Role of Sulfation in CD44-Mediated Hyaluronan Binding Induced by Inflammatory Mediators in Human CD14⁺ Peripheral Blood Monocytes

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_J Immunol_ 2001; 167:5367-5374; doi: 10.4049/jimmunol.167.9.5367
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Role of Sulfation in CD44-Mediated Hyaluronan Binding Induced by Inflammatory Mediators in Human CD14+ Peripheral Blood Monocytes

Kelly L. Brown, Arpita Maiti, and Pauline Johnson

Activation of T cells by Ag or stimulation of monocytes with inflammatory cytokines induces CD44 to bind to hyaluronan (HA), an adhesion event implicated in leukocyte-leukocyte, leukocyte-endothelial cell, and leukocyte-stromal cell interactions. We have previously shown that TNF-α induces CD44 sulfation in a leukemic cell line, which correlated with the induction of HA binding and CD44-mediated adhesion. In this study, we establish that TNF-α and IFN-γ induce HA binding and the sulfation of CD44 in CD14+ PBMC, whereas no induced HA binding or CD44 sulfation was observed in CD14− PBMC stimulated with TNF-α. Treatment of cells with NaClO3, an inhibitor of sulfation, prevented HA binding in a significant percentage of CD14+ PBMC, whereas no induced HA binding or CD44 sulfation was observed in CD14− PBMC stimulated with TNF-α. In contrast, the transient induction of HA binding in T cells by PHA was not affected by NaClO3, suggesting that activated T cells do not use sulfation as a mechanism to regulate HA binding. Overall, these results demonstrate that inducible sulfation of CD44 is one mechanism used by CD14+ peripheral blood monocytes to induce HA binding in response to inflammatory agents such as TNF-α and IFN-γ. The Journal of Immunology, 2001, 167: 5367–5374.
cytokines can induce changes in CD44 expression and induce HA binding, but the relationship between the two events is unclear. Likewise, activation of T cells by Ag transiently induces CD44 to bind HA by mechanisms that are, at present, poorly understood.

It is well established that molecules such as the selectins and integrins play an important role in leukocyte-endothelial cell interactions, a necessary event for both lymphoid recirculation and extravasation to inflammatory sites (21–24). Interactions between the selectins and their ligands are responsible for the initial contact between the leukocyte and the endothelial cell. Subsequent firm adhesion is thought to occur via chemokine-activated integrins. Leukocyte transmigration across the endothelial layer is not well defined, nor is the migration within the inflamed tissue. Inflammatory cytokines produced during an immune response can stimulate an increase in HA expression on endothelial cells (25) and HA binding by some activated leukocytes. It is possible that CD44-HA interactions occur to promote leukocyte-endothelial cell interactions and extravasation at an inflammatory site and/or facilitate leukocyte migration and adhesion within the inflamed tissue. Analysis of selectin ligands revealed the importance of sialylation and sulfation for their recognition by L- and P-selectins (26–28). Sulfation is a critical element necessary for L- and P-selectin binding and there is some evidence in leukemic cells that sulfation may regulate ligand binding by CD44. TNF-α induced the sulfation of CD44 in a leukemic cell line, and this correlated with induced HA binding and adhesion to an endothelial cell monolayer (20). Culture-differentiated, adherent, monocytic-derived macrophages induced a heparan sulfate-modified form of CD44V3, but, in this case, HA binding was not analyzed (29).

Given the importance of sulfation in mediating the interaction between selectins and their ligands in leukocyte-endothelial cell interactions and its induction on CD44 in leukemic cells in response to TNF-α, we wanted to determine whether sulfation was an inducible posttranslational mechanism occurring in monocytes to regulate the interaction between CD44 and HA in response to inflammatory agents.

In this work, we establish that TNF-α and IFN-γ induce the sulfation of CD44 in CD14+ PBMC, and we demonstrate that sulfation contributes to the induction of HA binding by these cells. We also show that induction of HA binding in CD14+ monocytes by LPS and IL-1β is significantly inhibited by NaClO₃, a sulfation inhibitor. In contrast, the transient HA binding of CD3+ T cells activated by PHA was not reduced by NaClO₃, suggesting that T cells use sulfate-independent mechanisms to regulate HA binding.

