Hepatocyte growth factor, Met, and CD44. Amenage a trois in B cells
van der Voort, R.

Citation for published version (APA):
van der Voort, R. (2000). Hepatocyte growth factor, Met, and CD44. Amenage a trois in B cells
Chapter 5

Regulation of cytokine signaling by B cell antigen receptor and CD40 controlled expression of heparan sulfate proteoglycans.

Robbert van der Voort, Robert M.J. Keehnen, Esther A. Beuling, Marcel Spaargaren, and Steven T. Pals

Department of Pathology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

SUMMARY

Recently, biochemical, cell biological, and genetic studies have converged to reveal that integral membrane heparan sulfate proteoglycans (HSPGs) are critical regulators of growth and differentiation of epithelial- and connective tissues. Since a large number of cytokines involved in lymphoid tissue homeostasis or inflammation contain potential heparan sulfate-binding domains, HSPGs presumably also play important roles in the regulation of the immune response. In the present report, we explored the expression, regulation and function of HSPGs on B lymphocytes. We demonstrate that activation of the B cell antigen receptor (BCR) and/or CD40, induces a strong transient expression of HSPGs on human tonsillar B cells. By means of these HSPGs, the activated B cells can bind hepatocyte growth factor (HGF), a cytokine that regulates integrin-mediated B cell adhesion and migration. This interaction with HGF is highly selective since the HSPGs did not bind the chemokine stromal cell-derived factor 1α (SDF-1α), even though the affinities of HGF and SDF-1α for heparin are similar. On the activated B cells, we observed induction of a specific HSPG isoform of CD44 (CD44-HS), but not of other HSPGs such as syndecans or glypican-1. Interestingly, the expression of CD44-HS on B cells strongly promotes HGF-induced signaling, resulting in a heparan sulfate-dependent enhanced phosphorylation of Met, the receptor tyrosine kinase for HGF, as well as downstream signaling molecules including the Grb2 associated binder 1 (Gab1) and Akt/protein kinase B (PKB). Our results demonstrate that the BCR and CD40 control the expression of HSPGs, specifically CD44-HS. These HSPGs act as functional co-receptors which selectively promote cytokine signaling in B cells, suggesting a dynamic role for HSPGs in antigen-specific B cell differentiation.

INTRODUCTION

Proteoglycans are proteins that are covalently linked to sulfated glycosaminoglycan (GAG) chains composed of repeating disaccharide units (1). These molecules, which are widespread throughout mammalian tissues as extracellular matrix (ECM) components and membrane-bound molecules, have been implicated in several important biological processes including cell adhesion and migration, angiogenesis, tissue morphogenesis, and regulation of blood coagulation (1-3). In these processes, proteoglycans are believed to function as scaffold structures, designed to accommodate proteins through non-covalent binding to their GAG chains. In particular heparan sulfate proteoglycans (HSPGs) have been shown to function as versatile protein co-receptors. Their ligand-binding sites reside within discrete sulfated domains formed by complex, cell-specific, chemical modifications.
of the heparan sulfate (HS) disaccharide repeat (4). Binding of proteins, including growth factors/cytokines, to HS chains may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of biological function (5, 6). This functional importance is illustrated by fibroblast growth factor 2 (FGF-2), whose binding to its signal-transducing receptors and consequent biological effects are critically dependent on its interaction with cell-surface HSPGs (7, 8). Recently, a number of cell biological and genetic studies have provided compelling evidence for an in vivo role of cell-surface HSPGs in growth control and morphogenesis in Drosophila, mice and humans (9-14).

