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J Immunol 2002; 169:4322-4331; doi: 10.4049/jimmunol.169.8.4322
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Synthesis and Surface Expression of Hyaluronan by Dendritic Cells and Its Potential Role in Antigen Presentation

Mark E. Mummert, Diana Mummert, Dale Edelbaum, Francis Hui, Hiroyuki Matsue, and Akira Takashima

Hyaluronan (HA) is a large glycosaminoglycan consisting of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine. HA is known to act as a filling material of extracellular matrices and as an adhesive substrate for cellular migration. Here we report that dendritic cells (DC) express mRNAs for HA synthases and hyaluronidases, actively synthesize HA, and display HA on their surfaces. Interestingly, HA expression levels on DC were not significantly altered by their maturation states. With respect to physiological function, three specific HA inhibitors, i.e., bovine proteoglycan, a 12-mer HA-binding peptide (GAI WQENALTVR) termed Pep-1, and an oligomeric Pep-1 formulation, all interfered with DC-induced activation of CD4+ T cells isolated from DO11.10 TCR transgenic mice. For example, Pep-1 oligomer efficiently inhibited DC-dependent cluster formation, H-2 and IFN-γ production, and proliferation by DO11.10 T cells in vitro without affecting the viabilities of DC or T cells, DC function to uptake exogenous proteins, or DC-T cell conjugate formation at earlier time points. These observations suggest a paracrine mechanism by which DC-associated HA facilitates some of the late changes in T cell activation. Although T cells constitutively expressed mRNAs for HA synthases and hyaluronidases, their surface HA expression became detectable only after activation. Oligomeric Pep-1 and bovine proteoglycan both inhibited mitogen-triggered T cell activation in the absence of DC, suggesting an autocrine mechanism by which HA expressed by T cells assists their own activation processes. Finally, adoptively transferred DO11.10 T cells showed progressive mitosis when stimulated with Ag-pulsed DC in living animals, and this clonal expansion was inhibited significantly by administration of Pep-1 oligomer. Our findings may introduce a new concept that relatively simple carbohydrate moieties expressed on DC and perhaps T cells play an important immunomodulatory role during Ag presentation. The Journal of Immunology, 2002, 169: 4322–4331.

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Received for publication April 18, 2002. Accepted for publication August 7, 2002.

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This work was supported by National Institutes of Health Grants RO1AI46755, RO1AR35068, RO1AR43777, RO1AI44232, and RO3AR47402.

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Abbreviations used in this paper: HA, hyaluronan; BM, bone marrow; bPG, bovine proteoglycan; DC, dendritic cell; DNPβ, dinitrofluorobenzene; FN, fibronectin; GlcA, glucuronic acid; GlcNAc, N-acetylgalactosamine; HAS, HA synthase; Hyal, hyaluronidase; LC, Langerhans cell; LYVE-1, lymphatic vessel endothelial HA receptor-1; PEG, polyethylene glycol; PI, propidium iodide; RP, random peptide.

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0022-1767/02/502.00
surface HA receptors (receptor for HA-mediated motility and LYVE-1) has further supported this concept (10, 11, 20). More recently, degradation products of HA ranging from 1.5 kDa (hexa- saccharides) to 500 kDa have been shown to trigger the secretion of various cytokines and chemokines by macrophages (21–24). Thus, HA may potentially regulate diverse cellular functions. On the other hand, due to the unavailability of specific inhibitors, the physiological functions of HA have been mostly deduced from the biological changes caused by HA receptor antagonists, such as anti-CD44 mAb and CD44-Fc fusion proteins. This approach, however, may not be legitimate, especially from the viewpoint that CD44 also binds other molecules, including collagens, fibronectin, chondroitin sulfates, heparin, heparan sulfate, and serylglucins (25–28). As an initial step toward studying HA function more directly, we have recently developed a 12-mer peptide inhibitor of HA using the phage display technique; this peptide, termed Pep-1, binds to HA in soluble, immobilized, and cell surface-associated forms and inhibits HA-mediated cell adhesion and migration (29). Taking advantage of this unique inhibitor, we now test the hypothesis that HA may regulate Ag presentation processes.

Materials and Methods

Animals and cells

Female BALB/c mice and DO11.10 transgenic mice (6–10 wk old) were used in this study. The phenotypic and functional features of X52 and X5106 DC lines were described previously (30, 31). Splenic DC and bone marrow (BM)-derived DC were prepared from BALB/c mice as previously described (32, 33). The B1W547 thymoma, B16-F10 melanoma, and KM18 B cell hybridoma lines were purchased from American Type Culture Collection (Manassas, VA). CD4+ T cells were isolated from spleens of DO11.10 transgenic mice (BALB/c background) by positive selection with Dynabeads (Dynal, Lake Success, NY), followed by depletion of Ia+ cells with MACS microbeads (Miltenyi Biotec, Auburn, CA). CD3+ T cells were isolated from spleens of BALB/c mice using a T cell isolation column (R&D Systems, Minneapolis, MN), followed by depletion of Ia+ cells. All animal experiments were approved by the institutional review board at University of Texas Southwestern Medical Center and conducted according to the guidelines from the National Institutes of Health.