Materials and Methods
Reagents and Abs
Complete and sulfated-free RPMI 1640 (Cell Culture Facility, University of California, San Francisco, CA) were supplemented with 10% FCS (HyClone, Life Technologies, Basingstoke, Hampshire, UK), 2 mM L-glutamine (Calbiochem-Novabiochem, San Diego, CA), and 50 μg/ml 2-ME (Sigma-Aldrich, Oakville, Ontario, Canada). Rooster comb HA (Sigma-Aldrich) was conjugated to fluorescein isothiocyanate (FL-HA) according to the method of de Belder and Urfe (30). Tissue sulfation of CD44 in CD14+ monocytic leukemia-derived macrophages (J774A.1, provided by M. Davis) was analyzed (31) by a fluorometric assay. Tissue sulfation was defined as the ratio of tissue sulfation to the total amount of sulfation, measured by radioactivity in the tissue. All reagents and Abs were purchased from the manufacturer’s instructions.

Cells
Whole blood (40–80 ml) was collected from healthy volunteers, treated with sodium heparin, and separated by centrifugation over a Ficoll-Paque Plus (Amersham Pharmacia Biotech, Baie d’Urfé, Quebec, Canada) density gradient. White blood cells were isolated from the buffy coat and contaminating RBCs were lysed with 10 mM Tris (pH 7.5), 0.83% NH₄Cl. PBMC were resuspended and complete RPMI 1640/10% FCS, and cell concentration was determined by trypan blue exclusion. PBMC (5 × 10⁶) were seeded into 6-well tissue culture dishes (Nunc; Life Technologies, Burlington, Ontario, Canada) at 2.5 × 10⁵/ml for 72 h at 37°C, 5% CO₂ with or without various recombinant cytokines: 20 ng/ml TNF-α, 20 ng/ml IL-1β, 500 U/ml IFN-γ (all from R&D Systems, Minneapolis, MN), and 1 μg/ml LPS (Sigma-Aldrich). PBMC were simultaneously incubated with 0–50 mM NaClO₃ (Sigma-Aldrich). Alternatively, PBMC (3 × 10⁶) were incubated in 6-well tissue culture dishes at 1.5 × 10⁵/ml for 12–48 h at 37°C, 5% CO₂ in the presence or absence of 5 μg/ml PHA (Sigma-Aldrich) and 50 mM NaClO₃.

Flow cytometry analysis
Following incubation, PBMC were resuspended in PBS containing 2 mM EDTA and 2% FCS (PBS/EDTA/FCS). PBMC were incubated with 200 μl of 1 μM biotinylated human CD14 antibody to block Fe receptors. Cells were washed twice in PBS/EDTA/FCS, then incubated with 100 μl of 3 μg/ml PE-conjugated streptavidin and/or 10 μg/ml HRP-conjugated goat anti-mouse Abs, 1/2000 PE anti-CD14, 1/300 PE streptavidin, and/or 10 μg/ml biotinylated anti-CD3 for 10 min on ice to block Fe receptors. Cells were washed twice and incubated with 100 μl of 1/100 FITC goat anti-mouse Abs, 1/2000 PE anti-CD14, 1/300 PE streptavidin, and 1/5000 biotinylated anti-CD3 on ice for 10 min followed by 10 μg/ml 1% FCS (HyClone) containing 5 μg/ml propidium iodide (Sigma-Aldrich). Alternatively, cells were incubated with buffer, isotype controls, or secondary Ab alone. A minimum of 5 × 10⁵ cells were used per labeling reaction. Ten thousand live PBMC, 5000 live CD3+ cells, or 2000 size-gated events (large cells based on forward and side scatter profiles) were collected on a FACScan flow cytometer (BD Biosciences, Mississauga, ON) and analyzed using CellQuest software (BD Biosciences).

