Immunomodulatory Functions of Low-Molecular Weight Hyaluronate in an Acute Rat Renal Allograft Rejection Model

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Abstract. Low molecular weight hyaluronate (LMW-HA) blocks interactions between T lymphocyte CD44 and hyaluronate (HA), a heteropolysaccharide that is expressed on the surface of endothelial cells and ubiquitously in the extracellular matrix. This study was undertaken to assess the ability of LMW-HA to modify the course of experimental acute renal allograft rejection. Lewis (LEW) rats were bilaterally nephrectomized and received an orthotopic, fully MHC-mismatched, Wistar-Furth (WF) kidney transplant. Animals received either no treatment, low doses of cyclosporin A (CsA) on days 0 to 5, LMW-HA on days 0 to 5, or CsA plus LMW-HA on days 0 to 5 after transplantation. With no treatment, CsA monotherapy, or HA monotherapy, animals rejected their allografts at a median of 15, 13, and 7.5 d, respectively (P = NS). In contrast, combined CsA plus LMW-HA therapy prevented acute rejection and significantly prolonged graft survival (P = 0.008) to a median of 49.0 d. CsA/LMW-HA-treated grafts also demonstrated better preservation of renal function at day 30 (serum creatinine level, 1.38 ± 0.8 mg/dl), compared with surviving animals treated with CsA alone (2.9 ± 0.55 mg/dl, P < 0.05). Histologic graft analysis of CsA/LMW-HA-treated animals at day 7 after transplantation showed minimal rejection and leukocyte infiltration, compared with all other groups. Intragraft gene expression analysis, using semiquantitative reverse transcription-PCR, at the same time point showed reductions of CD4, CD8, and interferon-γ transcript levels in the combined-treatment group. This is the first study demonstrating the immunomodulatory functions of LMW-HA in vivo in the setting of organ transplantation. Defining the exact mechanisms that underlie this immunomodulation may provide the rationale to develop novel strategies for use in clinical transplantation.

The activation of leukocytes and their subsequent recruitment into transplanted organs are integral components of the rejection response. Adhesive interactions between leukocytes and endothelial cells mediate the recruitment of leukocytes into graft tissue. Initial interactions involve the selectin family of adhesion molecules, which facilitate rolling of the leukocytes along the endothelium. Subsequent events, such as firm adhesion of leukocytes to the endothelium, diapedesis, and transmigration, are mediated by members of the integrin and Ig superfamilies of adhesion molecules (1). Recently, a new protein-carbohydrate ligand interaction between certain isoforms of the cell surface receptor CD44 and its principal endothelial ligand, hyaluronate (HA), has also been shown to be involved in this adhesion and extravasation process (2,3).

CD44 is a transmembrane glycoprotein that is expressed on a wide variety of cells, including CD4+ and CD8+ T cells, B cells, natural killer cells, and mononuclear phagocytes. Various isoforms of this molecule are involved in cell-cell and cell-extracellular matrix (ECM) interactions, cell trafficking, lymph node homing, presentation of chemokines and growth factors to traveling cells, and growth signal transmission (4,5). T cell costimulatory functions have also been proposed (6). The principal ligand for CD44 in vivo is endogenous HA, but binding to the ECM components collagen, fibronectin, laminin, and chondroitin sulfate may also occur.

HA is a large linear polymer consisting of repeated disaccharide units of N-acetylgalactosamine and glucuronic acid. It is secreted by cells of mesenchymal origin and plays an important role in organ and tissue development (7). HA is also found in body fluids and is a ubiquitous component of the ECM. More recently, the role of HA in inflammatory responses has been recognized (8). ECM molecules such as laminin, fibronectin, and HA are known to be expressed at high levels in rejecting solid-organ allografts. Increased levels of HA in the graft interstitium were correlated with rejection in experimental rat (9) and human (10) renal allografts. Increased serum levels of HA have also been suggested to serve as a marker for a number of pathologic conditions, including allograft rejection (11,12).

