD40 that markedly influences alloimmunity. Its presence in platelets suggests that its release and subsequent immune effects are driven by trauma and thus could be relevant following organ transplantation. However, the release of platelet derived CD154 and its consequences have not been investigated in a clinical transplant setting. To better characterize the relationship between platelet activation and CD154 release, we investigated CD154 release by platelets obtained from normal individuals, and patients with two genetic defects that influence platelet granule development. Using these unique patient populations and immune-electron microscopy, we confirmed that CD154 was an alpha granule and not a cell surface protein, and thereafter optimized the methods for its in vivo measurement in humans. We then investigated plasma CD154 levels in kidney and liver transplant recipients and found no evidence that CD154 levels fluctuated systemically as a result of kidney or liver transplant procedures. Paradoxically, we found that kidney transplant patients had significantly lower systemic CD154 levels during episodes of rejection. These data suggest that the immune effects of CD154 are likely mediated through local and not systemic mechanisms, and discourage the use of CD154 as a peripheral biomarker in organ transplantation.

Keywords

platelets; co-stimulation; trauma

Introduction

The transmembrane protein CD154 (also known as CD40 Ligand,) is a TNF-alpha family member that has well-documented immunostimulatory properties (1). It was originally identified (2, 3) on T-helper cells and subsequently on stimulated mast cells and basophils. CD154 acts by binding to its receptor CD40, which is expressed on many cells of the adaptive immune system including B cells, monocytes/macrophages, endothelial cells, and...
dendritic cells (4). Interactions between T cell CD154 and B cell CD40 are necessary for normal humoral immune responses; defective T cell CD154 ineffectively supports normal antibody class switching and provides the physiologic basis of the hyper-IgM syndrome (5).

More recently, CD154 has been recognized as a component of platelets (6), suggesting it has a role in initiating adaptive immune responses to trauma, particularly trauma involving platelet activation. Significant data support a critical role for CD154 in facilitating post-traumatic activation of the adaptive immune response. Specifically, platelet-derived CD154 activates endothelial cells and antigen presenting cells in vitro (6–8), and stimulates both protective and pathologic immune responses in vivo (9). Importantly, CD154 appears highly influential in controlling alloimmunity, since CD154-specific monoclonal antibodies markedly delay the rejection of transplanted organs in mice and non-human primates (10, 11). The surgical trauma inherent in organ transplantation carries with it an unavoidable propensity for platelet activation. Consequently, platelet-derived CD154 has been extensively investigated and shown to initiate allograft rejection independent of other sources of CD154 (9). Subsequent clinical studies have demonstrated an apparent correlation between blood levels of soluble CD154 and inflammatory disease processes, including acute coronary syndrome and autoimmunity (12).

The clinical correlations between ambient CD154 levels and various disease processes present a paradoxical situation. Specifically, the clear immunostimulatory potency of CD154 suggests that its release should be highly regulated, and its association with platelets suggests that its release would be localized to discrete areas of trauma rather than dispersed systemically. Although several investigators have reported associations between important diseases and soluble CD154, the central role of CD154 in initiating an adaptive immune response appear inconsistent with its existence as a ubiquitous ambient soluble protein. Indeed, such a potent instigator of adaptive immunity would teleologically be best sequestered unless immune activation was required. Given this paradox, and the general observation that the mechanisms for storage and release of CD154 from platelets remain incompletely understood, we investigated the specific means by which platelets store and release CD154, and applied that knowledge to the study of organ transplant recipients.

Platelets have two major types of secretory granules, alpha and dense (or delta) granules. Alpha granules store and release a myriad of protein growth factors, cytokines, and adhesion molecules (13). They are the most abundant (approximately 80 per platelet) and the largest (200–500 nm) platelet granules. Dense granules store and release high concentrations of small molecules such as ADP/ATP, calcium, and polyphosphates, have a dark appearance, and are less abundant (3–9 granules per human platelet) (14). To determine which platelet organelle stores CD154, we employed platelets, serum, and plasma from normal volunteers and from patients with the rare genetic diseases Hermansky-Pudlak Syndrome (HPS) and Gray-Platelet Syndrome (GPS). HPS is a group of genetic diseases characterized by defects in intracellular protein trafficking that result in ocuclorutaneous albinism and the absence of platelet dense (15) granules. GPS is an inherited disorder of platelets characterized by thrombocytopenia and enlarged platelets that lack alpha granules; a mutation in NBEAL2 has recently been implicated in its development (16). A unique feature of this syndrome is that proteins synthesized or endocytosed by the parent megakaryocyte cannot enter the secretable storage pool of the alpha-granules (17) (18).