[35S]Sulfate labeling and CD44 immunoprecipitation of CD14+ and CD14− cells
PBMC (5 × 10⁶) were cultured in 2 ml of sulfate-free RPMI 1640/10% FCS in 6-well tissue culture plates in the presence or absence of stimuli and 50 mM NaClO₃ at 37°C, 5% CO₂ for 72 h. Cells were supplemented with 200 μCi/ml [35S]sulfate as Na₂SO₄ (specific activity ~ 43 Ci/mmol (ICN Biomedicals, St. Laurent, Quebec, Canada) for the entire incubation period and re-fed every 24 or 48 h with label at 0, 24, 48, and 72 h. Alternatively, PBMC in complete medium were labeled with [35S]sulfate in the presence of TNF-α or IFN-γ with various NaClO₃ concentrations. Following sulfate labeling, PBMC were washed twice in PBS, then resuspended in PBS/EDTA/FCS at −1 × 10⁵ cells/ml. Cells were rotated end over end at 4°C for 1 h with 25 μg/ml anti-CD14-conjugated magnetic beads (M450; Dynal, Lake Success, NY). Beads were washed three times with 1 ml of PBS, washed (CD14+ and CD14− cells) or resuspended in 1 ml of lysis buffer (1% Triton X-100, 10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA) containing 200 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin for 10 min on ice then centrifuged at 16,000 × g for 10 min at 4°C. The lysate was preclarified with 15 μl/ml Sepharose CL-4B beads (Sigma-Aldrich) for 1 h at 4°C, rotating end over end. CD44 was immunoprecipitated from preclarified lysates with 15 μl/ml IM7.8.1-conjugated cyanogen bromide-activated Sepharose beads (4 mg/ml packed beads) for 2 h, rotating at 4°C. Immunoprecipitates were washed three times in 1 ml of lysis buffer containing 500 mM NaCl, twice in 1 ml of lysis buffer, then boiled for 2 min in sample buffer.

Autoradiography and Western blot analysis
Immunoprecipitated CD44 was resolved on a 7.5% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) Immobilon-P Membrane (Millipore, Ontario, Canada). Membranes were exposed to Kodak BioMax MR film (InterScience, Markham, Ontario, Canada) with an intensifying screen at ~80°C for 7–21 days. Subsequently, PVDF membranes were incubated with a 1/4 dilution of mouse anti-human CD13 mAb, OKT3, was obtained from the American Type Culture Collection (#CRL 8001; Rockville, MD). TCS was purified with protein G and biotinylated with E. coli NHS-LC-biotin (succinimidyl-6-endo-biotinamide) hexaate (Pierce, Rockford, IL) according to the manufacturer’s instructions.
Far Western blot analysis
CD44 immunoprecipitated from ~5 × 10^5 CD14^+ PBMC was resolved on a 7.5% SDS-polyacrylamide gel under nonreducing conditions and transferred to PVDF membranes. Membranes were incubated with 2 μg/ml FL-HA in TBST/milk for 1 h at room temperature with agitation. Membranes were washed six times for 10 min in TBST then incubated with 1 μg/ml HRP-conjugated goat anti-fluorescein Abs (in TBST/milk) for 30 min at room temperature with agitation. Membranes were washed six times for 5 min in TBST and developed with ECL Plus (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. [35S]sulfate incorporation was normalized with respect to CD44 protein by spot densitometry using National Institutes of Health image software.

Results

TFN-α-induced HA binding in PBMC is significantly inhibited by NaClO_3
PBMC were isolated from healthy individuals and incubated in RPMI 1640/10% FCS with or without 20 ng/ml TNF-α. Approximately 20% of freshly isolated PBMC bound low levels of FL-HA (data not shown), which decreased after 72 h in culture to ~5% of PBMC (Fig. 1A). Incubation of PBMC with TNF-α for 72 h induced a population of cells (~15% of PBMC) to bind high levels of FL-HA. Size selection of the larger cells (≥85% CD14^+) revealed that all of the TNF-α-induced HA-binding cells resided within this population (Fig. 1B). TNF-α increased the percentage of CD14^+ cells in the PBMC population and significantly up-regulated CD44 expression in CD14^+ cells (~4-fold, Fig. 1B). Gating on the CD14^+ population confirmed that these were the cells induced to bind to HA (Fig. 1C). HA binding was observed after 24 h with TNF-α (data not shown) but reached a maximum after 72 h when ~60% of the CD14^+ cells bound FL-HA (Fig. 1C and Table I). NaClO_3 is a sulfation inhibitor that blocks the transfer of sulfate to 3'-phosphoadenosine 5'-phosphosulfate, a universal sulfate donor (34). Coincubation of PBMC with TNF-α and 50 mM NaClO_3 for 72 h significantly inhibited the number of CD14^+ PBMC that bound HA (reduced FL-HA-binding cells by 47% ± 17%; n = 26). These results suggested that sulfation is one mechanism induced by TNF-α to promote HA binding in PBM.

