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*J Immunol* 2005; 175:2807-2813; doi: 10.4049/jimmunol.175.5.2807

http://www.jimmunol.org/content/175/5/2807

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Follicular Dendritic Cells Catalyze Hepatocyte Growth Factor (HGF) Activation in the Germinal Center Microenvironment by Secreting the Serine Protease HGF Activator

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Ag-specific B cell differentiation, the process that gives rise to plasma cells and memory B cells, involves the formation of germinal centers (GC). Within the GC microenvironment, multiple steps of B cell proliferation, selection, and maturation take place, which are controlled by the BCR in concert with cytokines and contact-dependent signals from follicular dendritic cells (FDCs) and T cells. Signaling by the multifunctional cytokine hepatocyte growth factor (HGF) and its receptor MET has been shown to induce integrin-mediated adhesion of B cells to VCAM-1, which is expressed by FDCs. In the present study we have examined the expression of regulatory components of the HGF/MET pathway, including HGF activator (HGFA), within the secondary lymphoid organ microenvironment. We show that MET is expressed by both centroblasts and plasma cells, and that HGFA is expressed by plasma cells. Because we have shown that HGF is a potent growth and survival factor for malignant plasma cells, HGF may also serve as a survival factor for normal plasma cells. Furthermore, we demonstrate that FDCs are the major source for HGF and its activator within the GC microenvironment. Both HGF and HGFA are expressed by FDCs in the GC dark zone (CD21high/CD23low), but not in the light zone (CD21high/CD23high). These findings suggest that HGF and HGFA provided by dark zone FDCs help to regulate the proliferation, survival, and/or adhesion of MET-positive centroblasts.

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Received for publication December 29, 2004. Accepted for publication June 10, 2005.

The Journal of Immunology, 2005, 175: 2807–2813.
conversion of sHGF to the heterodimeric active form is essential (21). Plasminogen activators, particularly urokinase plasminogen activator and factor XIIa, have been shown to activate sHGF, although at low rates (22, 23). More recently, HGF activator (HGFA), a factor XIIa-related serine protease with efficient HGF-activating activity, was identified (21, 23). HGFA has been implicated in HGF activation at sites of inflammation and tissue repair (24) as well as by tumor cells (25), including multiple myeloma (MM), a malignancy of plasma cells (26). In this study we have examined the mechanism of HGF activation within the GC microenvironment. We show that FDCs derived from the GC dark zone, in contrast to those from the GC light zone, produce HGFA and in this way are able to catalyze HGF activation. In addition, we demonstrate that normal plasma cells express HGFA.

Materials and Methods

Abs and reagents

The mAbs used were FITC-conjugated anti-human IgD (DakoCytomation); allophycocyanin-conjugated anti-human CD38 (IgG1; BD Biosciences); PE-conjugated anti-human CD20 (DakoCytomation); anti-human MET DO-24 (IgG2a; Upstate Biotechnology); anti-HGFA (A1 and P1-4, IgG1) (24); anti-factor XIIa, OT-2 (IgG1; Sanquin); anti-human CD21 long isomorph (dendritic reticulum cell (DRC)-1); anti-human CD21 (clone IFB), FITC-conjugated anti-human CD21 (clone IFB), and anti-CD20 (clone L243) (DakoCytomation); anti-CD11 (IgG1 M3, IgG2b); anti-CD3 (Leu 4, IgG1; BD Biosciences); and isotype controls anti-IgG1, anti-IgG2a, and anti-IgG2b (DakoCytomation). Secondary Abs were used were post-Ab for powerwision (Immunovision Technologies), HRP-conjugated goat-anti-mouse/rat IgG, and HRP-conjugated anti-FITC (DakoCytomation).

Purification of B cell populations and FDCs

B cells were purified from human tonsils obtained from children undergoing routine tonsillectomy as previously described (13). Briefly, mononuclear cells were isolated by Ficol-Isoaque density gradient centrifugation. Monocytes and T cells were depleted by plastic adherence and SRBC rosetting, respectively. The total B cells fraction was >97% pure as determined by FACS analysis. To obtain the different B cell populations (naive B cells, memory B cells, GC B cells, and plasma cells), total B cells were stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD20, and allophycocyanin-conjugated anti-CD38 and were sorted using a FACS aria (BD Biosciences).