In this study, we solidify previously published quantitative immunofluorescence mapping data (19) that CD154 is an alpha granule protein, and that its release depends upon alpha granule function. In the absence of platelet degranulation, CD154 is neither found on the cell surface of platelets nor present as an ambient human plasma protein. Using carefully designed methods for CD154 assessment, we find that systemic levels of soluble CD154 do
not change as a result of the transplant procedure, and significant amounts of CD154 are not systemically liberated during allograft rejection.

Materials and methods

Blood Collection

Whole blood was collected at the National Institutes of Health Department of Transfusion Medicine and at Emory University Hospital after written informed consent had been obtained. We examined sera and plasma samples from 6 patients with the HPS, 3 patients with the GPS, 38 normal human volunteers, and 100 stable kidney transplant recipients. We also examined plasma samples obtained intraoperatively from 10 kidney transplant recipients and 4 liver transplant recipients including samples obtained immediately proximal and distal to the transplanted organ post reperfusion. Plasma samples from 13 kidney transplant patients with no rejection (drawn simultaneous to confirmatory protocol biopsies) and 10 patients with biopsy proven rejection were also examined. These patients were selected for matched immunosuppressive regimens consisting of tacrolimus, mycophenolate mofetil and prednisone with samples obtained prior to biopsy and rescue therapy. All patients were enrolled in clinical protocols approved by the National Human Genome Research Institute and/or National Institutes of Diabetes and Digestive and Kidney Diseases Institutional Review Boards or the Emory Institutional Review Board and gave written, informed consent.

Platelet granule purification and CD154 detection

Platelets were purified using sucrose gradient purification. Platelets were resuspended in 250 mM sucrose and lysed by ultrasonication (Microson ultrasonic cell disruptor; Misonix, Farmingdale, NY). Non-lysed platelets were pelleted by centrifugation at 700g for 6 min. The supernatant containing platelet organelles was loaded onto six preformed, 10-mL linear sucrose gradients (14) (20–50% sucrose; 60% sucrose cushion). The gradients were centrifuged at 217,000g in a Beckman SW41 Ti rotor for 16 h at 4°C. Nine fractions were collected using careful pipetting from the top. Fractions from three gradients were combined, transferred to polycarbonate tubes, diluted in 6% sucrose, and centrifuged at 140,000 g in a Beckman 70.1 Ti rotor for 1 h at 4°C. The pellets were resuspended in 50 uL of Tyrode’s I buffer (pH 6.5) and frozen at 20°C. Protein concentration was determined using the BioRad Protein Assay with bovine serum albumin used as a standard. CD154 was detected using the Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Immuno-electron microscopy (EM)

Whole blood was collected into tubes containing citrate buffer (25 g sodium citrate, 8 g citric acid, 500 ml H2O). Centrifugation at 150g for 15 minutes at room temperature was performed, and supernatant containing platelet rich plasma was carefully isolated and 1/10 volume of ACD anticoagulant (6.25 g sodium citrate, 3.1 g citric acid anhydrous, 3.4 g D-glucose in 250 ml H2O) was added. Platelets were pelleted by centrifugation at 900 g for 5 minutes at room temperature, and resuspended in Heps-Tyrode buffer, pH 7.4 (134 mM sodium chloride, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, 0.34 mM sodium phosphate monobasic, 1 mM magnesium chloride, 5 mM Heps, 5 mM glucose, 1 % bovine serum albumin). Platelets were stimulated using a thrombin receptor activator peptide (Sigma-Aldrich, St. Louis, MO). Stimulated and unstimulated platelet pellets were immediately fixed with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After washing, samples were frozen, cryosectioned and immunogold labeled with a rabbit polyclonal anti-CD154 IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) according to established procedures (20).
Results