Other known cell surface receptors for HA include the receptor for HA-modified motility and intercellular adhesion molecule-1. There is evidence that the interaction between the receptor for HA-modified motility and HA plays a role in...
leukocyte locomotion (13) and in the migration of smooth muscle cells after wounding injuries (14); intercellular adhesion molecule-1 may function as an endocytosing receptor for HA in the liver (15). In addition, a new intracellular HA-binding protein has recently been described (16).

Blockade of cell-ECM and cell-cell interactions has been used for modulation of immune responses and has been shown to be effective in both animal models and clinical transplantation (17). Monoclonal blocking antibodies directed against the integrins leukocyte function-associated antigen-1 and very late activation-4 prolonged graft survival in a rat cardiac transplantation model. This was associated with a marked decrease in intragraft inflammatory cells (18).

No studies to date, however, have examined the effects of blocking CD44-HA interactions in solid-organ transplantation. We tested the hypothesis that soluble low molecular weight HA (LMW-HA) can modulate the immune response, diminish mononuclear cell recruitment in vivo, and influence graft survival in an experimental renal transplantation model. For this purpose, a low molecular weight form of HA was used. There is strong evidence from tumor models that hyaluronan oligomers can disrupt the interaction between CD44 and endogenous HA (19,20).

Materials and Methods
Mixed Leukocyte Reaction Studies
Cervical and axillary lymph nodes were collected from naive Lewis (LEW) and ACI animals, and leukocytes were isolated by standard techniques. LEW cells (3 × 10⁵) were incubated with 3 × 10⁵ irradiated ACI cells and serial concentrations of HA or control sugar for 96 h, with [3H]thymidine added for the last 6 h of culture. Proliferation was assayed by measuring [3H]thymidine incorporation. Results were expressed as the mean cpm values for quadruplicate samples, and percentage proliferation relative to the mixed leukocyte reaction (MLR) was calculated as follows: % Proliferation = [cpm (experimental) – cpm (medium)/cpm (unmodified MLR) – cpm (medium)] × 100, where cpm/medium represents the background values obtained from responder cells incubated with medium alone.

Experimental Design
Inbred LEW and Wistar-Furth (WF) rats (8 to 12 wk of age, weighing between 200 and 250 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Kidney allotransplants were from WF donors to LEW recipients. LEW to LEW kidney transplants served as isograft controls (21). The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Microsurgical Technique
The donor kidney was transplanted extraperitoneally and anastomosed side-to-end to the abdominal aorta and inferior vena cava immediately after unilateral nephrectomy on day 0. Contralateral nephrectomy of the naive kidney was performed on day 5 after transplantation. A ureterocystostomy was performed in all cases. All transplantsations were performed by the same surgeon. Renal allograft rejection was defined as uremic death (21).

Reagents and Treatment Protocols
A digested HA preparation consisting of six or seven disaccharide subunits of N-acetylgalactosamine and glucuronic acid was obtained from Sangstat Medical Corp. (Menlo Park, CA). Cyclosporin A (CsA) was obtained from Sandoz Research Institute (East Hanover, NJ).

Control animals received no treatment (n = 4). Single-treatment groups received either CsA (5 mg/kg per d, subcutaneously) for 5 d (n = 13) or HA (100 μg/d, intraperitoneally) for 5 d (n = 4) or 60 d (n = 4). Combined-treatment groups received CsA (5 mg/kg per d, subcutaneously) for 5 d after transplantation plus either HA (100 μg/d, intraperitoneally) for 5 d (short course, n = 19) or HA (100 μg/d, intraperitoneally) for 60 d (long course, n = 4). Isograft control animals (n = 8) received no treatment. The dose of 100 μg/d HA was chosen on the basis of pilot studies performed by the authors using a cardiac transplantation model (A. Knoflach, M. Sayegh, unpublished data).

Measurements of Graft Function
Blood samples were collected at 7, 30, 50, and 60 d after transplantation, for serum creatinine measurements. Creatinine was measured by a modified Jaffe’s reaction, using a Hitachi 911 autoanalyzer (Hitachi, Indianapolis, IN).