Reagents and Abs

OVA peptide 323–339 was synthesized at the Biopolymers Facility, University of Texas Southwestern Medical Center. OVA protein was purchased from Sigma-Aldrich (St. Louis, MO). LPS (Escherichia coli 026: B6) and Con A were obtained from Sigma-Aldrich and Amersham Pharmacia Biotech (Piscataway, NJ), respectively. The mAb KJ1.26, which recognizes the transgenic TCR expressed by DO11.10 T cells, and its isotype-matched control IgG were purchased from Caltag (Burlingame, CA). Other mAbs were purchased from BD PharMingen (San Diego, CA). Other reagents and Abs were purchased from BioWhittaker, Walkersville, MD.

RT-PCR analyses

The following primer sets were used: 5′-CTTCTCAAGGACCTGCG GAC-3′ and 5′-CAGGCTTCTAGATGCTAC-3′ (first reaction for HAS1), 5′-GGAGAAGCTTGACTGACAACAGGAC-3′ and 5′-AGGGAA TTGCTATGACCCCCAGG-3′ (second reaction for HAS1), 5′-TGG AACACCAGGAATGAAGAAG-3′ and 5′-GAGGCCAGCCGCTGTAT TTAGTTCG-3′ (HAS2), 5′-ATGACATGGCGGATGGAAAGAG-3′ and 5′-ATGGGGCCACGCTGTAAGAAGTGTG-3′ (HAS3), 5′-TATCCAC CGGACATCCTACAGT-3′ and 5′-ATACCCCCGTTGTACACACT TG-3′ (HAS4), 5′-CATCCATTCCGAGATGGAGATGG-3′ and 5′-CAGTGGGAGCGCTGTAAGAGTGTG-3′ (HAS5), 5′-TGGCACTGAGTGCACACCGAGG-3′ and 5′-GACCGCGGACTGAGGAAGAAG-3′ (HAS6). PCR products were separated by 2% agarose and visualized with ethidium bromide.

HA biosynthesis in DC

To study HA synthesis, X5106 DC were labeled for 16 h with [3H]Glucosamine (25 μCi/ml). After collecting culture supernatants (secreted fractions), the cells were incubated for 10 min on ice in a hypotonic buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.4, 1 mM l-glutamine, and 1 mM PMSF), pH 7.4 and harvested with a rubber policeman. The cellular fractions were then freeze-thawed twice, homogenized, and centrifuged at 400 × g for 5 min to remove nuclear materials. The supernatants were further centrifuged at 25,000 × g for 60 min to obtain pellets (membrane fraction) and supernatants (cytoplasmic fraction). High m.w. materials were precipitated from each fraction with 1% cetylpyridinium chloride in 0.02 M NaCl in the presence of carrier HA (200 μg/ml) and then digested with papain (1 U/ml; Sigma-Aldrich) for 90 min at 60°C. After 10 min boiling, one-half of each sample was digested with Streptomyces Hyal (80 U/ml; Sigma-Aldrich) for 1 h at 37°C, while the other half was mock-digested. Hyal-resistant glycosaminoglycans were precipitated as described above, and the pellets were dissolved in 4.0 M guanidine hydrochloride, ethanol precipitated, and measured for radioactivity. The amount of newly synthesized HA in each fraction was then calculated by subtracting the Hyal-resistant radioactivity from the total radioactivity.

Surface HA expression

To examine surface HA expression, DC preparations were incubated for 30 min on ice with biotinylated Pep-1 (100 μg/ml), washed extensively, and labeled with PE-conjugated streptavidin. For splenic DC and BM-derived DC preparation, Pep-1 binding was analyzed within CD11c+ populations. To test the specificity of Pep-1 binding, samples were pretreated with Hyal or heparinase III (Sigma) before incubation with biotinylated Pep-1. The carbohydrate specificity of the two enzymes was determined by measuring their abilities to release biotinylated HA polymers that had been immobilized onto the Amine Covalink plates (Nalge Nunc International, Rochester, NY). In some experiments BM-derived DC were tested for Pep-1 binding and CD40 and CD86 expression after 16-h incubation in the presence or the absence of 100 ng/ml LPS. CD3+ T cells isolated from BALB/c mice were examined for Pep-1 binding after 16-h incubation in the presence or the absence of 4 μg/ml Con A.

Preparation of HA inhibitors

Pep-1 (GAHWQFNALTVR) and a control peptide (SATPASAPYPLA), termed random peptide (RP), were synthesized with the amidated GGGG linker sequence at the C terminus as previously described (29). To prepare oligomeric formulations, a 40-fold molar excess of Pep-1 or RP was incubated for 7 days with the tetravalent polyethylene glycol (PEG) derivative, bis[polyethylene bis(midaiazolyl carbonyl)] at 37°C in DMSO. Triethylamine (0.5%) was added to the reaction to ensure maximum nucleophilicity of the peptide α-amine. Reactions were terminated by dialysis against dH2O or PBS, and the density of substitution was estimated by the bicinechinonic acid assay (to determine peptide content) and dry weight analysis. Bovine proteoglycan (bPG) from nasal cartilage (ICN Bio- medicals, Costa Mesa, CA) was dissolved in PBS. None of the HA inhibitors contained detectable amounts of endotoxin.