HA binding induced in CD14^+ PBMC by TNF-α, LPS, IL-1β, and IFN-γ is significantly inhibited by NaClO_3 and is mediated by CD44
LPS and IL-1β, like TNF-α, have previously been shown to enhance HA binding in PBM in the presence of human serum (18, 29). To determine whether sulfation played a role in the regulation of HA binding by other inflammatory stimuli, PBMC were treated with LPS, IL-1β, or IFN-γ for 72 h in the presence or absence of 50 mM NaClO_3, and HA binding by CD14^+ PBMC was analyzed by flow cytometry. Fig. 2A illustrates that like TNF-α, LPS, IL-1β, and IFN-γ induce HA binding in a population of CD14^+ cells that is significantly inhibited by NaClO_3. On average, ~50–70% of the CD14^+ cells were induced to bind HA and ~40% of the HA binding was inhibited by treatment with NaClO_3 (Table I). In all cases, the induced HA binding was inhibited by a CD44 mAb, Hermes-1 (Fig. 2B). Therefore, TNF-α, IL-1β, LPS-, and IFN-γ-induced HA binding by CD14^+ PMBC is CD44-mediated and is significantly reduced in the presence of NaClO_3.

TNF-α and IFN-γ induce CD44 sulfation in CD14^+ cells
To determine whether TNF-α and IFN-γ induced the sulfation of CD44 in CD14^+ cells, and to establish whether this was inhibited by NaClO_3, unstimulated and stimulated PBMC were labeled with [35S]sulfate during the 72 h incubation period in the presence or absence of 50 mM NaClO_3. After the incubation period, CD44 was immunoprecipitated from either CD14^+ or CD14^- PBMC, subjected to SDS-PAGE, and analyzed for [35S]sulfate incorporation by autoradiography and CD44 expression by Western blot analysis. Fig. 3A demonstrates that low levels of [35S]sulfate were incorporated into CD44 in unstimulated CD14^+ and CD14^- PBMC, and [35S]sulfate incorporation could be significantly reduced in the presence of 50 mM NaClO_3. Neither CD44 expression nor sulfate incorporation was increased in CD14^- PBMC upon TNF-α treatment (1.1 ± 0.1-fold increase in [35S]sulfate incorporation per unit of CD44H; n = 3). In contrast, treatment of CD14^+ cells with...
TNF-α or IFN-γ caused a significant increase in $[^{35}S]$sulfate incorporation into CD44 (2.8 ± 1.0 (n = 9)-fold increase in sulfation per unit of CD44H for TNF-α and 2.3 ± 0.6 (n = 3)-fold increase in sulfation per unit of CD44H for IFN-γ; Fig. 3, A and B). Both TNF-α and IFN-γ stimulation increased expression of the 85-kDa form as well as higher and lower molecular mass forms of CD44. It is possible that the lower molecular mass forms are degradation products and the higher forms are glycosaminoglycan-modified or alternatively spliced isoforms of CD44. To further illustrate the increase in CD44 sulfation in TNF-α-stimulated CD14$^+$ cells, approximately equivalent amounts of CD44 were loaded on the gel after incubation with TNF-α for various times (Fig. 3C, lower panel). In this example, the $[^{35}S]$sulfate was added for the final 2 h of the incubation. In Fig. 3C there is an ~3-fold increase in $[^{35}S]$sulfate incorporation on CD44H at both the 24 and 48 h time point. The addition of 50 mM NaClO₃ significantly inhibited the $[^{35}S]$sulfate incorporation, as illustrated by the 72-h time point.