CD154 is an alpha granule protein

Serum samples (serum being derived from clotted blood and containing products of platelet degranulation) from normal individuals (n=38) had easily detectable CD154 (12,674pg/ml +/− 6,399pg/ml). Serum samples from patients with HPS (n=6) had comparable levels of CD154 (15,519pg/ml +/− 1,565pg/ml). In stark contrast, serum from patients with GPS (n=3) had no detectable CD154 (<50pg/ml). These results suggested that platelets lacking alpha granules either could not release CD154 into the serum or did not contain CD154. Therefore, we prepared platelet pellets from three individuals with GPS and assessed CD154 levels in lysed GPS platelets. These pellets had high concentrations (>50,000 pg/ml) of CD154, indicating that the GPS platelets contained CD154 but could not release it upon activation by thrombosis. This could be readily explained if CD154 were an alpha granule protein. To pursue this, we performed sucrose gradient purification of alpha granules using platelets from two normal human volunteers. The alpha granule fraction was rich in CD154 compared to the cytosolic fraction (6,397 +/− 159pg/ml versus 431 +/− 282pg.ml).

To anatomically confirm that the alpha granule was the location of CD154, we performed EM using gold-stained CD154-specific monoclonal antibodies. In quiescent platelets, CD154-specific antibodies exclusively decorated the alpha granules, but not the cytosol or dense granules (Figure 1a). To demonstrate that this was a dynamic location, platelets were thrombin stimulated and reassessed using EM. Stimulated platelet preparations demonstrated a dispersed CD154 pattern as opposed to a localized pattern (Figure 1b). Quantitative counting of gold particles demonstrated that the ratio of gold-labeled CD154 inside alpha granules compared to extracellular was significantly lower in stimulated platelets than in unstimulated platelets (p=0.01, Figure 2). Furthermore, we observed that CD154 was free floating in the extracellular space rather than lining the platelet membrane, implying a degranulation mechanism of CD154 release by the platelets. Thus, CD154 is an alpha granule protein and dependent on alpha degranulation for its release.

CD154 is not a prevalent systemic plasma protein

Typically, alpha granule proteins are released as an ordered process solely upon platelet stimulation (21). However, this mechanism, when applied to CD154 release, appears at odds with reports suggesting a clinical association between disease states and substantial (e.g., μg/ml) concentrations of ambient blood CD154. We therefore investigated aspects of cell preparation, such as ex vivo platelet activation, and analysis that could explain this apparent discrepancy. First, we determined the relationship of serum CD154 levels and platelet counts, surmising that as serum is a product of clotted blood, it contents would merely reflect the initial platelet content. For 100 clinical serum samples from stable renal transplant patients, CD154 levels were strongly correlated with the platelet count (p <0.0001; Figure 3). Furthermore, for 20 patients who provided monthly samples, fluctuations in serum CD154 concentrations correlated with fluctuations in platelet counts (p<0.0001; Figure 4) indicating that as the platelet count changes, so changes the serum levels of CD154. Thus, reports indexing CD154 in serum samples perhaps relate more to platelet count than to any physiological in vivo release of CD154.

Freeze and thaw cycles are known to activate platelets, and platelets can contaminate samples spun at g-forces used to remove red blood cells and lymphocytes (22). To investigate these variables on CD154 levels, we subjected plasma samples to centrifugation of varying intensities (200, 800, 1600g) and subjected these samples to freeze thaw cycles. As demonstrated in Figure 5, platelet rich plasma obtained through 200g centrifugation had the highest level of CD154 detected by ELISA (p=0.002). A stepwise decrease in serum
CD154 was noted with increased removal of platelets from plasma, obtained with 800g and 1600g centrifugation. For a fixed centrifugation speed, increasing the number of freeze thaw cycles in platelet trended towards increasing CD154 levels, but some variability was noted among patients. These data support that soluble CD154 levels correspond to the amount of platelets in plasma and the amount of activation to which the platelets are subjected. Thus, CD154 is not a prevalent ambient plasma protein, existing at most in picogram per ml rather than microgram per ml ranges, and its measurement can be heavily influenced by contaminating platelets in the measured plasma sample.

**CD154 is not systemically released during organ transplantation or rejection**

The CD40/CD154 pathway is of paramount importance in T cell activation. Furthermore, trauma and platelets activation are inseparable from transplantation surgery. This has led to a reasonable speculation that the trauma of transplant surgery could provide substantial amounts of CD154 and in turn facilitate the initiation of an alloimmune response. We therefore studied patients intraoperatively to determine whether significant amounts of CD154 were released during transplant surgical procedures. We studied patients undergoing kidney transplantation (Figure 6a), as well as those undergoing liver transplantation (Figure 6b), an operation of considerably greater magnitude. Despite the clear presence of surgical trauma, we found no significant fluctuation in systemic plasma CD154 during either procedure, with pre- and immediately post-operative levels statistically unchanged, and indeed numerically decreased postoperatively (Figure 6). To assess whether the operative field was a source of concentrated CD154 release that was perhaps diluted to be imperceptible systemically, we assessed blood samples directly from the iliac vein downstream from the renal vein anastomosis in renal transplant recipients (Figure 6a, intraoperative measurements), and similarly found no increase in CD154. Thus, when measured appropriately, we could demonstrate no evidence that the surgical trauma of kidney or liver transplantation appreciably altered the ambient levels of plasma CD154.