Ag presentation assays

Immunoregulatory activities of HA inhibitors were tested using an in vitro Ag presentation system in which CD4+ T cells (2.5 × 105 cells/ml; >95% CD4+ cells) purified from DO11.10 TCR transgenic mice and gamma-irradiated splenic DC or BM-derived DC preparations (2.5 × 105 cells/ml, >70% CD11c− cells) isolated from BALB/c mice were cocultured in the presence or the absence of 2 μg/ml OVA323–339 peptide. T cell proliferation was measured by 3HThymidine uptake on day 3 as previously described (30, 31, 33). Viabilities of DC and T cells were examined by propidium iodide (PI) uptake by the CD11c+ and KJ1.26+ populations, respectively. In some experiments DO11.10 T cells were labeled with CFSE (Molecular Probes, Eugene, OR) and examined for their fluorescence profiles on day 2. Cytokine production was examined by ELISA on day 3 (33). To study DC-T cell conjugate formation, BM-derived DC labeled with a red fluorescent dye KJ1.26 (Sigma-Aldrich) and CFSE-labeled DO11.10 T cells were incubated for 30 min in the presence or the absence of OVA peptide, followed by FACS analyses in which DC-T cell conjugates were detected as double-positive events (34). In vivo effects of HA inhibitors were examined in adoptive transfer experiments. Briefly, BALB/c mice received i.p. injections of CFSE-labeled DO11.10 T cells (105 cells/mouse), OVA peptide-pulsed BM-DC (3 × 105 cells/mouse), and oligomeric Pep-1 or RP (20 μg/mouse), and the cells recovered from the peritoneal cavity on day 2 were examined for CFSE profiles.

Other functional assays

To study potential roles of T cell-associated HA, CD3+ T cells isolated from BALB/c mice were stimulated by Con A or the combination of anti-
CD3 mAb and anti-CD28 mAb as described previously (33). The ability of DC to uptake exogenous proteins was examined using FITC-conjugated BSA (Sigma-Aldrich). Briefly, BM-derived DC were incubated for 10 min with FITC-BSA and then examined for FITC profiles within the CD11c+ populations. Cell adhesion was examined as described previously (29). Con A-activated splenic T cells and B16-F10 melanoma cells were labeled with [35S]methionine/cysteine (ICN Biomedicals) and incubated for 30 min at room temperature on Nunc plates coated with HA or for 60 min at 37°C on tissue culture plates coated with bovine fibronecin (FN; Sigma-Aldrich). After removal of nonadherent cells, the adherent cells were solubilized in 1% SDS and counted for radioactivity. The percentages of adherent cells were calculated by dividing the recovered counts per minute by the total counts per minute added to each well.

Statistical analyses

Comparisons between two groups were performed with Student’s two-tailed t test, and more than two groups were compared by ANOVA. Each experiment was repeated at least twice to assess reproducibility.

Results

HA synthesis in DC

We first examined whether DC express mRNAs for HA synthases (HAS1 through HAS3) and Hyal (Hyal-1 through Hyal-3), the enzymes known to be involved in HA synthesis and degradation (13, 35). All tested HAS and Hyal mRNAs were detected in mouse BM-derived DC cultures (Fig. 1A). Stable DC lines (XS series) established from mouse epidermis (30, 31) showed similar profiles, except that HAS1 or HAS2 mRNA was undetectable in the mature DC line XS106 or the immature line XS52, respectively. Underlying mechanisms or biological consequences of this finding (differential expression of HAS1 and HAS2 mRNAs) remain unknown at present. Nevertheless, our observations suggest the potential of DC to synthesize and catabolize HA polymers.

When cultured in the presence of [3H]glucosamine, XS106 DC showed marked incorporation of radioactivities, and significant portions (15–20%) of the total cell-associated radioactivities were found to be susceptible to HA-mediated digestion (data not shown). To study the subcellular distributions of newly synthesized HA, we measured Hyal-sensitive radioactivities in the membrane fractions, cytoplasmic fractions, and culture supernatants. Consistent with the current idea that HA polymer is synthesized at the inner face of the plasma membrane and then extruded to the cell surface, most of the newly synthesized HA molecules were recovered from the membrane fractions (Fig. 1B). These results suggest that DC actively synthesize HA and perhaps express the resulting HA polymer at the outer surface.