FDC clusters (enriched FDC preparation) were isolated from human tonsils as described previously by Liu et al. (27). Tonsils were cut into pieces and treated with collagenase/DNase mix (200 U/ml collagenase IV and 10 U/ml DNase I; Roche), followed by Ficol-Isoaque density gradient centrifugation and density sedimentation on a BSA gradient (1.5% BSA).

FDCs (CD21high) were stained with FITC-conjugated anti-CD21 and sorted using FACS aria (BD Biosciences). PE-conjugated anti-CD23 together with anti-CD21 were used to obtain the CD23 FDC populations. For immunocytochemical staining, FDCs were isolated from an enriched FDC preparation by cell sorting of large cells. Cell purity was >60–80%, as judged by staining for DRC-1, a FDC-specific marker.

Assay for HGF activation

HGF activation was assayed as described previously (25). In brief, sHGF (R&D Systems) was incubated with FDC-conditioned medium. Conditioned medium was obtained as described previously (28). For HGF activation, 20 μl of conditioned medium were pretreated with 1 U of thrombin and added to 0.1 μg of sHGF. Inhibitor studies were performed in the presence of leupeptin (500 μg/ml), neutralizing Ab against FXII (OT-2; provided by E. Hack, Sanquin, Amsterdam, The Netherlands), or neutralizing Ab against HGFA (P1-4; 40 μg/ml).

FDC sarcoma

The FDC sarcoma, which is a rare neoplasm that arises from lymph nodes as well as extranodal regions (29), was obtained from a 38-year-old male patient and was diagnosed at the Department of Pathology, Academic Medical Center (Amsterdam, The Netherlands). The tumor showed >95% CD21- and DRC-1-positive cells, indicating almost complete replacement of the normal follicle structure by the neoplasm.
To confirm and extend these observations, we also performed immunocytochemistry on human tonsillar B cells depleted of T cells and monocytes (Fig. 2C). This population consisted of \(97\%\) CD20-positive cells and did not contain monocytes (CD14\(^{-}\)) or T cells (CD3\(^{-}\)). Of note, scattered cells within this population showed a strong expression of HGFA (Fig. 2C). Apart from a few cells with a plasmacytoid morphology, these HGFA-positive cells showed morphological features of FDCs, i.e., large oval cells with an eccentric or double nucleus (Fig. 2C). Indeed, immunocytochemical staining of FACS-sorted FDCs showed a distinct granular HGFA expression pattern (Fig. 2D), whereas immunoblotting confirmed the expression of HGFA (Fig. 2E). Hence, both plasma cells and FDCs express HGFA.

**HGFA and HGFA expression is a feature of dark zone FDCs.**

HGFA and HGFA expression is a feature of dark zone FDCs. In addition to differential HGFA and HGFA expression, we also observed differential expression of CXCL13 mRNA in the CD23-sorted FDCs, confirming the study by Allen et al. (9) showing that this chemokine is predominantly expressed by GC light zone FDCs.

**Expression of HGFA and HGFA by FDC sarcoma**

To corroborate our finding that FDCs express both HGFA and HGFA, we also studied the expression of HGFA and HGFA mRNA in FDC sarcoma, a rare neoplasm composed of malignant FDCs (29). As in normal dark zone FDCs, both HGFA and HGFA were prominently expressed by this tumor (Fig. 3B). We were unable to detect MET expression on the FDC sarcoma cells (data not shown), indicating the absence of an autocrine HGF/MET loop in this tumor.

**FDCs convert HGF by producing HGFA**

Subsequently, we investigated whether FDCs are able to process scHGF to its active form. Conditioned medium of FDCs effectively converted scHGF (Fig. 4). This required thrombin, whereas the conversion was completely inhibited by the serine protease inhibitor leupeptin (Fig. 4A). Because proteases other than HGFA are, although with low efficiency, capable of activating scHGF in vitro, we explored whether the conversion of scHGF by FDCs could be inhibited by specific interference with HGFA activity. We observed that the anti-HGFA mAb P1-4, which blocks HGFA
function, effectively inhibits scHGF conversion by FDCs (Fig. 4A). These findings identify HGFA as the (major) serine protease responsible for the conversion of scHGF by FDCs and identify FDCs as important regulators of HGF activity in the GC microenvironment.