Morphologic Analysis
Additional kidneys from unmodified control, CsA (alone)-treated, and CsA/LMW-HA-treated animals were collected at day 7 after transplantation for histologic and reverse transcription (RT)-PCR analysis. Sagittal sections of allografts were fixed in 10% formalin and stained with hematoxylin/eosin and periodic acid-Schiff reagent for morphologic analysis. This evaluation was performed by an independent experienced pathologist, in a blinded manner. The degree of rejection was ranked according to the Banff criteria (22).

Gene Transcript Level Measurements
Total RNA extraction from snap-frozen tissue sections was performed (RNAeasy total RNA kit; Qiagen, Chatsworth, CA). Total RNA was assessed for purity using optical density measurements and then formaldehyde-agarose gels. cDNA was synthesized using the SuperScript single-stranded cDNA synthesis kit (Life Technologies, Gaithersburg, MD). The RT-PCR assay was used to measure transcript levels, according to standard protocols. Glyceraldehyde-3-phosphate dehydrogenase gene transcript levels were used to assess cDNA amounts in samples from different treatment groups. Primer pairs used in amplification were obtained from Integrated DNA Technologies (Coralville, IA) and Clontech (Palo Alto, CA) or were synthesized by Genosys Biotechnologies (The Woodlands, TX). Primer sequences, annealing temperatures, and cycle numbers for monocyte chemotactic protein-1, inducible nitric oxide synthase, transforming growth factor-β, interferon-γ, granzyme-B, perforin, interleukin-4, CD4, CD8, and tumor necrosis factor-α are shown in Table 1.

PCR was performed in a PTC-100Tm programmable thermal controller (MJ Research, Inc., Boston, MA) under standard conditions (denaturation at 94°C for 15 s, annealing at 50°C to 60°C for 30 s, and extension at 72°C for 60 s). Ten microliters of the PCR products were analyzed on a 2% agarose gel. The densitometric band intensities were measured using computerized imaging software (Alpha-imager, version 0.1.12; Alpha Innotech Corp., San Leandro, CA). Glyceraldehyde-3-phosphate dehydrogenase gene product levels were used to assess variations in cDNA amounts in different samples. Densitometric values obtained were adjusted according to the amounts of cDNA.
used in the reaction. Reactions were repeated for consistency (in triplicate).

Statistical Analyses

Kaplan–Meier actuarial survival estimates were calculated using JMP software (version 2.3; SAS Institute, Inc., Cary, NC), and the log-rank test was used to compare different groups. Long-term graft survival was defined as survival beyond day 80 after transplantation. For comparisons of serum creatinine levels and PCR densitometric results among the various groups, data were analyzed first by one-way ANOVA and then by the Tukey–Kramer post hoc test. A P value of <0.05 was considered significant.

Results

LMW-HA Inhibition of the Rat MLR in Vitro

First, we tested the effect of LMW-HA in vitro in the MLR, using a high-responder rat strain combination. Dose-dependent inhibition of the LEW/ACI rat MLR by LMW-HA (50% inhibition at a dose of approximately 20 μg/ml) is shown in Figure 1. The control sugar did not inhibit the MLR.

Significant Prolongation of Renal Allograft Survival with the Addition of HA to CsA Therapy

Control untreated recipients exhibited a median graft survival time of 15.0 d. Median graft survival times in the groups treated with CsA and short-course LMW-HA monotherapy were 13.0 and 7.5 d, respectively (P = NS). Addition of short-course HA to low-dose CsA significantly prolonged graft

