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Stem Cell Factor Programs the Mast Cell Activation Phenotype

Tonomobu Ito,*1 Daniel Smrž,*1 Mi-Yeon Jung,* Geethani Bandara,* Avanti Desai,* Šárka Smržová,* Hye Sun Kuehn,* Michael A. Beaven,† Dean D. Metcalfe,* and Alasdair M. Gilfillan*

Mast cells, activated by Ag via FcεRI, release an array of proinflammatory mediators that contribute to allergic disorders, such as asthma and anaphylaxis. The KIT ligand, stem cell factor (SCF), is critical for mast cell expansion, differentiation, and survival, and under acute conditions, it enhances mast cell activation. However, extended SCF exposure in vivo conversely protects against fatal Ag-mediated anaphylaxis. In investigating this dichotomy, we identified a novel mode of regulation of the mast cell activation phenotype through SCF-mediated programming. We found that mouse bone marrow-derived mast cells chronically exposed to SCF displayed a marked attenuation of FcεRI-mediated degranulation and cytokine production. The hyporesponsive phenotype was not a consequence of altered signals regulating calcium flux or protein kinase C, but of ineffective cytoskeletal reorganization with evidence implicating a downregulation of expression of the Src kinase Hck. Collectively, these findings demonstrate a major role for SCF in the homeostatic control of mast cell activation with potential relevance to mast cell-driven disease and the development of novel approaches for the treatment of allergic disorders. The Journal of Immunology, 2012, 188: 5428–5437.

Antigen-mediated mast cell (MC) activation, via FcεRI, results in the release of an array of inflammatory mediators that underlies allergic reactions in atopic disease (1, 2). MCs are derived from bone marrow progenitors that migrate into the circulation and peripheral tissues where they expand and mature under the influence of cytokines contained within the surrounding milieu (3). KIT activation, as a consequence of binding of its ligand stem cell factor (SCF) produced within tissues by stromal cells, is critical for the development of MCs from bone marrow progenitor cells and their subsequent accumulation, maturation, and survival in tissues (4). Unlike human MCs (5), mouse MC development and survival in culture are supported by IL-3 in the absence of SCF (6). However, the supplementary presence of SCF markedly enhances the rate of growth of these cells and is described to skew the development of the cells to that of a serosal/connector tissue phenotype. In contrast, cells grown in IL-3 alone are considered to more resemble the mucosal phenotype based on the types of proteases expressed (7–10).

Little is known about how SCF and other extrinsic factors, or the combination thereof, may dictate the MC phenotype with regard to responsiveness to Ag and other stimulants. Under acute experimental conditions, SCF is one of several endogenous agents known to potentiate Ag-mediated MC degranulation and cytokine production (11–13). Nevertheless, in contrast to its acute effects on MC activation in vivo (14), it is reported that repetitive s.c. injection of SCF over a period of 21 d into mice may actually protect against fatal anaphylactic reactions (15). Indeed, at the sites of injection, the MCs exhibited little morphological evidence of degranulation after induction of anaphylaxis via IgE in these mice (figure 2 in Ref. 15), suggesting that chronic exposure to SCF may have a profoundly different impact on MC activation than does short-term exposure. Thus, we investigated the hypothesis that prolonged exposure of MCs to SCF, as likely occurs in vivo to maintain MC homeostasis, may lead to transcriptional modifications that alter the underlying activation properties of the cells.

As reported in this article, these studies led us to identify a novel mechanism for the regulation of the extent of MC activation through SCF-dependent induction of a hyporesponsive phenotype with respect to both cytokine production and degranulation. This phenotype was not due to downregulation of the expression of either FcεRI or KIT, but it could be explained by an inability of the cells to undergo the cytoskeletal reorganization required for mediator release, potentially as a consequence of decreased expression of the Src kinase Hck. These findings reveal that the sensitivity of MCs to IgE/Ag stimulation is highly regulated by SCF and presumably other cytokines in the surrounding tissue milieu. This may have important implications for understanding how the activation capacity of tissue MCs may be phenotypically modified in health and in disease.