Overall, these results support the analysis from flow cytometry that TNF-α or IFN-γ increase the expression of CD44 (Fig. 1 and

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**Table I. FL-HA binding induced by inflammatory mediators in CD14$^+$ PBMC and the effect of NaClO₃.$^*$**

<table>
<thead>
<tr>
<th>CD14$^+$ PBMC That Bind FL-HA</th>
<th>Loss in FL-HA Binding + NaClO₃</th>
<th>No. of times experiment was performed</th>
<th>% ± SD</th>
<th>No. of times experiment was performed</th>
<th>% ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td>63 ± 15</td>
<td>34</td>
<td>47 ± 17</td>
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<tr>
<td>LPS</td>
<td></td>
<td></td>
<td>69 ± 18</td>
<td>7</td>
<td>42 ± 7</td>
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<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td>51 ± 17</td>
<td>7</td>
<td>36 ± 23</td>
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<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td>73 ± 23</td>
<td>23</td>
<td>41 ± 18</td>
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$^*$ Values were subtracted for background FL-HA binding by unstimulated CD14$^+$ PBMC incubated in the presence or absence of NaClO₃.

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**FIGURE 2.** CD44-mediated FL-HA binding of CD14$^+$ PBMC after 72 h in response to various inflammatory stimulants and in the presence of the sulfation inhibitor, NaClO₃, as determined by flow cytometry. The first panel in A and B represents FL-HA binding of unstimulated CD14$^+$ cells. The second panel shows FL-HA binding in response to various stimuli (indicated on the left in A and B). The third panel is FL-HA binding of stimulated CD14$^+$ cells in the presence of 50 mM NaClO₃ (+ NaClO₃) in A or anti-CD44 mAb (+ anti-CD44) in B. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. The results are representative of one experiment repeated more than three times.
data not shown) and establish that both TNF-α and IFN-γ increase the incorporation of [35S]sulfate in CD44 in CD14⁺ PBMC. TNF-α treatment did not alter the sulfation level of CD44 and did not induce HA binding in CD14⁺ PBMC. This work therefore establishes a correlation between the induction of CD44 sulfation and the induction of HA binding by TNF-α and IFN-γ.

**Transient binding of HA in PHA-stimulated CD3⁺ T cells is not sulfation-dependent**

Inflammatory cytokines induced HA binding in CD14⁺ monocytes in a sulfate-dependent manner, but TNF-α did not induce HA binding in other PBMC. To determine whether sulfation played a role in the induction of HA binding in other leukocyte populations, PBMC were activated with PHA and the HA binding ability of CD3⁺ T cells was monitored over time by flow cytometry (Fig. 4A). Following culture with PHA, ~20% of CD3⁺ cells were induced to bind HA. Maximum HA binding was observed after 18–24 h in culture, but was transient and significantly reduced by 48 h. Coincubation of PBMC with PHA and 50 mM NaClO₃ for 24 h had no effect on the induction of HA binding, implying that sulfation is not regulating HA binding in PHA-activated T cells (Fig. 4B).

**Decreasing levels of CD44 sulfation correlate with decreasing FL-HA binding in TNF-α- or IFN-γ-stimulated CD14⁺ PBMC**

To strengthen the correlation between sulfation and FL-HA binding ability, PBMC were stimulated with TNF-α or IFN-γ for 72 h in complete medium in the presence or absence of [35S]sulfate and increasing concentrations of NaClO₃. Unlabeled CD14⁺ PBMC were analyzed for FL-HA binding ability by flow cytometry (Fig. 5A) and CD44 sulfation was analyzed from radiolabeled cells after CD44 immunoprecipitation and autoradiography (Fig. 5B). The percentage of FL-HA-binding cells and CD44 sulfation both decreased with increasing concentrations of NaClO₃ (Fig. 5C). The loss of HA binding and decrease in CD44H sulfation were not linear, decreasing rapidly then reaching a plateau between 30 and 50 mM NaClO₃, at which concentration ~30% of CD44H sulfation and 50–60% of HA-binding monocytes remained. These data show that the titration of CD44 sulfation correlates with HA binding in both TNF-α- and IFN-γ-induced CD14⁺ PBMC and further supports the notion that sulfation is one contributing factor regulating HA binding in CD14⁺ PBMC.