We then investigated CD154 levels during biopsy proven acute kidney allograft rejection, to determine whether CD154 release was a characteristic of the rejection process. Interestingly, despite the known influence of CD40:CD154 interactions on allograft rejection, we found that patients experiencing an acute rejection event had a significantly lower level of CD154 in their plasma when compared to patients on a similar immunosuppressive regimen but with biopsy proven stable kidney function (Figure 7). Indeed, the numerical trend toward decreased CD154 in the setting of the surgical procedure, and its decrease during rejection, suggests that CD154 consumption, perhaps through local savaging by CD40, and not release, is most related to clinical transplant processes.

**Discussion**

CD154 is well established as a potent immunostimulatory molecule that evokes robust antigen presenting cell and B cell activation through binding to CD40 (3, 4). Blockade of CD154/CD40 interaction has profound inhibitory effects on a variety of immune responses, including auto-, protective and allo-immunity (1, 23, 24). Importantly, CD154 is relatively ubiquitous, with platelets serving as a seemingly substantial reservoir of active CD154, storing quantities sufficient (1, 6, 7, 9) for in vivo immune activation. As such, strict regulation of this molecule’s release would appear to be required to avoid inappropriate immune activation. However, several reports have suggested that CD154 can be readily detected in the peripheral circulation and that ambient levels of CD154 associate with a variety of disease states. Given this apparent discrepancy, we sought to better understand the mechanisms involved in CD154 storage and release, and specifically to evaluate systemic CD154 in relevant clinical transplant settings. Our general finding is that CD154, when measured cognizant of potential artifactual in vitro liberation from platelets, is present if at
all in low levels that do not elevate significantly in the setting of organ transplantation or transplant rejection. These data suggest that the profound effects of CD154 on transplant rejection are mediated at the local level, and that as would be expected, this potent molecule is not released in substantial amounts as a result of surgical trauma of the magnitude anticipatable in kidney and liver transplantation.

By studying platelets from individuals with defined genetic defects, we found that CD154 is stored in platelet alpha granules and importantly is released in an alpha degranulation dependent manner. These results have several implications. Platelets are fundamentally involved in the initial response to trauma, particularly that initiated by endothelial cell disruption. Platelet alpha degranulation mediates multiple platelet dependent phenomena promoting thrombosis and vasoregulation (25, 26), and in the case of CD154, immune activation, as an innate response to trauma, linking physiological hemostasis to adaptive immune activation. This is consistent (6, 7, 9) with several previous papers, but assigning CD154 specifically to alpha granules helps to clarify the local deposition and regulation of this potent molecule, making it similar to many thrombogenic and vasoregulatory proteins that are delivered locally.

Our experiments indicate that CD154 is not released systematically even with major, though controlled, surgical trauma. This is consistent with the strict control required to prevent catastrophic immune activation that would likely result from broad systemic CD154 release. Henn et al reported that CD40 is co-expressed on platelets (27), and this may provide a scavenging mechanism to limit platelet CD154 release beyond the local environment. A similar scavenging effect of CD40 on other cell types may also serve in this way, and indeed, the trend toward decreased CD154 presence in the periphery during known states of rejection suggests that CD40 presence is an important regulated factor in determining the impact of CD154. Our data show that plasma levels of CD154 during renal allograft rejection are if anything lower than those drawn from patients with stable kidney transplant function. This too may relate to upregulation of CD40 and increased scavenging, or be an inconsequential association. However, it diminishes enthusiasm for the use of CD154 as a biomarker of rejection.

Similarly, the localization of CD154 to the alpha granule rather than the surface of platelets suggests that the thromboembolic complications associated with the clinical use of CD154-specific antibodies are activation dependent, with direct antibody-CD154 binding interactions predicated on some degree of primary platelet degranulation, or interactions with sources of CD154 other than platelets.