Most studies concerning the expression and function of cell-surface HSPGs have focussed on epithelial cells and fibroblasts, but these molecules presumably also play important roles in the immune system. A vast number of cytokines involved in lymphoid tissue homeostasis or inflammation bind to heparin, a GAG structurally related to HS. These cytokines, which include chemokines, as well as interleukins and hematopoietic growth factors, e.g. interleukin (IL) 3, IL-8, granulocyte-monocyte colony stimulating factor (GM-CSF), and HGF (15-20), can thus be potentially immobilized by HSPGs. HSPGs expressed on the luminal surface of endothelial cells have been shown to bind chemokines produced at sites of inflammation (21), thereby preventing their immediate dilution by the blood stream. Presentation of HSPG-bound chemokines, e.g. macrophage inflammatory protein (MIP) 1ß and IL-8, to leukocytes plays a crucial role in activating the leukocyte integrins that mediate stable adhesion to and transmigration across the vessel wall (22, 23). However, chemokines and other heparin-binding cytokines do not exclusively act at the endothelial-blood interface. They also play key roles in the regulation of lymphocyte trafficking within lymphoid tissues and are involved in the control of lymphocyte growth, differentiation and survival (24). This suggests that cell-surface HSPGs on cells of the immune system, such as lymphocytes and antigen-presenting cells, might also be involved in the regulation of cytokine responsiveness. To explore this hypothesis, we have studied the expression, identity, regulation and function of HSPGs on human tonsillar B cells. We show that ligation of the BCR or CD40, two key receptors in the initiation of antigen-specific B cell differentiation (25-29), induces a strong upregulation of cell-surface HSPGs, specifically of CD44-HS. These HSPGs enable B cells to selectively bind hepatocyte growth factor (HGF), a growth factor that induces integrin-dependent adhesion and migration of B cells (30, 31). Moreover, we show that CD44-HS strongly potentiates HGF-induced signaling in B cells.
MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibodies used were anti-pan CD44, Hermes-3 (IgG2a) (32); anti-CD44v3, 3G5 (IgG2b) (R&D Systems, Abingdon, UK); anti-heparan sulfate, 10E4 (IgM) (Seikagaku, Tokyo, Japan); anti-desaturated uronate from heparitinase treated heparan sulfate (anti-ΔHS-stub), 3G10 (IgG2b) (Seikagaku); anti-HGF, 24612.111 (IgG1) (R&D Systems); anti-Met, D024 (IgG2a) (Upstate Biotechnology, Lake Placid, NY); anti-CXCR4, 12G5 (IgG2a) (Pharminigen, San Diego, CA); anti-syndecan-1, 1D4 (IgG1) (CLB, Amsterdam, The Netherlands); anti-syndecan-2, 8G3 (IgG1) (34); anti-syndecan-4, 8G3 (IgG1) (34); anti-glypican-1, S1 (IgG1) (35); anti-phosphotyrosine, PY-20 (IgG2b) (Affiniti, Nottingham, UK); and IgG1, IgG2a, IgG2b and IgM control antibodies (ICN Zoetermeer, The Netherlands). Polyclonal antibodies used were rabbit anti-Met, C-12 (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-Gab1 (Upstate Biotechnology); rabbit anti-SDF-1α (R&D Systems, Minneapolis, MN); rabbit anti-Akt (H-136) (Santa Cruz Biotechnology); phospho-specific rabbit anti-Akt (Ser 473) (New England Biolabs, Hiten, UK); RPE-conjugated goat anti-mouse (Southern Biotechnology Associates, Birmingham, AL); biotin-conjugated swine anti-rabbit (DAKO); HRP-conjugated goat anti-rabbit (DAKO), and HRP-conjugated rabbit anti-mouse (DAKO). In addition we used RPE-conjugated streptavidin (DAKO).

B cell isolation and culturing. B cells were isolated from human tonsils as described previously (36). Total B cell fractions were ≥97% pure as determined by FACs analysis. B cells were cultured in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all Gibco BRL/Life Technologies, Breda, The Netherlands). Some media were supplemented with either 50 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma), 0.002% Staphylococcus aureus strain Cowan I (Sac) (Calbiochem Novabiochem, La Jolla, CA), 1 μg/ml Immunobeads with covalently bound rabbit anti-human Ig (Irving Scientific, Santa Ana, CA), 100 μM IL-2 (Eurocetus, Amsterdam, The Netherlands), 100 U/ml IL-4 (Genzyme Diagnostics, Cambridge, MA), 0.5 ng/ml IL-6 (CLB), or 25 ng/ml IL-10 (Genzyme Diagnostics).