Detection of surface HA expression on DC and T cells

Based on our previous observation that Pep-1 binds to HA molecules in soluble, immobilized, and membrane-associated forms (29), we thought that Pep-1 might serve as a unique tool to study HA expression on DC. Pep-1 in a biotinylated form showed marked binding to the surfaces of XS106 DC (Fig. 2A). By contrast, a 12-mer control peptide termed RP showed no detectable binding. Moreover, surface binding of Pep-1 was abolished almost completely by Hyal pretreatment of XS106 DC, indicating that HA moieties are the major target of Pep-1. Biotinylated Pep-1, but not RP, also bound significantly to other tested DC preparations, including XS52 DC, splenic DC, and BM-deriv DC (Fig. 2B). Once again, Hyal pretreatment of each DC preparation markedly reduced Pep-1 binding (data not shown). To determine whether HA expression on DC might be regulated by their maturation states, we incubated BM-derived DC in the presence or the absence of LPS. As shown in Fig. 2C, HA expression levels remained relatively unchanged after LPS treatment, although surface expression of CD40 and CD86 was markedly elevated in LPS-stimulated DC. These observations indicate that DC in both immature and mature states express detectable amounts of HA on their surfaces.

CD3+ T cells isolated from BALB/c mice expressed mRNAs for HAS1, HAS3, and Hyal-3, and Hyal-2 mRNA became detectable after Con A activation, suggesting that T cells might express HA under certain conditions (Fig. 1A). In fact, Con A-activated T cells showed significant surface binding of biotinylated Pep-1, whereas only marginal, if any, binding was observed in the absence of Con A stimulation (Fig. 2D). Although the extent of binding to activated T cells was relatively modest, Hyal pretreatment markedly diminished Pep-1 binding, indicating the specificity. Thus, we concluded that T cells express detectable amounts of HA on their surfaces after activation.

Because CD44 is generally considered to function as a primary HA receptor, we next studied the distributions of HA and CD44 on the surfaces of XS106 DC. Pep-1 (green) bound diffusely and uniformly to the cell bodies (Fig. 2E, left). This Pep-1 binding pattern showed only occasional overlap with the binding pattern of anti-CD44 mAb (red). Once again, no detectable binding was observed for the RP control (Fig. 2E, right). These observations imply that some of the HA molecules on DC are not colocalized with CD44.

Molecular targets of Pep-1 on DC

Heparan sulfate proteoglycans are major proteoglycans found in the extracellular matrix (especially in basement membrane) and at the cell surface. Although heparan sulfate polysaccharide side chains generally consist of repeated units of GlcA and N-deacetylated/N-sulfated Glc, a unique subunit composition of GlcA and N-unsubstituted Glc has also been identified (36, 37). Thus, we examined whether Pep-1 might also recognize heparan sulfates through the above HA-like epitope. Pretreatment of XS106 DC with heparinase III, which is known to selectively degrade heparan sulfates (38), reduced the extent of Pep-1 binding, albeit modestly even at the highest concentration (330 U/ml; Fig. 3A). Importantly,
Hyal pretreatment at the lowest concentration (37 U/ml) caused more prominent effects, suggesting that HA moieties are the major target for Pep-1 on DC. On the other hand, our finding that heparinase III showed no detectable enzymatic activity to digest HA substrates (Fig. 3B) strongly suggests that heparan sulfates serve as a second carbohydrate target for Pep-1 binding on DC.

Impact of HA inhibitors on DC-dependent, Ag-specific T cell proliferation

A majority of CD4\(^+\) T cells purified from DO11.10 TCR transgenic mice showed a characteristic phenotype of naive T cells: CD25\(^+\) (>95%), CD69\(^+\) (>97%), CD62L\(^\text{high}\) (>84%), CD44\(^\text{low}\) (>91%), and CD45RB\(^\text{high}\) (>98%) (data not shown). These CD4\(^+\) T cells, which express the transgenic TCR- and -chains specific for OVA peptide 323–339 (39), allowed us to study the potential contribution of HA to the primary T cell activation process. DO11.10 T cells showed robust [\(^3\)H]thymidine uptake when stimulated by splenic DC in the presence of OVA 323–339 peptide (Fig. 4A). By contrast, no significant T cell proliferation was detected in the absence of DC or OVA peptide, indicating DC dependency and Ag specificity. Purified bPG, which has been used as a specific probe and a standard inhibitor of HA (40), completely abolished DC-induced proliferation of DO11.10 T cell proliferation, whereas the same reagent showed no inhibitory potential after heat inactivation. It should be noted that HA, but not heparan sulfates, efficiently competed with bPG in the binding to HA-coated plates, indicating that bPG does not cross-react with heparan sulfate (Fig. 4B). Moreover, DC-dependent, Ag-specific proliferation of DO11.10 T cells was also inhibited by Pep-1, but not by RP control (Fig. 4C). Thus, the two tested HA inhibitors with totally different chemical structures exhibited the same pharmacological activity (i.e., to block DC-induced T cell activation), with the implication that HA molecules associated with DC and/or T cells may play an indispensable role in Ag presentation.