**Direct effect of CD44H sulfation on its ability to bind FL-HA**

We have shown that TNF-α and IFN-γ induced CD44 sulfation and CD44-dependent HA binding in CD14⁺ PBMC. Treatment of PBMC with NaClO₃ decreased the percentage of cells that bound FL-HA, which correlated with the decrease in CD44 sulfation. To demonstrate that the sulfation on CD44 directly affects HA binding, CD44 was immunoprecipitated from TNF-α- and IFN-γ-treated CD14⁺ PBMC in the presence or absence of 50 mM NaClO₃. CD44 from equivalent cell numbers was resolved under nonreducing conditions then analyzed for FL-HA binding by far Western blotting. Results indicated that CD44H isolated from TNF-α- or IFN-γ-stimulated CD14⁺ PBMC bound FL-HA, whereas CD44H from unstimulated cells did not (Fig. 6). CD44H from TNF-α-stimulated CD14⁺ PBMC bound 2.7 ± 0.7 (n = 4) times more FL-HA than CD44H immunoprecipitated from TNF-α-stimulated CD14⁺ PBMC treated with 50 mM NaClO₃. Similarly, 3.0 ± 1.5 (n = 4) times more FL-HA bound to CD44H isolated from IFN-γ-stimulated CD14⁺ PBMC compared with CD44H from stimulated cells treated with NaClO₃. This demonstrates that the sulfation of CD44H induced by TNF-α or IFN-γ has a direct and positive effect on FL-HA binding by CD14⁺ PBMC.

**Discussion**

In this paper, we have demonstrated that sulfation plays a role in regulating HA binding induced in CD14⁺ monocytes by the inflammatory agents TNF-α, IL-1β, LPS, and IFN-γ. We have shown that both TNF-α and IFN-γ increase the amount of sulfation on CD44 3-fold in CD14⁺ PBMC and have correlated this sulfation with induced HA binding. Finally, we have shown that CD44 sulfation directly affects the HA-binding ability of CD44. In a previous study, incubation of PBMC with autologous human serum induced CD44-mediated HA binding (16), which was further enhanced by the inflammatory cytokines TNF-α, IL-1, and LPS (18). In the present study, PBMC were cultured in RPMI 1640/10% FCS in the absence of human serum. Under these conditions, FL-HA binding was not induced during the 72-h culture period (Fig. 1).

Although induced HA binding was only observed in the CD14⁺ population, all PBMC were exposed to the inflammatory stimuli, raising the possibility that these stimuli could act indirectly on the
CD14+ cells. However, TNF-α, IL-1β, and LPS can induce HA binding by acting directly on PBM, but IL-1β- and LPS-induced HA binding may be partially dependent on monocyte secretion of TNF-α (18). Therefore, TNF-α may be responsible for a component of the induced HA binding observed with IL-1β, LPS, and possibly IFN-γ. However, in this study, IFN-γ consistently induced HA binding in a larger percentage of CD14+ cells than was observed after TNF-α stimulation, implying that TNF-α cannot be entirely responsible for the observed effect with IFN-γ. The induction of HA binding in PBM by IFN-γ has also been observed by others (17). However, this is in contrast to one study in PBM (18) and another in the leukemic cell line, SR91 (20). IFN-γ is a potent activator of monocytes and can up-regulate the expression of several molecules associated with Ag presentation and with co-stimulation of an immune response, in addition to activating the phagocytic capacity and antibacterial function of macrophages (35). In this study, both IFN-γ and TNF-α up-regulated CD44 expression on CD14+ PBM and induced HA binding, but only IFN-γ significantly up-regulated expression of B7, MHC II, and CD40 (data not shown). Although there is evidence to suggest a role for CD44-HA interactions in mediating T lymphocyte-endothelial cell interactions at inflammatory sites in vivo (11, 36), there is only a correlation between the activation of PBM and the induction of HA binding. Activated monocytes and macrophages are present in synovial joints under inflammatory conditions, where CD44 and HA levels are up-regulated (Ref. 37 and reviewed in Ref. 38). The culture of PBM in the presence of inflammatory agents may mimic the differentiation of PBM into macrophages, which occurs in vivo once the monocytes have migrated into the tissues. Therefore, the up-regulation of CD44-mediated HA binding by PBM induced by inflammatory agents may occur to facilitate monocyte-endothelial cell interactions and extravasation.