For CD40 ligation, B cells were cultured on irradiated (7000 rad) CD40L (CD154)-transfected or, as a control, wild-type L cells (37).

Cell lines and transfectants. The Burkitt's lymphoma cell line Namalwa was purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in RPMI 1640 (Gibco BRL/Life Technologies) supplemented with 10% Fetal Clone 1 serum (HyClone Laboratories, Logan, UT), 10% FCS (Integro, Zaandam, The Netherlands), 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (all Gibco BRL/Life Technologies). The Namalwa cell lines transfected with CD44s (Nam-SM) or CD44v3-10 (Nam-V3M) were described previously (30, 38).

Enzyme treatments. For enzymatic cleavage of HS, cells or tissue sections were treated with 10 mU/ml heparitinase (Haemobacterium heparinum, EC 4.2.2.8, ICN Biomedicals, Aurora, OH) in RPMI 1640 (Gibco BRL/Life Technologies) at 37°C for 3 h.
The cleavage of HS by heparitinase was determined by the loss of cell-surface expressed HS (mAb 10E4), and the simultaneous gain of HS-stub expression (mAb 3G10). Chondroitinase treatment was used as a specificity control.

**FACS analysis.** FACS analyses using a single or triple staining technique were described previously (39, 40). For cytokine binding assays, cells were incubated with saturating concentrations (20 nM) of recombinant human HGF or recombinant human SDF-1α (both R&D Systems), in PBS for 1 h. prior to the antibody incubations. This step was followed by washing with FACS buffer. For blocking studies, cells were incubated with 125 nM recombinant human FGF-2 or recombinant human SDF-1α (both R&D Systems) in PBS at 4°C for 1 h. prior to the incubation with HGF.

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation and Western blotting were performed as described (39). For the immunodepletion experiments, the lysates were immunoprecipitated twice and the lysate remaining after the second immunodepletion and the immunoprecipitate obtained during the first immunoprecipitation were analysed by Western-blotting.

**RESULTS**

**Expression and Regulation of HSPGs on Human B Cells.** We investigated the expression of HSPGs on the cell-surface of resting and activated human tonsillar B cells by means of FACS analysis, employing mAb 10E4, against an epitope on HS-chains. In addition, we used mAb 3G10, recognizing the ΔHS-stubs that remain present on HSPG-core proteins after heparitinase treatment. HSPGs were hardly detectable on freshly isolated tonsillar B cells. However, upon stimulation of these cells with the phorbol ester PMA, we observed a strong induction of HSPGs (data not shown). This observation prompted us to explore whether HSPGs can also be induced by physiological B cell activators. Since engagement of the BCR and CD40 plays a key role in the initiation of T cell-dependent B cell responses and in the formation of germinal centers (GC) (25-29), we assessed whether activation via these receptors also leads to HSPG upregulation. Tonsillar B cells were cultured on CD40 ligand (CD40L) transfected L cells or, as a control, on wild-type L-cells, in the presence or absence of BCR stimuli (anti-Ig antibodies or *Staphylococcus aureus* Cowans strain I [SAC]). As is shown in Fig. 1, concurrent ligation of CD40 and the BCR induced a strong induction of HSPGs on the B cells. Single triggering of either the BCR or CD40 also led to enhanced HSPG expression, although the HSPG levels were lower than those obtained upon dual receptor ligation. In contrast, stimulation by various cytokines including IL-2, IL-4, IL-6, and IL-10 did not lead to a significant induction of HSPGs (data not shown). These data identify activation via the BCR and CD40 as major signals for the induction of HSPG expression on tonsillar B cells.
**HSPGs on Activated B Cells Selectively Bind HGF.** HSPGs are capable of highly selective cytokine binding and presentation (4). To explore the cytokine-binding ability and specificity of the HSPGs expressed on activated B cells, we tested their capacity to bind two distinct cytokines with established heparin-binding capacity, *i.e.* HGF and stromal cell-derived factor (SDF) 1α (18, 41). Although they are structurally unrelated, HGF belongs to the plasminogen-related growth factor family (42) and SDF-1α is a chemokine (43), these cytokines have both been implicated in the regulation of B cell adhesion and migration (30, 31, 41, 43). In agreement with the data presented in Fig. 1, culturing of tonsillar B cells on CD40L transfected, but not on wild-type, L-cells, led to a strong induction of HS (Fig. 2A). In parallel, these B cells acquired a vast capacity to bind HGF (Fig. 2A). This HGF binding was largely dependent on HS, since over 80% was lost after heparitinase