Improvement of pharmacological activity of Pep-1 by multimerization

While testing the impact on DC-dependent T cell activation, we noticed that the extent of inhibition achieved with Pep-1 was rather modest (<40%) even at a relatively high concentration of 300 \(\mu\)M (Fig. 4C). To improve its biological avidity, we conjugated Pep-1 molecules to a tetravalent PEG derivative. The resulting preparations showed an estimated molecular ratio of Pep-1/PEG of 2.3–2.9 (data not shown). We first compared this oligomeric Pep-1 formulation with the original monomeric Pep-1 for their pharmacological activities using two standard assays (29). First, the PEG-conjugated Pep-1 inhibited the adhesion of CD44-expressing B16-F10 melanoma cells to HA-coated plates almost completely.
tively low affinity (K_d = 1.7 μM) and in a nonsaturated fashion (29). These features most likely reflect the chemical property

Effects of PEG-Pep-1 on DC-induced, Ag-specific T cell proliferation

We next added PEG-Pep-1 to the cocultures of splenic DC and DO11.10 T cells to test its impact on Ag presentation. As shown in Fig. 5C, PEG-Pep-1 inhibited T cell proliferation almost completely (80–100%) at 50–150 μM, whereas the original Pep-1 caused only modest (30%) inhibition at 300 μM. Likewise, PEG-Pep-1, but not PEG-RP, efficiently inhibited DO11.10 T cell proliferation triggered by BM-derived DC (a second DC preparation) at a relatively low concentration of 60 μM (Fig. 5D). Moreover, tetravalent PEG alone showed no inhibitory effect on DC-induced DO11.10 T cell proliferation, formally excluding the possibility that free arms of PEG molecules might affect DC-T cell interaction nonspecifically (Fig. 5E). Taken together, our observations with the multimeric Pep-1 formulation further support our conclusion that HA is functionally involved in DC-induced T cell activation processes.

In an attempt to determine the relevant cellular target(s) of PEG-Pep-1, we pretreated BM-derived DC or DO11.10 T cells with PEG-Pep-1 or PEG-RP, washed the cells extensively, and then cocultured them in the presence of OVA peptide. Nontreated DO11.10 T cells showed similar levels of [3H]thymidine uptake when stimulated with DC pretreated with PEG-Pep-1 (141,000 ± 6,400 cpm) vs PEG-RP (114,000 ± 18,000 cpm). Conversely, nontreated DC induced similar degrees of proliferation by DO11.10 T cells pretreated with PEG-Pep-1 (140,000 ± 25,000 cpm) vs PEG-RP (142,000 ± 1,700 cpm). Our failure to block the function of HA by pretreatment is not totally unexpected, because we have observed previously that Pep-1 binds to HA with a relatively low affinity (K_d = 1.7 μM) and in a nonsaturated fashion (29). These features most likely reflect the chemical property

FIGURE 3. Pep-1 binding to heparin sulfate moieties on DC. A, XS106 cells were pretreated with either Hyal (top) or heparinase III (bottom) at the indicated concentrations. The samples were then examined for binding of biotinylated Pep-1 (filled histograms). Open histograms, Baseline staining indicated concentrations. The samples were then examined using streptavidin-conjugated alkaline phosphatase. Data shown are the mean ± SD (n = 3) percent digestion.

HA, which is a long, homogenous polymer consisting of repeating disaccharide, thus providing no discrete or isolated binding sites for Pep-1. Alternatively, PEG-Pep-1 pretreatment may have caused no inhibition simply due to rapid turnover of surface HA molecules. Nevertheless, we interpreted our observations to imply that Pep-1, even in a multimeric form, must be present continuously to block the function of HA.

An obvious question concerned the identity of the putative counter-receptor for HA. CD44 is generally thought to serve as a primary surface receptor of HA, and CD44 expression is detectable on the majority of DO11.10 T cells. Anti-CD44 mAb (KM811) significantly inhibited DC-induced activation of DO11.10 T cells in a dose-dependent manner, whereas an isotype-matched control IgG caused no inhibition (Fig. 6A, left panel). On the other hand, the extent of inhibition achieved with anti-CD44 mAb was relatively modest (<40%) even at the highest tested concentration of 70 μg/ml. By contrast, the same anti-CD44 mAb completely blocked the adhesion of activated T cells to HA-coated plates at a much lower concentration of 7 μg/ml (Fig. 6A, right panel). In this regard we have observed previously that adhesion of activated T cells to HA-coated plates is blocked almost completely by the monomeric Pep-1 (29). To assess the target specificity of the above inhibitors, we crossed two different adhesive substrates, HA and FN. As shown in Fig. 6B, adhesion of B16-F10 melanoma cells to HA-coated plates, but not FN-coated plates, was inhibited by PEG-Pep-1. By contrast, PEG-RP or PEG alone showed no inhibitory activity. Anti-CD44 mAb inhibited the adhesion of activated T cells to HA-coated plates without affecting their binding to FN-coated plates (Fig. 6C). Having confirmed the target specificity of anti-CD44 mAb and PEG-Pep-1, we interpret our data (shown in Fig. 6A) to suggest that CD44 is fully responsible for the adhesive interaction of activated T cells with HA substrates, whereas CD44 is one, but not the only, relevant receptor mediating HA-dependent