Materials and Methods

Cell culture and coculture

Experiments on mice were conducted under a protocol approved by the Animal Care and Use Committee at National Institutes of Health (NIH). Bone marrow-derived MCs (BMMCs) were developed from bone marrow...
obtained from femurs of C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), as described (16). Essentially, the cells were cultured for 4–6 wk in media containing mouse rIL-3 (30 ng/ml; PeproTech, Rocky Hill, NJ) or a combination of mouse rIL-3 (30 ng/ml) and mouse recombinant SCF (unless otherwise indicated: 100 ng/ml; PeproTech). The cells were maintained at 37°C in a humidified incubator gassed with 95% air and 5% CO2. The purity of the cultures, as assessed by toluidine blue staining (17) and FcεRI and KIT expression, was >95% cell viability. The NIH 3T3 mouse fibroblast cell line (American Type Culture Collection, Manassas, VA) was grown or cocultured (18) with BMMCs in the same media as for BMMCs but in the absence of IL-3 and SCF.

Cell sensitization, activation, degranulation, and cytokine/chemokine release

BMMCs were sensitized overnight in cytokine-containing or cytokine-free media (as indicated) with mouse anti–DNP-IgE (100 ng/ml; clone SPE-7; Sigma). After sensitization, the cells were processed and activated as described (16). Degranulation after a 30-min activation was monitored by the release of the granule component α-hexosaminidase (α-hex) into the supernatants, as described (19), and expressed as a percentage of θ-hex released into supernatant. The amount of cytokines released from cells after a 6-h activation was determined by Quantikine ELISA kits (R&D Systems, Minneapolis, MN). To measure cytokine content within the cytoplasm, the activated cells were lysed by adding distilled water followed by freezing (1 h)/thawing, the supernatants were collected, and the amount of cytokines was determined as above.

Cell fractionation, immunoblotting, and intracellular calcium measurement

Sensitized BMMCs were stimulated with Ag for 2 min at 37°C, and cell fractionation was performed, as described (16). The cells were lysed as described (20, 21), and proteins were separated by electrophoresis on 4–12% NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA) and probed for immunoreactive proteins using the following protein-specific Abs: β-actin (Sigma), Hck (Santa Cruz Biotechnology, Santa Cruz, CA), and other phosphoprotein- and protein-specific Abs (Cell Signaling, Beverly, MA). The immunoreactive proteins were visualized by probing with rabbit IgG-specific Ab (Amersham Biosciences, Piscatway, NJ), or mouse IgG Fc-specific Ab (Sigma) conjugated with HRP. To monitor the intracellular calcium during cell activation, the sensitized BMMCs were pretreated, activated, and analyzed, as described (22).

Toluidine blue and alcian blue/safranin staining

Cytoplasm of 4-wk-old BMMCs were prepared, fixed, and stained with toluidine blue or alcian blue/safranin, as described (17, 23).

Flow cytometric analysis of FcεRI and KIT surface expression

Following blocking of FCyRs with 2.4G2 (BD Pharmingen), cells were stained with FITC–anti-FcεRI (eBioscience) and PE–anti-KIT (BD Pharmingen) Abs to examine surface expression of FcεRI and KIT, respectively. Cells were incubated for 1 h at 4°C and, after washing with PBS, the stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

F-actin and α-tubulin

Sensitized BMMCs were stimulated with DNP-human serum albumin (HSA) (10 ng/ml) for the indicated times, fixed with 4% paraformaldehyde/5 mM EGTA and EDTA in PBS for 15 min at room temperature (RT) and then F-actin (polymeric) was stained with FITC-labeled phalloidin (Sigma) in 2% BSA/0.1% saponin/PBS for 1 h in dark at RT. The stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Retroviral transduction

Retroviral vector pMX-puro with/without the mouse hck cDNA (a kind gift from Dr. Toshiaki Kawakami, La Jolla Institute for Allergy and Immunology, San Diego, CA) was transduced into PLAT-E cells (Cell Biolabs, San Diego, CA) with PUGENE 6 transfection reagent (Roche Applied Science) in Opti-MEM (Invitrogen). Transfected cells were grown in DMEM containing FBS (10%) and l-glutamine (4 mM), and the media were replaced 16–20 h posttransfection with fresh media containing penicillin (100 U/ml) and streptomycin (100 µg/ml). After 24 h, virus particles in the media were collected by centrifugation (8000 rpm, overnight, 4°C), and the pellet was resuspended in complete BMMC medium. Viral titer was determined using a QuickTiter retrovirus quantitation kit (Cell Biolabs). A total of 0.5–1 × 109 BMMCs (2–3 wk old) was transduced with 4.5 × 10^10 virus particles in hexadimethrine bromide (10 µg/ml). After 72 h, the medium was replaced with fresh BMMC medium with/without SCF, and transduced cells were selected in the presence of 1.2 µg/ml puromycin for 2 wk.