![Figure 1](image-url)

**Figure 1.** Activation via the BCR and CD40 induces strong expression of HSPGs on B cells. Tonsillar B cells were cultured on CD40L-transfected L cells or wild-type L cells, in the presence or absence of BCR stimuli, *i.e.* anti-Ig immunobeads (Ig) or *Staphylococcus aureus* Cowans strain I (SAC), for 0, 24, or 48 h, and analysed by FACS. Expression of HSPGs is given as the mean fluorescence intensity (MFI) of the anti-ΔHS-stub staining after heparitinase treatment minus its staining before treatment.
HSPGs regulate cytokine signaling in B cells

Figure 2. HSPGs on activated B cells bind HGF but not SDF-1α. A, CD40 stimulation induced HS expression and HGF binding, but not SDF-1 binding. Tonsillar B cells were analyzed by FACS for HS expression and for their capacity to bind HGF or SDF-1α after being cultured on CD40L-transfected L cells or wild-type L cells for 48 h. To determine the involvement of HS, the cells were analyzed after control or heparitinase treatment. Expression of HS is given as the MFI after staining with anti-HS mAb 10E4 minus staining with an isotype-matched control mAb. Binding of HGF or SDF-1α is given as the MFI of cells that were incubated with one of the cytokines, washed, and stained with a cytokine-specific Ab, minus the MFI of identically stained control cells. B, Effect of CD40 stimulation on Met and CXCR4 expression. Expression of the receptor for HGF, Met, and the receptor for SDF-1α, CXCR4, on unstimulated or CD40L-stimulated tonsillar B cells was analysed by FACS. Expression of Met or CXCR4 is given as the MFI after staining with receptor-specific mAbs, minus the MFI after staining with an isotype-matched control mAb.

treatment of the B cells (Fig. 2A). The HGF that remained present on the cells after heparitinase-treatment most probably was bound to its receptor tyrosine kinase Met, as Met was also induced by CD40 ligation (Fig. 2B). In contrast to HGF, binding of SDF-1α to the B cells was completely independent of HS: The HSPG low B cells that were cultured on wild-type L cells had a much greater SDF-1α binding capacity than the HSPG high B cells cultured on CD40L-transfected L cells (Fig. 2A). Moreover, heparitinase treatment did not have any effect on SDF-1α binding. The differences in SDF-1α binding between resting and activated cells were directly related to differential expression of CXCR4, the high affinity receptor
Figure 3. CD40 stimulation induces expression of CD44-HS on tonsillar B cells. A, CD44-HS, but not syndecan-1, is induced by CD40 stimulation. Expression of syndecan-1, or CD44v3 by tonsillar B cells cultured on CD40L-transfected L cells, or wild-type L cells for 0, 24, or 48 h, was analyzed by FACS. Expressions are given as the MFI after staining with anti-syndecan-1 or anti-CD44v3, minus the staining with isotype-matched control mAbs. B, CD44v3 isoform(s) on CD40-activated B cells are decorated with HS. CD44 was immunoprecipitated from tonsillar B cells that had been cultured on wild-type L cells or CD40L-transfected L cells for 48 h, and immunoblotted with anti-pan CD44 (left panel), or with anti-AHS-stub (right panel). To allow the detection of AHS-stubs, the cells had been treated with heparitinase before immunoprecipitation. Namalwa B cells transfected with the HSPG CD44v3-10 (Nam-V3M) were used as a positive control.
HSPGs regulate cytokine signaling in B cells