FIGURE 5. CD44 sulfation correlates with FL-HA binding by CD14+ PMBC. A–C, CD14+ PMBC were stimulated with 20 ng/ml TNF-α (top panels) or 500 U/ml IFN-γ (bottom panels) for 72 h. A, FL-HA binding by stimulated CD14+ PMBC in the presence of increasing concentrations of NaClO3 (mM concentration noted on the left-hand side) as analyzed by flow cytometry. The x-axis is fluorescence intensity (log scale) and the y-axis is cell number. B, CD44 was immunoprecipitated from stimulated CD14+ PMBC in the presence of 0–50 mM NaClO3 and [35S]sulfate. Sulfation was analyzed by autoradiography (left panels) and CD44 expression by Western blotting (right panels). Prestained molecular mass markers are on the right in kDa. C, Spot densitometry was used to determine the sulfate incorporation per CD44 as represented on the y-axis (filled bars) with the percentage of CD14+ PMBC that bind FL-HA (open bars) in the presence of increasing concentrations of NaClO3 (x-axis). All results are representative of one experiment repeated more than three times.

FIGURE 6. FL-HA binding to isolated CD44. A, CD44 was immunoprecipitated from unstimulated or TNF-α-stimulated cells (20 ng/ml; top panel), or IFN-γ-stimulated (500 U/ml; bottom panel) CD14+ PMBC in the presence or absence of 50 mM NaClO3, FL-HA binding was analyzed by far Western blotting (left panels) and CD44 expression was determined by Western blotting (right panels) on the same membrane. Spot densitometry was used to determine the relative amount of bound HA per CD44. The prestained markers indicate the relative molecular mass on the right. Results are representative of one experiment repeated four times.
and/or may play a role in retaining the macrophage in the inflamed tissue, where HA is a major component of the extracellular matrix.

TNF-α, IL-1β, LPS, or IFN-γ induced CD44-mediated HA binding in a significant population of CD14+/H11001 PBMC (on average, between 50–70%), but not in all CD14+ cells. Likewise, treatment with 50 mM NaClO₃ did not inhibit the induced HA binding in all cells (50 mM NaClO₃ abolished HA binding in ~40% of the binding population). The reasons for this are presently not understood; however, it is quite possible that other factors in addition to sulfation contribute to the induced HA binding in monocytes by inflammatory agents. For example, CD44 isoform expression, heparan sulfate addition, and decreased sialylation all can occur upon the activation of monocytes by inflammatory agents (16, 19, 29) and may contribute to the regulation of HA binding, as indicated in Ref. 19. It is also possible that a threshold of sulfation is required to achieve HA binding and perhaps some CD14+ cells do not reach this threshold. NaClO₃ would then have to suppress sulfation to levels below the threshold value to prevent HA binding. In the presence of complete medium, treatment of cells with 50 mM NaClO₃ reduced the incorporation of [³⁵S]sulfate by ~70%, but did not abolish it (Fig. 5). A threshold for binding may also explain why we observe only two populations of CD14+ cells, those that bind HA and those that do not. This is consistent over the time course of the stimulation where we observe an increase in the number of FL-HA binding cells, not an increase in FL-HA binding per cell (data not shown). Even with the titration of NaClO₃, we do not see a significant intermediate HA binding population, just a decrease in the HA binding population and a corresponding increase in the nonbinding population. However, additional experiments are required before we can establish whether there is a threshold of sulfation required for HA binding.