Quantitative real-time PCR

Total RNA was isolated using an RNeasy Plus mini kit (QIAGEN, Valencia, CA). One microgram of RNA was used for reverse transcription reaction using random hexamers and Superscript III reverse transcriptase (Invitrogen). The 20-µl reaction from microtiter plate of the resulting cDNA was used for real-time PCR using an HCK TaqMan gene expression assay, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA).

Affymetrix gene chip analysis

Bone marrow cells were harvested from six 3-mo-old male littermates in a randomized order on a single day, and cells were cultured for 4 wk in either IL-3 (30 ng/ml) alone or IL-3+SCF (100 ng/ml); cell suspensions were centrifuged, and each cell pellet was lysed in 600 µl RLT buffer (QIAagen). Cell lysates were then homogenized for 2 min at 21,000 × g at using a QiA shredder homogenizing column (QIAGEN). Each sample lysate aliquot (100 µl) was combined with 100 µl RLT buffer, 140 µl 100% ethanol, and 2-ME (0.145 mM). RNAs were extracted using an RNeasy 96 kit (QIAGEN), as described (24), except that each sample was treated with 27 U DNase I during the extraction process. RNA concentrations were determined by spectrophotometry measuring absorbance at 260 and 280 nm, and RNA quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The sample RNA integrity number values ranged from 8.7 to 9.8, with an average of 9.4. Affymetrix GeneChip targets were made by amplification of 50 ng RNA using the WT-Ovation RNA Amplification System (Nugen, San Carlos, CA). The resulting amplified single-stranded cDNAs were purified according to the QIAquick homogenizing column (QIAGEN). Each sample lysate aliquot (100 µl) was combined with 100 µl RLT buffer, 140 µl 100% ethanol, and 2-ME (0.145 mM). RNAs were extracted using an RNeasy 96 kit (QIAGEN), as described (24), except that each sample was treated with 27 U DNase I during the extraction process. RNA concentrations were determined by spectrophotometry measuring absorbance at 260 and 280 nm, and RNA quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies). Amplified cDNAs were fragmented and labeled following the FL-Ovation cDNA Biotin Module V2 protocol (Nugen).

Hybridization, fluids, and scanning were performed according to standard Affymetrix protocols (http://www.affymetrix.com). GeneChip Operating Software (GCOS v1.4) was used to convert the image files to cell intensity data (cel files). All cel files, representing individual samples, were normalized using the scaling method within expression console (EC v1.1: http://www.affymetrix.com) and a scaled target of 1250 to produce the analyzed cel files (chp files) along with the report files. The cel files were imported into Partek Genomics Suite (Partek, St. Louis, MO) and quantile normalized to produce the principal components analysis graph. An ANOVA was performed within Partek to obtain multiple test-corrected p values using the false discovery rate method at the 0.01 significance level and was combined with fold-change values, signal confidence (above background), and call consistency (as a percent) as calculated using custom Excel templates for each comparison (25). The data have been deposited in Gene Expression Omnibus under accession number GSE35332 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pngzzescaqkwta&acc=GSE35332).

Statistical analysis

Data are presented as the mean and SEM. Statistics were analyzed using the two-tailed Student t test. Differences were considered significant at p < 0.05; n represents the number of preparations from individual mice.
Results

Prolonged exposure of developing BMMCs to SCF enhances IL-3–mediated growth but subsequently inhibits Ag-mediated degranulation

BMMCs were cultured in media containing IL-3, SCF, or IL-3 in combination with SCF for up to 8 wk. As reported (10), the combination of IL-3 and SCF (100 ng/ml) significantly enhanced the number of MCs in culture compared with IL-3 alone (Supplemental Fig. 1A). Culturing in SCF alone also supported the growth of mouse BMMCs; however, the yields were inconsistent and significantly lower than those obtained with either IL-3 alone or IL-3 in combination with SCF (data not shown).

In contrast to the ability of SCF to enhance Ag-mediated degranulation on acute exposure (12, 13), cells cultured in IL-3 in the presence of SCF throughout the culture period, hereafter termed chronic SCF-cultured (cSCF)-BMMCs, displayed a marked reduction in the capacity of Ag to induce degranulation compared with the cells grown in IL-3 alone, regardless of whether the cells were nonstarved (Fig. 1A) or starved overnight (Fig. 1B) of cytokines prior to Ag challenge. The attenuated degranulation observed in the cSCF-BMMCs was not a consequence of reduced surface expression of FcεRI or KIT (Supplemental Fig. 1B, 1C), and there were no noticeable differences in the purity, staining characteristics, or morphology of the cells cultured in IL-3 or IL-3 plus SCF (Supplemental Fig. 1D, 1E), other than that cells exposed to SCF were more granulated than were cells cultured in IL-3 alone. Further, the histological examination of cells revealed that, although the cells cultured in IL-3 alone appeared depleted of granules following Ag challenge, the cSCF-BMMCs did not (Supplemental Fig. 2). These latter data are consistent with the conclusion that the reduced Ag-mediated degranulation in the cSCF-BMMCs was due to a defect in the process of degranulation rather than a defect in granule formation.