FIGURE 4. Impact of two currently available HA inhibitors on DC-dependent T cell proliferation. A, DO11.10 T cells were cultured with splenic DC and/or OVA peptide in the presence of intact bPG (250 μg/ml), heat-denatured bPG, or PBS alone. Data shown are the mean ± SD (n = 3) [3H]thymidine uptake. B, bPG (50 μg/ml) was incubated for 16 h with the indicated doses of HA (●) or heparan sulfate (○) and then added to HA-coated plates. The data shown are the mean ± SD (n = 3) percent inhibition of bPG binding as measured with anti-bPG mAb. C, DO11.10 T cells were cultured with splenic DC at the indicated numbers and with OVA peptide in the presence of Pep-1 (300 μM), RP (○), or PBS alone (□). Data shown are the mean ± SD (n = 3) [3H]thymidine uptake. All data in this figure are representative of three independent experiments showing similar results. Statistically significant differences are indicated by asterisks (*, p < 0.05; **, p < 0.01).
T cell activation. Alternatively, T cells may employ different CD44 isoforms to recognize HA moieties naturally expressed on DC vs those artificially immobilized on plates. Nevertheless, our data indicate that DC-induced T cell activation can be inhibited almost completely by interfering with HA function by specific inhibitors (e.g., bPG and multimeric Pep-1) or partially by blocking CD44 function with anti-CD44 mAb.

**Differential influences of PEG-Pep-1 on different aspects of DC-induced T cell activation**

We first examined whether PEG-Pep-1 might inhibit T cell proliferation by simply killing DC and/or T cells. To test this possibility, we added PEG-Pep-1, PEG-RP, or PEG alone to the cocultures of BM-derived DC and DO11.10 T cells in the presence of OVA\textsubscript{322-339} peptide or native OVA protein. None of the reagents significantly affected the viability of either DC or T cells in this second system (Fig. 7A, right panels). Likewise, although PEG-Pep-1 inhibited DO11.10 T cell proliferation triggered by OVA protein (data not shown), none of the reagents caused detectable death of DC or T cells in this second system (Fig. 7A, right panels). To visually examine the impact of PEG-Pep-1 on T cell division, we labeled DO11.10 T cells with a fluorescence dye, CFSE. When stimulated by BM-derived DC in the presence of OVA\textsubscript{322-339} peptide, CFSE-labeled DO11.10 T cells showed marked reduction in fluorescence intensity, reflecting their progressive mitosis (Fig. 7B). By contrast, DC failed to trigger T cell mitosis in the absence of OVA peptide, indicating Ag specificity. Consistent with our observations in \textsuperscript{3}Hthyimidine uptake assays, PEG-Pep-1, but not PEG-RP, completely prevented DC-induced, Ag-specific clonal expansion of DO11.10 T cells. It should be noted that we examined CFSE profiles within the PI-negative populations to further address the cytotoxicity issue.
Does PEG-Pep-1 affect other changes that accompany T cell activation? As shown in Fig. 7C, DO11.10 T cells secreted significant amounts of IL-2 and IFN-γ when cocultured with BM-derived DC in the presence of OVA peptide, whereas neither cytokine was detected in the absence of DC or Ag. Importantly, production of IL-2 and IFN-γ was inhibited efficiently by PEG-Pep-1, but not by PEG-RP. DO11.10 T cells exhibited characteristic cluster formation only when cocultured with DC in the presence of OVA peptide (Fig. 7D). This morphological change was also inhibited by PEG-Pep-1, but not by PEG-RP. Thus, PEG-Pep-1 is capable of inhibiting multiple events that accompany DC-induced T cell activation.

The experiments described above examined relatively late events during Ag presentation, i.e., T cell cluster formation at 16 h, cytokine production at 24 h, T cell mitosis at 48 h, and [3H]thymidine uptake at 72 h. We next sought to examine the potential influence of PEG-Pep-1 on earlier events. As shown in Fig. 8A, PEG-Pep-1, PEG-RP, or PEG alone did not inhibit FITC-BSA uptake by BM-derived DC. To test the impact on early formation of DC-T cell aggregates, we labeled BM-derived DC with a red fluorescence dye (PKH-26) and DO11.10 T cells with a green fluorescence dye CFSE. Significant numbers of DC-T cell conjugates were detected as double-positive events when the two populations were incubated for 30 min in the presence of OVA peptide (Fig. 8B). Importantly, PEG-Pep-1 even at relatively high concentrations (up to 75 μM in the experiment shown in Fig. 8B and 150 μM in a different experiment) failed to inhibit this early event. Thus, PEG-Pep-1 efficiently inhibits relatively late events that accompany DC-induced T cell activation, without affecting the viability of DC or T cells, the ability of DC to uptake exogenous...
proteins, or Ag-specific DC-T cell conjugate formation at earlier time points.