**Figure 4.** HGF, but not SDF-1α, binds to CD44v3-10 (CD44-HS). A. Namalwa B cells stably transfected with CD44v3-10 (Nam-V3M) express HS, whereas those transfected with CD44s (Nam-SM) do not. B. Namalwa B cells stably transfected with CD44v3-10 (Nam-V3M) express Met, and CXCR4, and show HS-dependent binding of HGF but not of SDF-1α. Nam-V3M cells were analyzed by FACS for their expression of HS, Met, or CXCR4. In addition, their capacity to bind HGF or SDF-1α is shown. To determine the involvement of HS, the cells were analyzed after control or heparitinase treatment. Expression of HS, Met, or CXCR4 is given as the MFI after staining with specific mAbs minus the MFI after staining with an isotype-matched control mAb. Binding of HGF or SDF-1α is given as the MFI of cells that were incubated with one of the cytokines, washed, and stained with a cytokine-specific Ab, minus the MFI of identically stained control cells. C. HGF binding to CD44v3-10 is cross-blocked by FGF-2 but not by SDF-1α. HGF binding to Namalwa CD44v3-10 transfectants was analyzed by FACS as described above. Prior to the incubation with HGF, the cells were incubated with a range of concentrations of either FGF-2 or SDF-1α.
for SDF-1α: Expression of this receptor strongly decreased as a result of CD40 ligation (Fig. 2B).

**Activated B Cells Express HSPG forms of CD44.** The above data show that, upon their activation, B cells acquire cell-surface expressed HS-chains that are capable of selective growth factor binding. This may be the consequence of either upregulation of proteoglycan core protein(s) or upregulation or activation of the enzymes involved in HS-synthesis. To address this issue and to identify the proteoglycan core proteins carrying the HS-chains, we employed mAbs against a panel of defined proteoglycan core proteins, *i.e.*, the syndecans-1, -2, and -4, glypican-1, and CD44. As is shown in Fig. 3A, B cell activation enhanced expression of CD44 splice variants containing epitopes encoded by exon v3, which can be decorated with HS (39, 44). By contrast, no basal expression, nor induction of the distinct syndecans or glypican-1 was observed after B cell activation via CD40 and/or the BCR (Fig. 3A, and data not shown).

To ensure that the CD44 isoforms expressed by activated tonsillar B cells are indeed decorated with HS-chains, CD44 was immunoprecipitated from resting and activated B cells and the immunoprecipitates were analysed on Western blot for the presence of CD44 and HS. Whereas both unstimulated and CD40L-stimulated B cells expressed the 90 kDa 'standard' isoform of CD44 (CD44s) (Fig. 3B), activation via CD40, in addition, induced expression of a 200 kDa CD44 isoform (Fig. 3B). Upon re-staining the blot, only this 200 kDa CD44 isoform was found to be modified with HS. By its size, the CD44-HS isoform on activated B cells resembles the HS-modified CD44v3-10 isoform expressed on Namalwa cells (Fig. 3B), suggesting that they are the products of similar or identical transcripts.