Titration with increasing concentrations of NaClO₃ decreased cellular FL-HA binding, and this was paralleled by a decrease in CD44 sulfation. This demonstrates a correlation between induced CD44 sulfation and HA binding. This conclusion relies on the fact that CD44 sulfation. This demonstrates a correlation between induced CD44 sulfation and HA binding. This was paralleled by a decrease in the HA binding population and a corresponding increase in the nonbinding population. However, additional experiments are required before we can establish whether there is a threshold of sulfation required for HA binding.

HA binding induced by the activation of PBMC with human serum and TNF-α has been shown to involve protein synthesis and possible glycosylation changes, and can be inhibited by IL-4 (39). In vitro culture of PBMC in human serum under adherent conditions induces expression of alternatively spliced forms of CD44 (16), including the CD44 isoform containing variant exon 3 (V3) which is modified by heparan sulfate (29). In this study we have identified that the major 85-kDa form of CD44, CD44H, is sulfated (Fig. 3, A and B). It is possible that CD44 isoform expression or the addition of chondroitin sulfate or heparan sulfate contributes to the sulfation of the higher molecular mass forms of CD44, because higher molecular mass species between 85 and 175 kDa were observed. However, the sulfation of the 85-kDa form of CD44 implies that there are other types of sulfation present on CD44. Clearly, it will be important to establish the precise nature and location of the sulfated moiety. Sulfation has also been shown to play a role in regulating CD44-mediated cell adhesion in fibroblasts, keratinocytes, lung epithelial-derived cancer cells, and colon carcinoma cells (9, 40–42). In these examples, the sulfation was thought to reside primarily on chondroitin sulfate, heparan sulfate, or keratan sulfate attached to CD44. Thus, there is increasing evidence to support a role for sulfation and sulfated glycans in the regulation of HA binding by CD44. In this work, we demonstrate that sulfation contributes to the regulation of HA binding by CD44 in CD14+ PBMC in response to various inflammatory agents.

By far Western blotting, we were able to establish that reduction of CD44H sulfation by NaClO₃ treatment directly affected its ability to bind HA. CD44 isolated from NaClO₃-treated cells bound ~3-fold less HA than sulfated CD44H. CD44 isolated from unstimulated cells did not bind to HA, thereby mimicking the situation on unstimulated CD14+ cells. However, if more CD44 was loaded on the gel, then HA binding was observed under all conditions, which does not reflect HA binding at the cellular level observed by flow cytometry. We conclude that sulfation of CD44 contributes to FL-HA binding in CD14+ PBMC induced by certain inflammatory cytokines, but we do not exclude the possibility that other factors can also contribute to the induction of FL-HA binding. Indeed, HA binding to cell-surface CD44 is described as a complex interplay of multivalent binding events (43) which can be affected by the quantity, density, and activation state of CD44 on the cell. We speculate that sulfation acts in two ways: specifically enhance the affinity of CD44 for HA and to increase the avidity of CD44 on the cell surface, possibly by facilitating CD44 aggregation. A v10-containing CD44 isoform can bind to chondroitin sulfate-modified CD44 (44), suggesting that chondroitin sulfate may facilitate CD44-CD44 interactions.

In summary, TNF-α and IFN-γ stimulation of CD14+ PBMC increased the sulfation of CD44 and induced HA binding. Sulfation of CD44H directly affected HA binding. Reduction of sulfation by NaClO₃ significantly inhibited HA binding in a population of CD14+ PBMC stimulated by TNF-α, IFN-γ, LPS, or IL-1β, indicating that sulfation is one mechanism to regulate HA binding. In contrast, the induction of HA binding by PHA-stimulated T cells was not affected by NaClO₃, suggesting that T cells regulate HA binding by sulfate-independent mechanisms. This work establishes that inflammatory cytokines TNF-α and IFN-γ induce the sulfation of CD44H in CD14+ PBMC and that this promotes CD44-mediated HA binding.

Acknowledgments

We thank G. Dougherty, S. Hemmerich, J. Lesley, and R. Hyman for their generous donation of anti-CD44 mAbs. We sincerely thank the numerous volunteers who donated blood and without whom this study could not have been done.

References