**Potential function of T cell-associated HA**

It has been an established concept that immunologically naive T cells are activated most efficiently by DC (41, 42). From this viewpoint, our findings illustrate functional roles of HA in Ag presentation under physiological conditions. On the other hand, it remains unclear whether Pep-1 primarily acts on HA moieties constitutively displayed on DC or whether it may also inhibit the function of HA being expressed by T cells upon activation. To test the latter possibility, we employed nonphysiological experimental systems in which T cells were stimulated by mitogens in the absence of DC or Ag. When stimulated by both anti-CD3 mAb and anti-CD28 mAb, CD3⁺ T cells showed robust proliferation (Fig. 9A). T cell responses to this mitogenic stimulation were almost completely inhibited by PEG-Pep-1, but not by PEG-RP. Likewise, Con A-triggered proliferation of CD3⁺ T cells was almost completely inhibited by bPG (Fig. 9B). These observations imply a second mechanism by which HA moieties expressed by activated T cells facilitate their own proliferation in an autocrine manner.

**In vivo ability of PEG-Pep-1 to inhibit DC-induced, Ag-specific T cell expansion**

With respect to the in vivo impact of Pep-1, we have successfully inhibited allergic contact hypersensitivity responses to dinitrofluorobenzene (DNFB) by injecting the original Pep-1 formulation before or after sensitization (29). Although these results may first appear to support our working model that HA facilitates DC-induced T cell activation, it should be stated that administered Pep-1 also blocked hapten-triggered migration of epidermal DC, known as Langerhans cells (LC), from the epidermis at the sensitization phase and skin-directed homing of effector T cells at the elicitation phase. Thus, our previous observations may mainly reflect the ability of Pep-1 to block HA-dependent, two-way trafficking of leukocytes from and to the skin, but not its potential impact on the Ag presentation processes in which DNFB-pulsed LC trigger clonal expansion of DNFB-reactive T cells. To study the direct impact of PEG-Pep-1 on DC-induced T cell activation, we sought to develop a new in vivo model that would not require DC or T cell homing. When OVA peptide-pulsed DC and CFSE-labeled DO11.10 T cells were injected i.p., a significant reduction in the fluorescence intensity was detected in cells recovered from the peritoneal cavity 48 h later (Fig. 10A). No significant change was observed in the CFSE profiles of DO11.10 T cells when injected with PBS-pulsed DC (data not shown). Importantly, a substantial reduction in T cell mitosis was detected following i.p. administration of PEG-Pep-1, but not PEG-RP. In two independent experiments the panel receiving PEG-Pep-1 injection showed statistically significant impairment in the extent of clonal expansion of adoptively transferred T cells compared with the control panel receiving PBS alone and the second control panel receiving PEG-RP (Fig. 10B). Our model, which may be seen as in vivo cocultures of Ag-pulsed DC and T cells in the peritoneal cavity, does not represent physiological immune responses or diseases in living animals. However, we believe that our observation in this model at least reflects direct influences of PEG-Pep-1 on DC-dependent, Ag-specific clonal expansion of T cells in living animals.

**Discussion**

We have demonstrated in this study that DC constitutively express mRNAs for various enzymes involved in HA synthesis and degradation, actively synthesize HA, and uniformly express HA on their surfaces. Moreover, we have detected HA expression on activated T cells, albeit at modest levels. These findings, which imply an immunoregulatory role for HA, challenge the classic view of HA as an extracellular matrix component or a filling material being produced by fibroblasts, chondrocytes, epithelial cells, and endothelial cells. In fact, DC-induced, Ag-specific activation of T cells was blocked significantly by all tested HA inhibitors (i.e., monomeric and multimeric Pep-1 formulations and bPG). PEG-Pep-1, for example, efficiently inhibited DC-induced, Ag-specific T cell clustering, cytokine production, [³H]thymidine uptake, and mitosis. Complementary observations have been reported by Gallandrini et al. (43), who found that exogenous HA polymers in an

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**FIGURE 9.** HA-mediated autocrine mechanism promoting mitogen-triggered T cell activation. A, CD3⁺ T cells were stimulated with anti-CD3 mAb and/or CD28 mAb in the presence of PEG-Pep-1 (60 μM), PEG-RP, or PBS alone. B, CD3⁺ T cells were stimulated with Con A in the presence of bPG (250 μg/ml) or PBS alone. Data shown are the mean ± SD (n = 3) [³H]thymidine uptake. Statistically significant differences are indicated by asterisks (**, p < 0.01).

**FIGURE 10.** In vivo impact of PEG-Pep-1 on DC-dependent clonal expansion of T cells. A and B, BALB/c mice received i.p. injections of CFSE-labeled DO11.10 T cells, OVA peptide-pulsed BM-derived DC, and the indicated reagent. The cells recovered from the peritoneal cavity 2 days later were examined for CFSE fluorescence profiles within the PI-negative populations and for percentage of dividing cells (A). Results obtained from three animals per each panel were examined for the mean ± SEM percentage of dividing cells and for statistical difference (*, p < 0.05; B). All data in this figure are representative of two independent experiments showing similar results.
immobilized form augment T cell proliferation and IL-2 production triggered by anti-CD3 mAb or phorbol ester. These findings made in opposing directions (i.e., loss of function and gain of function) introduce a new concept that HA polymers being synthesized and expressed by DC and T cells facilitate Ag presentation processes.