Since we demonstrated in Fig. 2 that the HSPGs which were induced upon B cell activation specifically bound HGF but not SDF-1α, we investigated the ability of CD44-HS to bind HGF and SDF-1α. For this purpose we employed Namalwa B cells transfected with either the CD44-HS isoform CD44v3-10, or the isoform CD44s, which does not contain an HS-attachment site (44). Indeed, as shown in Fig. 4A, did only the Namalwa cells transfected with CD44v3-10 express HS. Moreover, in contrast to HGF, SDF-1α did not bind to these cells in a HS-dependent manner (Fig. 4B). Furthermore, in contrast to the heparin-binding growth factor FGF-2, SDF-1α did not compete with HGF for binding to the cells, even at concentrations exceeding those of FGF-2 by more than a factor of ten (Fig. 4C). Taken together, these results indicate that ligation of CD40 on tonsillar B cells induces the expression of CD44-HS, most likely the CD44v3-10 isoform. This CD44 isoform is capable of selectively recruiting HGF to the B cell surface.
Figure 5. CD44-HS promotes HGF/Met signaling in B cells in a HS-dependent fashion. A, CD44-HS promotes HGF-induced tyrosine phosphorylation of Met. Namalwa B cells, stably transfected with either CD44v3-10 (v3), which is HS-decorated, or CD44s (s), were treated with heparitinase, as indicated, and subsequently stimulated with HGF. Anti-Met immunoprecipitates were immunoblotted with anti-phosphotyrosine (PY-20) (upper panel), or with anti-Met (lower panel). B, CD44-HS promotes HGF-induced tyrosine phosphorylation of proteins downstream of Met. An immunoblot of total cell lysates prepared from cells treated as described in A, was stained with anti-phosphotyrosine. The arrow indicates (a) highly phosphorylated protein(s) at 115-120 kDa. C, HGF-induced Gab-1 phosphorylation is enhanced in the presence of CD44-HS. An immunoblot of immunodepleted Namalwa CD44v3-10 cell lysates (left panels) or their corresponding immunoprecipitates (right panels) is shown. Immunoprecipitation was performed with anti-Gabl or with control mAbs (C) as indicated. The blots were stained with anti-phosphotyrosine (upper panels) or with anti-Gabl (lower panels). The arrowhead indicates a band probably representing co-immunoprecipitated Met, while the thin and thick arrow indicate an unidentified protein of about 115 kDa, and Gab1, respectively. D, CD44-HS promotes HGF-induced activation of Akt/PKB. Control or heparitinase-treated cells were incubated with HGF for 0, 2, or 10 min as indicated. Total cell lysates from the transfectants described in A were immunoblotted with anti-phospho-Akt (upper panel), or anti-Akt (lower panel).
Chapter 5

Cell Surface HSPGs Regulate Met Signaling in B Cells. The above data suggest a role for HSPGs, specifically CD44-HS, in the regulation of HGF/Met signaling. To address this hypothesis, we employed Met positive Namalwa B cells transfected with either a CD44-HS isoform (CD44v3-10), or, as a control, with a CD44 isoform which can not be decorated with HS (CD44s) (39). Upon HGF stimulation a strong phosphorylation of Met was induced in the cells expressing CD44-HS, whereas phosphorylation in the cells expressing CD44s was weak (Fig. 5A). HS-moieties decorating CD44 were responsible for the strongly enhanced Met phosphorylation in the cells carrying CD44-HS, as heparitinase treatment reduced the HGF induced phosphorylation to the control level observed in the cells expressing CD44s (which lack HS) (Fig. 5A). The strong potentiation of Met signaling by HS on the B cell surface was not only present at the level of receptor phosphorylation: As is show in Fig. 5B, upon HGF stimulation a broad band representing (at least) two bands (Fig. 5C), the lower of these bands was identified as Grb2-associated binder 1 (Gab1) (Fig. 5C), an adapter protein that can associate with the cytoplasmic docking site of Met (45). Indeed, in anti-Gab1 immunoprecipitates, we detected a phosphorylated protein of about 145 kDa which probably represents Met (Fig. 5C). In addition to Gab1 phosphorylation, a strongly enhanced activation of Akt, also referred to as protein kinase B (PKB), was found upon HGF-stimulation in cells expressing CD44-HS as compared to cells expressing CD44s (Fig. 5D). Again, enhanced Akt activation was HS-dependent, as it could be abrogated by heparitinase treatment of the cells expressing CD44-HS but not CD44s (Fig. 5D). Taken together, these data demonstrate that CD44-HS is capable of regulating HGF/Met signaling in B cells in a HS-dependent fashion.