Discussion

Proteolytic activation of HGF in the extracellular milieu is a critical limiting step in HGF/MET signaling (21). In this study we present data indicating an important role of the serine protease

FIGURE 2. A, Expression of HGFA in B cell subsets. Tonsillar B cell populations were isolated by FACS sorting using CD20/CD38/IgD triple staining. After RNA isolation and cDNA synthesis, RT-PCR for HGFA and HGF was performed. β2-Microglobulin was used as housekeeping gene control. B, Plasma cells express HGFA. Highly purified B cell populations were collected after FACS sorting, and the expression of HGFA was analyzed using immunoblotting. The MM cell line LME-1 was used as a positive control. C, HGFA expression in total tonsillar B cell preparations. Immunocytochemical staining of unsorted B cell preparations for HGFA, the B cell marker CD20, and the monocyte marker CD14 was performed. The arrowhead indicates a plasmacytoid cell (inset), whereas the arrow indicates a FDC. D, HGFA is expressed by FDCs. FDCs were isolated from an enriched FDC preparation by cell sorting of large cells. The cell purity was determined by staining for the FDC marker DRC-1 and the B cell marker CD20. The mAb A-1 against HGFA was used to detect HGFA expression. The arrow indicates a FDC. E, MET and HGFA expression in sorted FDC preparations. Purified FDC preparations were analyzed by immunoblotting for the expression of MET and HGFA.

FIGURE 3. A, Dark zone FDCs express HGF and HGFA. Highly purified FDCs were collected from enriched FDC preparation after FACS sorting using the markers CD21 and CD23. The purity of both sorted FDC fractions was determined by restaining the cells with FITC-conjugated anti-human CD21, followed by HRP-conjugated anti-FITC and detection with 3,3'-amino-9-ethylcarbazol. Inset, High magnification of the cells. B, CD21– FDCs, CD23low FDCs (GC dark zone), CD23high (GC light zone), and FDC sarcoma were analyzed for the expression of HGF, HGFA, CD21, and CXCL13 using RT-PCR. β2-Microglobulin was used as a housekeeping gene control.
HGFA in regulating HGF activation in normal lymphoid tissue. We demonstrate that HGFA is expressed by plasma cells, but not by other B-lineage populations. Furthermore, we show that HGFA is strongly expressed by FDCs and is confined to FDCs in the GC dark zone. These cells coexpress HGF and are in close contact with MET-positive centroblasts. Our findings suggest specific functions of the HGF/MET pathway in Ag-specific B cell differentiation, affecting plasma cell and centroblast functions.

Previous studies from other and our own laboratories have implicated the HGF/MET pathway in the pathogenesis of the plasma cell malignancy MM (34–37). Our current study represents the first report of HGF/MET pathway components in normal plasma cells. We observed that normal plasma cells express the receptor tyrosine kinase MET, but do not express its ligand, HGF (Figs. 1 and 2A). In addition, we show that HGFA is expressed by plasma cells, but not by other B-lineage populations (Fig. 2, A and B). The coexpression of MET and HGFA in plasma cells is of interest because it indicates that plasma cells are well equipped to receive paracrine HGF signals. Indeed, tonsillar as well as bone marrow stromal cells have been reported to produce HGF, and it is hence conceivable that plasma cells receive paracrine stimulation from the microenvironment (18, 20, 38). Contrary to their malignant counterparts (35), normal plasma cells do not express HGF and hence do not possess an autocrine HGF/MET loop. Although the functional consequences of HGF/MET signaling in the plasma cell have not yet been explored, as in B cells (18, 20, 39), the pathway might regulate integrin-mediated adhesion and promote migration. HGFA expression by plasma cells could thus play a role in their homing to the bone marrow and control their integrin-mediated interaction with bone marrow stromal cells. These stromal cells produce cytokines that support plasma cell survival, including IL-5, IL-6, TNF-α, and SDF-1. In view of the potent effects of HGF on the survival of MM plasma cells (37), it is conceivable that HGF produced by bone marrow stromal cells also contributes to plasma cell survival.