Table 1. Primer sequencesa

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>5' ATG CAG TCT TCT TCT ACG 3' CTA GGT TCT TGT CAT ACT</td>
<td>50°C, 28 cycles</td>
</tr>
<tr>
<td>iNOS</td>
<td>5' TGC CAG GGT CAC AAT TTT ACA AG 3' GGT CGA TGT CAC ATG CAG TTT TGT</td>
<td>60°C, 35 cycles</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5' TGA ACC AAG GAG ACG GAA TAC AGG 3' TAC TGT GTG TCG AGG CTA AAT</td>
<td>57°C, 26 cycles</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5' GGT TAT CTG GAG GAA CTG GCA AAA G 3' GCT AGA TTT TGG TGA CAG CTG GTG</td>
<td>60°C, 35 cycles</td>
</tr>
<tr>
<td>CD4</td>
<td>5' TGA GAG CTG GAG AAC AAG AAA GAG G 3' TCA CAG GTC AAA GGT TTT CTG TCG G</td>
<td>57°C, 26 cycles</td>
</tr>
<tr>
<td>CD4</td>
<td>5' TGC GAG CTG GAG AAC AAG AAA GAG G 3' TCA CAG GTC AAA GGT TTT CTG TCG G</td>
<td>24°C, 57 cycles</td>
</tr>
<tr>
<td>CD8</td>
<td>5' TCC ATG CAC CGA GAT GTT TGT ACC A 3' CTT TCA GTG TTT TGA GCG TGG ACT C</td>
<td>54°C, 32 cycles</td>
</tr>
<tr>
<td>IL-4</td>
<td>5' TCC ATG CAC CGA GAT GTT TGT ACC A 3' CTT TCA GTG TTT TGA GCG TGG ACT C</td>
<td>55°C, 40 cycles</td>
</tr>
<tr>
<td>Perforin</td>
<td>5' TGC TAC ACT GCC ACT GCC ACT CGG TCA 3' GCA TGC TCT GTG GAG CTA TTA</td>
<td>60°C, 35 cycles</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>5' GAC TTT GTG CTG ACT GCT GCT CAC 3' TTG TCC ATA GGA GAC GAT GCC CGC</td>
<td>60°C, 35 cycles</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5' GGC ATT GCT GTC CTG TGA TTA C 3' CCG TGA TGT CTA AGT ACT TGG</td>
<td>50°C, 28 cycles</td>
</tr>
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a MCP-1, monocyte chemotactic protein-1; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; IL-4, interleukin-4; TNF-α, tumor necrosis factor-α.

Figure 1. Effects of low molecular weight hyaluronate (LMW-HA) on T cell proliferation in the rat mixed leukocyte reaction (MLR). The concentration of LMW-HA is shown on the x-axis and the percent proliferation, relative to unmodified MLR, is shown on the y-axis. The actual radioactivity levels for the unmodified control MLR in the four experiments presented here were 28,333 cpm (background, 1,513 cpm), 37,196 cpm (background, 1,114 cpm), 70,621 cpm (background, 340 cpm), and 172,347 cpm (background, 893 cpm). The control compound was glucose. Cells from Lewis (LEW) lymph nodes served as responders and ACI splenocytes as stimulators. Data are shown as mean ± SD.
survival (median, 49.0 d; \( P = 0.008 \)), compared with all other groups. Approximately 30% of these animals exhibited long-term (>80 d) graft survival, whereas there was no long-term graft survival in the CsA-treated group (Figure 2). Monotherapy with LMW-HA for 60 d was ineffective in prolonging survival (median, 10.0 d). The combination of LMW-HA for 60 d and low-dose CsA for 5 d resulted in a median survival time of 47.5 d (\( P = 0.07 \), compared with CsA-treated group). The isograft control group (LEW to LEW) exhibited 100% long-term survival rates, as expected. No toxic effects of LMW-HA therapy were noted.

Functional Status of Kidney Grafts

At day 7 after transplantation, there was no significant difference in serum creatinine levels between the CsA-treated group (0.56 ± 0.11 mg/dl) and the CsA/LMW-HA (short course)-treated group (0.85 ± 0.36 mg/dl) (Table 2), reflecting the fact that the remaining native kidney was removed only on day 5. By day 30, however, serum creatinine levels were significantly lower in the CsA/LMW-HA (short course)-treated animals (1.38 ± 0.8 mg/dl), compared with surviving CsA-treated animals (2.9 ± 0.55 mg/dl, \( P < 0.05 \)). Creatinine levels at this time point were also lower in animals receiving CsA plus long-course LMW-HA, although this did not achieve statistical significance. Serum creatinine levels beyond day 30 are also shown in Table 2 for animals receiving CsA plus LMW-HA; too few animals treated with CsA alone were alive at later time points for comparative analysis.