The relevant cellular target(s) or molecular mechanism(s) for Pep-1-induced inhibition remain unclear at this time. PEG-Pep-1 and bPG both inhibited mitogen-triggered activation of T cells in the absence of DC, revealing a putative pathway in which HA molecules expressed by T cells may regulate their own activation in an autocrine manner. These observations, however, should not be interpreted to indicate that T cell-associated HA, but not DC-associated HA, plays a regulatory role in Ag presentation under physiological conditions. Perhaps the most unexpected observation was the failure of PEG-Pep-1 to inhibit DC-T cell conjugate formation at an early time point, despite the fact that the same reagent almost completely inhibited several later events in Ag presentation. Activation of naïve T cells has been reported to increase not only the extent of immunoreactivity to anti-CD44 mAb, but also the HA binding potential of CD44 molecules (44, 45). Thus, it is tempting to speculate that DC-associated HA may not be able to interact efficiently with CD44 expressed by naïve T cells in the absence of T cell activation signals. Conversely, DC maturation is accompanied by increased immunoreactivity to anti-CD44 mAb and elevated surface expression of selected CD44 variant isoforms (46, 47). An alternative explanation is, therefore, that DC may need additional maturation signals from T cells for their CD44-dependent interaction with T cell-associated HA. Although HAS2-knockout embryos die during midgestation due to severe cardiac and vascular abnormalities (48), HAS1/HAS3 double-knockout mice show no apparent developmental defect (48). Experiments are underway in our laboratories to study immunological functions of DC and T cells isolated from the HAS1/HAS3 knockout animals.

Our detection of HA molecules that did not colocalize with CD44 on DC is not totally surprising, because even a CD44-negative tumor cell line has been reported to elevate surface HA expression levels upon transfection with HAS2 or HAS3 cDNA (49). Perhaps HAS molecules at the inner face of the plasma membrane not only catalyze HA synthesis, but also serve as a membrane-anchoring mechanism for newly synthesized HA polymers (13). Our observation that anti-CD44 mAb caused only partial inhibition of DC-induced T cell activation is also in complete agreement with the current idea that HA binds many other surface receptors and extracellular matrix components (see the introduction). Thus, it seems reasonable to state that CD44 is one of multiple HA receptors involved in HA-mediated immunoregulation.

HA molecules, which are present along the migration path (e.g., intercellular space in epidermis and dermal matrix) for epidermal LC, have been considered to promote CD44-dependent LC homing. This concept was originally derived from the observations that 1) CD44 expression is detectable on epidermal LC in normal skin (50, 51); 2) LC elevate surface expression of selected CD44 isoforms upon hapten painting (47); and 3) antagonistic anti-CD44 mAb and Pep-1 both inhibit LC migration from the epidermis and the subsequent contact hypersensitivity responses (29, 47, 52). More recent reports, however, do not necessarily support the above view. Schmits et al. (53) reported that CD44 knockout mice generated by conventional homologous recombination show normal contact hypersensitivity responses to DNFB, indicating that CD44 expression by LC is not absolutely required for their hapten-triggered migration. By contrast, Kaya et al. (54) reported severely impaired contact hypersensitivity responses to DNFB in transgenic mice expressing an antisense CD44 cDNA under the keratin-5 promoter. Interestingly, the same transgenic animals also exhibited marked reduction in the amount of HA present in the epidermal intercellular space and massive accumulation of HA in the superficial dermis, with the implication that CD44 expressed on keratinocytes plays a critical role in HA turnover in skin (55). Thus, it is conceivable that anti-CD44 mAb may have inhibited LC migration indirectly by altering HA metabolism in skin, instead of directly blocking the function of LC-associated CD44. Our observations in this study suggest an even more extreme possibility, that HA polymers expressed on DC may direct their homing to the afferent lymphatics by binding to various putative receptors, including LYVE-1, a newly identified HA receptor expressed almost exclusively in the lymphatic vessels (10, 11).

Degradation products of HA are known to trigger cytokine and chemokine production by macrophages (21–24). More recently, Termeer et al. (56, 57) have reported the potential of small HA fragments of tetra- and hexa-saccharide to induce DC maturation by a Toll-like receptor 4-dependent mechanism. With respect to the physiological source of HA fragments, these investigators postulated that HA polymers in the extracellular matrices might be digested into oligosaccharides at the sites of inflammation. Our findings now suggest that DC (expressing mRNAs for HA syntheses and Hyal) may release degradation products of HA, thereby regulating their own maturational state in an autocrine manner. Alternatively, T cells (expressing similar mRNA profiles) may elaborate HA fragments during Ag presentation, triggering DC maturation in a paracrine manner. The heparan sulfate moiety detected on DC may also play an immunoregulatory role; in fact, heparan sulfate and its degradation products have been reported to deliver costimulatory signals to T cells and maturation signals to DC (58–60). Obviously, further studies are required to examine the metabolic activities of DC to synthesize and degrade HA and heparan sulfate and to characterize the immunoregulatory properties of DC-derived oligosaccharides. Nevertheless, the present study may introduce a new concept, that Ag presentation processes are regulated by relatively simple carbohydrates being produced by DC.

Acknowledgments

We thank Lesa Ellinger and Pat Adcock for their assistance.

References