Differential protein expression within the GC dark and light zones reflects the distinct processes that take place within these compartments. One of the key findings of our study is that FDCs are able to autocatalyze HGF activation by producing both HGF and HGFA (Fig. 4) and that HGFA and HGF expression by FDCs is confined to the CD23high subset, which resides in the GC dark zone (Fig. 3B). Combined with our previous observation that MET is selectively expressed by B cells in the GC dark zone, i.e., by centroblasts, these observations suggest specific functions for the HGF/MET pathway in the GC dark zone. HGF stimulation of B cells has been shown to mediate integrin activation, promoting B cell adhesion to VCAM-1 and ICAM-1 (11, 19), two important integrin ligands on FDCs (10, 11). Hence, active HGF in the GC dark zone could play a role in initiating physical contact of MET-positive centroblasts, which have down-regulated their BCR, with FDCs. Upon transition to the light zone, the B cells, now centrocytes, re-express their BCR, which engages in interactions with Ag presented by FDCs. At this stage, high-affinity interactions of the BCR with Ag could take over the regulation of integrin activity required for B cell-FDC interaction (12). Alternatively, MET-mediated growth and survival signals might directly contribute to the expansion of centroblasts. These signals could be crucial at a differentiation stage at which the cells are largely devoid of BCR.

FIGURE 4. FDCs autocatalyze HGF activation by producing HGFA. A, Conditioned medium of an enriched FDC preparation was incubated with scHGF for 6 h in the presence of thrombin combined with either the serine protease inhibitor, leupeptin, or neutralizing Ab against HGFA (P1-4) or against factor XII, as indicated. B, As a positive control, HGF conversion by recombinant HGFA is shown. HGF conversion was determined by immunoblotting with anti-HGF. α-HGF, α-chain of HGF; HC, H chain of Ig.

FIGURE 5. Components of the HGF/MET pathway in the GC and bone marrow microenvironments. During B cell differentiation, HGF and HGFA produced by dark zone (DZ) FDCs (CD23+) may act as proliferative and survival factors for the MET-positive centroblasts (CB). In the light zone (LZ), where the FDC network is more extensive, the centrocytes (CC) are found in tight association with FDCs (CD23+), and via direct physical contact, the CCs receive survival signals. After B cell differentiation, the plasma cells home to the bone marrow (BM), where BM stromal cells secrete HGF. The MET-positive plasma cells autoactivate HGF by producing HGFA, which may contribute to plasma cell survival.
expression and may not receive sufficient growth and survival signals.

Cell migration within the lymphoid organs is directed by chemokines and is essential during GC reaction. The chemokine CXCL13 produced by follicular stromal cells is required for recruiting Ag-activated B cells to the GC (8). We observed that CD23bright FDCs expressed high levels of CXCL13 mRNA compared with CD23low FDCs (Fig. 3B). This observation confirms that by Allen et al. (9), who, by immunohistochemical staining of the GC and by microdissection of the different GC regions, also demonstrated that CXCL13 is more abundantly expressed in the GC light zone than in the dark zone. Mice lacking CXCL13 (B6.CD19CreERT2; CXCL13−/−) show defects in GC localization and size, indicating an important role in GC organization (8, 40). CXCR4 and its ligand, SDF-1, also contribute to GC organization, specifically to sorting of centroblasts into the GC dark zone (9).

Several studies have implicated the HGF/MET pathway in the pathogenesis of B cell neoplasia (18, 19, 34–37, 39). The expression of MET has been demonstrated in MMs and a subset of non-Hodgkin’s lymphomas. Activation of the pathway in these tumors may involve autocrine stimulation, because coexpression of HGF and MET has been observed (35, 41–43). In MMs, MET activation promotes proliferation and survival (37). We observed that MMs compared with normal plasma cells, strongly expressed HGF as well as MET protein (Fig. 2B). Overexpression of HGF by tumor cells may enhance HGF/MET signaling, promoting cell growth and survival, and may contribute to disease progression.

In the FDC sarcoma tested (Fig. 3B), we observed strong expression of both HGF and HGFA, suggesting that the tumor was related to GC dark zone FDCs. However, the tumor cells did not express MET, excluding a role for autocrine HGF/MET signaling in this tumor.

In summary, our study indicates that HGFs regulate the bioavailability of HGF within the GC microenvironment, a function that may contribute to the control of B cell growth, survival, and adhesion during normal B cell differentiation within lymphoid tissue (Fig. 5).

Acknowledgments

We thank Berend Hoobrink for sorting the B cells and FDCs and the coworkers of the Department of Otolaryngology of the Saint Lucas/Andreas Hospital (Amsterdam, The Netherlands) for their kind support.

Disclosures

The authors have no financial conflict of interest.

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