Graft Morphologic Characteristics

Banff scores for the individual animals are presented in Table 3. Grafts from untreated animals exhibited severe acute rejection, as shown by severe parenchymal mononuclear cell infiltration, tubulitis, endothelialitis, and fibrinoid necrosis of

Table 2. Serum creatinine levels of animals at day 7, 30, 50, and 60 posttransplantation*

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Creatinine (mg/dl)</th>
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<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>CsA</td>
<td>0.56 ± 0.11</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
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<tr>
<td>CsA + HA (short course)</td>
<td>0.85 ± 0.36</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>CsA + HA (long course)</td>
<td>0.63 ± 0.25</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as mean ± SD.

b \( P < 0.05 \) compared with CsA-treated group.
vessels. Low-dose CsA therapy alone resulted in moderate tubulitis and parenchymal infiltration and early signs of endotheliitis. However, addition of LMW-HA to CsA therapy almost completely abrogated acute rejection at day 7. Although patchy parenchymal infiltration was present, minimal tubulitis and no endotheliitis were present. Representative light microscopic findings are shown in Figure 3.

**Gene Expression Analyses**

The densitometric data are presented in Figure 4. Grafts from animals treated with CsA plus LMW-HA showed significantly reduced interferon-γ gene transcript levels, compared with all other groups, including CsA-treated animals (P < 0.05) (Figure 4B). Expression of CD4 and CD8 (Figure 4A) was greatly reduced in the CsA/LMW-HA-treated group, compared with all other groups, but these differences did not achieve statistical significance in ANOVA and Tukey-Kramer post hoc testing (P = 0.08 and 0.1, respectively). There was also a trend toward lower granzyme-B, perforin, and tumor necrosis factor-α expression in the CsA/LMW-HA-treated animals, compared with all other groups (data not shown). Transcript levels for inducible nitric oxide synthase and monocyte chemoattractant protein-1 were significantly reduced with either CsA monotherapy or combination LMW-HA/CsA therapy, compared with untreated controls (P < 0.05) (Figure 4C).

**Discussion**

This is the first study of the immunomodulatory functions of LMW-HA in an in vivo allogeneic transplantation model. With the doses and protocols used in this aggressive acute rejection model, treatment with LMW-HA alone or low-dose CsA alone was unsuccessful in prolonging renal allograft survival. This lack of efficacy of LMW-HA monotherapy is not unexpected, because CD44-ligand interactions may be important but are not necessarily critical in the pathogenesis of acute rejection. Addition of LMW-HA to low-dose CsA, however, significantly prolonged transplant kidney survival, suggesting synergy between these two agents. In addition, long-term graft survival occurred only in animals treated with combination therapy. The beneficial effect of HA treatment was prolonged; renal function, as measured by serum creatinine levels, was significantly better in CsA/LMW-HA-treated animals, compared with CsA-treated animals, at day 30 after transplantation.

Our in vitro, morphologic, and RT-PCR studies addressed the mechanisms by which HA exerted its graft-protective effects. The marked reduction in mononuclear cell infiltration on day 7 in CsA/LMW-HA-treated grafts and the concomitant finding of clinically significant reductions in CD4 and CD8 transcript levels in these grafts provide evidence that LMW-HA prevents leukocyte migration into allografts in the early posttransplantation period. The predominant mechanism by which this occurs is likely via the antiadhesion effects of LMW-HA. A large body of evidence has indicated that CD44-HA interactions are important for leukocyte adhesion to
endothelium and subsequent recruitment into inflammatory sites (2,23). Furthermore, in a rat skin transplantation model, monoclonal antibodies directed against the CD44s isoform were shown to block homing of activated lymphocytes into the allograft (24). The importance of disrupting CD44 interactions with other ECM glycoprotein ligands, such as chondroitin-4-sulfate or osteopontin, in vivo has yet to be established.