Many papers have presented correlations between CD154 levels and clinical diseases, based upon serum CD154 levels. Our data confirm that of other reports in the clinical chemistry literature (28–31) that serum is an inappropriate fluid in which to assess biologically relevant CD154 and relates best to platelet presence and in vitro release upon clot formation, and not in vivo activation. This does not discount the potential for pathological states associated with CD154 release, but suggests that in these circumstances, the primary defect is one of ongoing platelet degranulation. Regardless, the detection of CD154 in the circulation requires strict attention to platelet integrity to avoid artifactual data and related associations. Pre-analytical factors causing differences in serum and plasma CD154 measurements have been studied previously, and our experiments are in keeping with these data. Technical issues are clearly paramount in the interpretation of data in this field (32).

CD154 is present in the cytosol of patients with GPS, marking it as distinct from other alpha granule proteins such as beta-thromboglobulin. Beta-thromboglobulin has decreased levels in the cytosol of GPS patients probably due to premature release or leaking (33). The high
levels of CD154 in the cytosol of alpha granule deficient platelets are most consistent with active production of CD154 by megakaryocytes. Indeed, CD154 mRNA was recently quantified in the megakaryocyte, and promoter analysis of the CD154 gene revealed that NFAT, a calcium-dependent transcriptional regulator, mediates megakaryocyte-specific CD154 expression (34). Thus, the packaging of CD154 in the alpha granule is an active process that may also be amenable to therapeutic inhibition. Interestingly, calcineurin inhibitors used in transplantation inhibit NFAT. We are investigating the effect of these agents on platelet CD154 packaging. Furthermore, the combination of defective alpha granule formation and substantial CD154 production and loading into platelets could result in inappropriate leakage of CD154 into the bone marrow. We speculate that this could contribute to the myelofibrosis observed in some patients with GPS.

These data are consistent with CD154 being viewed as one of many proteins released in a measured fashion from platelets at the time of degranulation. They suggest that CD154 serves its physiological role through local interactions, and that systemic release is controlled even in the setting of substantial operative procedures associated with organ transplantation.

Acknowledgments

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Abbreviations

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<tr>
<th>Abbr</th>
<th>Description</th>
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<tr>
<td>GPS</td>
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<td>HPS</td>
<td>Hermansky-Pudlak Syndrome</td>
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<td>EM</td>
<td>Immuno-electron microscopy</td>
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References


Figure 1.
CD154 localizes to the alpha granules of platelets. Shown are immuno-electron micrographs of normal human platelets decorated with gold-labeled CD154-specific monoclonal antibodies. The gold particles (black spheres marked by arrow) localize to the alpha granule, but not to the cytosol or plasma membrane in unstimulated platelets (1a), and are more dispersed in stimulated platelets (1b).
Figure 2.
Quantitative assessment of CD154 localization in resting and activated platelets shows that CD154 is largely extra-granular after activation.
Figure 3. Serum CD154 levels are a reflection of platelet count. Shown are CD154 levels (pg/mL) as determined by ELISA correlated with absolute platelet count per microliter whole blood as determined by complete blood count. Samples were drawn from clinically stable renal allograft recipients.
Figure 4.
Changes in platelet count over a 1 month time period correlate with proportional changes in serum CD154 levels over the same time period. Shown are simultaneous relationships between serum CD154 and platelet count for 20 normal healthy volunteers.
Figure 5. Soluble CD154 correspond to plasma platelet concentration and activation via freeze-thaw cycles. Shown are CD154 levels (pg/mL) as determined by ELISA on plasma samples obtained by low, medium, and high g centrifugation. Increasing speeds remove more platelets from the sample, leading to a stepwise decrease in soluble CD154. Repeated freeze-thaw cycles liberate increasing amounts of CD154.
Figure 6.
CD154 is not markedly influenced by the surgical trauma associated with kidney or liver transplantation. Shown are plasma CD154 levels derived from kidney transplant recipients (a) immediately prior to (pre) and at the conclusion of (post) kidney transplantation. Intraoperative samples drawn directly from the renal vein post reperfusion (intra) are similarly not elevated. The same lack of alteration is seen following liver transplantation (b). No intraoperative levels were drawn in these patients.
Figure 7.
CD154 is not systemically elevated during renal allograft rejection. Significantly lower CD154 is seen at the time of biopsy, prior to therapy, in patients undergoing acute cellular rejection.