DISCUSSION

Heparan sulfate proteoglycans are involved in regulating the growth, migration, and differentiation of epithelial cells and fibroblasts (1, 3, 7-9). During these processes HSPGs immobilize and oligomerize cytokines and present them to their high affinity receptors (5, 46-48). In this way, HSPGs create niches in the microenvironment and regulate cytokine responses. Since a large number of cytokines involved in lymphoid tissue homeostasis or inflammation contain potential heparan sulfate-binding sites, HSPGs presumably also play important roles in the regulation of the immune response. However, the expression and

118
function of HSPGs on the cell surface of lymphocytes, as well as within the ECM of the lymphoid tissues, has thus far remained largely unexplored. In the present study, we investigated the regulation and function of HSPGs on human B cells. We demonstrate that expression of HSPGs on human B cells is dynamic and that HSPGs are capable of selective cytokine binding and regulation of cytokine-induced signaling.

We observed that freshly isolated tonsillar B cells express low levels of HSPGs but that single or concurrent ligation of the BCR and CD40 induced a strong expression of HSPGs on the B cell surface (Fig. 1). These observations for the first time show that B cell triggering by physiological stimuli has a profound effect on their HSPG expression and suggests that B cells use cell surface HSPGs as a means to control their cytokine-binding capacity and responsiveness. To explore this hypothesis, we analyzed the binding of the cytokines HGF and SDF-1α to HSPGs on activated B cells. These cytokines were selected since they bind heparin, a heavily sulfated heparan sulfate proteoglycan, with similar affinities (41, 49, 50). Moreover, although HGF and SDF-1α are structurally unrelated, they have both been implicated in the regulation of B cell adhesion and migration (30, 31, 51, 52). HGF is a 90 kDa cytokine, which induces complex responses in target cells, e.g., stimulation of motility, growth and morphogenesis, by binding to the receptor tyrosine kinase Met (53-58). HGF is essential for vertebrate development, since knock-out of the HGF or Met genes is lethal and causes abnormal development of the liver and placenta and disrupt the migration of myogenic precursors to the limb buds (59-61). Other studies suggest important roles for HGF in tissue regeneration and in tumor growth, invasion, and metastasis (53, 62-64). The CXC chemokine SDF-1 was originally identified as a pre-B cell growth stimulating factor (65) and, more recently, has been implicated in a variety of processes, including hematopoiesis, cerebellar and vascular development, and cardiogenesis, by activating its receptor CXCR4 (66-69). SDF-1α is a potent chemoattractant for hematopoietic progenitors, and induces migration of naive and memory, but not germinal center, B cells (43, 52, 69, 70).

We observed that the inducibly expressed HSPGs on CD40-activated B cells are capable of binding large quantities of HGF, but not SDF-1α. Instead, B cell activation resulted in a strongly decreased binding of SDF-1α to the B cells (Fig. 2A). This could be explained by the observation that CXCR4, the SDF-1α receptor, was down-regulated in response to CD40 triggering, in analogy to BCR-induced down-regulation of CXCR4 (71) (Fig. 2B). The finding that HS-moieties on B cells do not bind SDF-1α was further corroborated by our observation that SDF-1α, unlike the heparin-binding cytokine FGF-2, does not compete with HGF for HSPG-binding (Fig. 4C). Hence, upon their activation via the BCR and CD40, B cells do not only gain expression of the HGF receptor Met (30) (Fig. 2B), but in addition acquire the appropriate HSPGs, i.e., HSPGs with the capacity to bind large
quantities of HGF. Thus, unlike interactions with heparin, the interaction between cytokines and the natural HSPGs that are induced during B cell activation appear to be highly selective, suggesting that HSPG contribute an additional level of specificity to B cell-cytokine interactions and may co-regulate B cell differentiation. Selectivity of HSPGs-protein interactions has also been observed in other biological systems, including blood coagulation and embryonic development (2-4, 9). It is determined by the structural modifications of the HS-chains, which take place within the Golgi complex, as well as by the nature of the core protein (4, 18).