A direct immunosuppressive effect of LMW-HA (as illustrated by its inhibitory effects on the MLR) in this transplantation model may also be important. The ability of HA to inhibit T cell proliferation in the MLR is unlikely to be attributable to antiadhesion effects alone; other effects, such as modulation of T cell activational signals, may play a role. Interestingly, this inhibition of proliferation occurred at much lower concentrations than that seen with monoculture of a murine melanoma cell line (20). Ligation of CD44 can activate intracellular signaling pathways that induce cell activation (25,26), and studies of CD40-CD40L interactions have sug-

![Figure 4. Semiquantitative analysis of gene expression in rat renal allograft tissue at day 7 after transplantation. Results are expressed as relative densitometric units (mean ± SD). (A) Relative levels of CD4 and CD8. (B) Interferon-γ (IFN-γ) and interleukin-4 (IL-4). (C) Inducible nitric oxide synthase (iNOS) and monocyte chemotactic protein-1 (MCP-1).]
gested that specific isoforms of CD44 may also possess co-stimulatory activity (6). Natural killer cell-mediated lysis of endothelial cells may also be associated with high levels of expression of CD44 (27). Therefore, multiple components of the immune response are potentially affected by CD44 blockade; it is probable that LMW-HA exerts a combination of immunomodulatory effects, depending on the phenotype and microenvironment of the CD44-expressing cell and the isotype of the expressed CD44 molecule itself.

The synergistic effects of CsA and HA in vivo presumably reflect their different mechanisms of action, with the former inhibiting antigen-mediated T cell activation and the latter inhibiting recruitment of inflammatory and immune cells into the graft. An effect of HA on CsA pharmacokinetics is unlikely, because HA is not known to affect the cytochrome P450 system, which is the principal pathway of CsA metabolism. To formally exclude this possibility, samples from additional animals treated with CsA alone or CsA plus LMW-HA were analyzed for whole-blood CsA levels on days 1, 3, and 6; there were no statistical or clinical differences in levels between the two groups (data not shown). Interestingly, in the transplantation model studied here, prolonging the course of LMW-HA yielded no additional survival benefit. The impressive effects of the short course of LMW-HA suggest that CD44 receptor-HA interactions exert their main effects in promoting rejection in the immediate posttransplantation period, at least in this model. This may reflect the increased production of endogenous HA resulting from the ischemia-reperfusion injury associated with the collection and transplantation of renal allografts (28).

Additional studies are required to determine the exact mechanisms of action of LMW-HA in vivo. Comparative studies with antibodies directed against specific isoforms of CD44 will be useful in this regard. Testing the compound alone in a less aggressive donor-recipient rejection model would establish whether coadministration of a calcineurin inhibitor is required for a meaningful immunosuppressive effect. There is ongoing interest in the application of antiadhesion strategies for renal allografts at high risk of developing ischemic acute tubular necrosis. Ischemia-induced upregulation of adhesion molecule expression on graft endothelium is thought to be important in facilitation of intragraft immune cell recruitment and in the high incidence of acute rejection associated with delayed graft function. LMW-HA-type therapies may prove to have a useful preventive role in this setting. It is important to realize, however, that translation of the results of rodent studies of immunomodulatory agents to the treatment of primates and human subjects has met with variable degrees of success in the past.

In conclusion, administration of LMW-HA has been shown for the first time to modify alloimmune responses in vivo. When combined with CsA, perioperatively administered LMW-HA was successful in preventing early acute rejection, prolonging allograft survival, and preserving organ function. Further investigation is required to delineate the precise means of action of LMW-HA in this setting. Such studies should yield clinically relevant information for the development of novel immunomodulatory strategies for use in organ transplantation.

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References