HSPGs consist of HS-chains covalently attached to a core protein. Ligation of CD40 resulted in a strong induction of cell-surface expressed CD44 splice variants containing the domain encoded by exon v3 (Fig. 3A). This domain contains a consensus motif for HS attachment (39, 44), and we indeed confirmed that CD44 isoforms on activated B cells are decorated with HS (Fig. 3B). The relative molecular mass of the HSPG form of CD44 on activated B cells was indistinguishable from that of a CD44v3-10 isoform expressed by Namalwa cells, suggesting that they are products of similar or identical transcripts (Fig. 3B). Although we cannot exclude a contribution of other (unknown) core proteins, our findings identify CD44 as an important cell surface HSPG on activated B cells. As of yet, data on the expression and function of HSPGs in lymphocytes are scarce. The HSPG syndecan-1 can be expressed by human plasma cells, myeloma cells, and Reed-Sternberg cells of classical Hodgkin's disease (72-74), and syndecan-4 expression has been demonstrated in mouse B cell (75). Apart from a possible role in growth factor presentation, analogous to that observed for CD44-HS in our present study, syndecan-1 and -4 may be important mediators of cell-cell adhesion since their transfection to B lymphoblastoid cell lines results in cell spreading and aggregation (76, 77).

Incubation of HGF with heparin or HS-derived oligosaccharides has been reported to promote phosphorylation of the HGF receptor Met (15). This prompted us to explore the impact of CD44-HS expression on HGF-induced signal transduction. Interestingly, we observed that the autophosphorylation of Met, as well as the phosphorylation of the kinase Akt/PKB, and of two proteins of 110-120 kDa is strongly promoted by expression of CD44-HS at the B cell surface (Fig. 5). Immunodepletion experiments indicated that the smaller of the two proteins represents Grb2-associated binder 1 (Gab1), an adapter protein which can associate with Met (45) (Fig. 5C). The other hyperphosphorylated protein might represent p120-Cbl, a protein tyrosine kinase that participates in signal transduction via receptor tyrosine kinases, and that has implicated in the regulation of integrin activation (78, 79). This is of particular interest, since stimulation of B cells with HGF results in enhanced integrin dependent adhesion (see below).
Our observations suggest a scenario in which B cells, upon their activation by antigen and T cells, become insensitive to the migration promoting activity of SDF-1α as a result of down-regulation of CXCR4. At the same time they acquire the receptor tyrosine kinase Met (30) as well as HSPG, viz. CD44-HS, which allow them to selectively recruit HGF to the B cell surface, resulting in efficient HGF/Met signaling. We have previously shown that HGF is produced by follicular dendritic cells (FDC) and enhances integrin-dependent adhesion of B cells to fibronectin and VCAM-1. Hence, activation of the HGF/Met pathway may strengthen B cell adhesion, specifically to FDC, which is mediated by α4β1–VCAM-1 (80). Interestingly, apart from establishing physical contact, outside-in signaling via α4β1 presumably contributes to the B cell selection process in the germinal center by inhibiting apoptosis of B cells (36, 40). Since both integrin engagement and Met stimulation may lead to activation of Akt/PKB, a pathway reported to suppress apoptosis (81), it will be of interest to explore the collaborative effects of integrin and Met signaling on B cell survival.

In conclusion, our data demonstrate that the BCR and CD40 control the expression of HSPGs, specifically CD44-HS, on B cells. By selectively binding HGF to the B cell surface, these HSPGs act as functional co-receptors for HGF promoting signaling through Met, which suggests a role for these HSPGs in the regulation of antigen-specific B cell differentiation.

ACKNOWLEDGEMENTS

We thank Drs G. David, C.G. Figdor and S. Jalkanen for mAbs. This study was supported by grants from the University of Amsterdam and the Association for International Cancer Research (AICR).

REFERENCES


HSPGs regulate cytokine signaling in B cells


123
Chapter 5


HSPGs regulate cytokine signaling in B cells


HSPGs regulate cytokine signaling in B cells