Both soluble and membrane-associated SCF downregulate MC activation

In addition to its soluble form, SCF exists as a membrane-associated moiety. Thus, fibroblast feeder layers, expressing membrane-bound SCF on the cell surface, support MC culture (26, 27). Therefore, we examined whether membrane-bound SCF, presented in this manner, has the same effect as does soluble SCF. Two-week-old BMMCs, cultured in IL-3 alone, were incubated for an additional 2 wk on a monolayer of NIH 3T3 fibroblasts, and Ag-mediated activation of BMMCs was examined. The cells grown on the feeder layer, although smaller, were densely granulated and had a similar morphological appearance to the cells exposed to soluble SCF (Supplemental Fig. 3). As for BMMCs cultured in the presence of soluble SCF, Ag-induced degranulation in the fibroblast-cultured BMMCs was significantly reduced (Fig. 1C). Together, these data support the conclusion that prolonged exposure of developing BMMCs to either soluble or membrane-bound SCF profoundly attenuates FcεRI-mediated degranulation.

Prolonged SCF exposure blocks enhanced degranulation mediated by acute KIT activation but not by PGE2 and adenosine G protein-coupled receptors

When added concurrently with Ag, SCF (12) via the tyrosine kinase-based receptor KIT, and PGE2 or adenosine via G protein-coupled receptors (GPCRs) (16), synergistically enhance Ag-mediated degranulation, albeit by different mechanisms (16, 28).

To examine whether the hypoactive phenotype observed in the cSCF-BMMCs was restricted to FcεRI-regulated MC activation, cells starved of cytokines overnight were acutely challenged with Ag, SCF, PGE2, or adenosine in the presence of Ag. To further examine whether the hypoactive phenotype was restricted to receptor-operated pathways, we examined degranulation in response to PMA/ionomycin, which circumvents receptor requirements, in BMMCs cultured in the absence or presence of SCF. As shown in Fig. 2A and 2B, the ability of acutely added SCF to enhance Ag-mediated degranulation in the cSCF-BMMCs was also attenuated compared with that observed in the cells grown in IL-3 alone. However, the synergistic actions of PGE2 (Fig. 2C, 2D) and adenosine (Fig. 2E, 2F) were minimally affected. In addition, there was no difference in the capacity of PMA/ionomycin to elicit a degranulation response in the BMMCs, regardless of whether they were cultured in SCF (Fig. 2G). Taken together, these data suggest that the ability of cSCF to alter degranulation may be restricted to receptors that signal through tyrosine kinases and not those that signal via GPCRs.

Downregulation of cytokine production by prolonged exposure to SCF

In addition to degranulation, MCs generate cytokines (29). Therefore, we examined whether sustained exposure to SCF downregulated the production of specific cytokines in the same way.
manner as did degranulation. As shown in Fig. 3, the production of IL-6 (Fig. 3A), TNF-α (Fig. 3B), and, although not statistically significantly, IL-13 (Fig. 3C) in response to Ag, alone or in combination with acute SCF, was reduced in the cSCF-BMMCs compared with BMMCs cultured in IL-3 alone. The production of the chemokine MCP-1 (CCL2) was also reduced in the cSCF-BMMCs when cells were stimulated with Ag alone, but there was little reduction in MCP-1 production when cells were challenged with Ag and acute SCF concurrently (Fig. 3D), potentially reflecting differential transcriptional regulation. In contrast to acute SCF, PGE2 was still capable of interacting synergistically with Ag to stimulate the production of IL-6 in the cells cultured in the presence of SCF (Fig. 3E), again suggesting that this GPCR agonist can overcome the defect induced by extended SCF exposure.

To verify that the diminution in cytokine release accurately reflected a reduction in cytokine production rather than retention within SCF-treated cells, intracellular and extracellular IL-6 levels were determined in cell lysates and media, respectively. The data showed that relatively little IL-6 was retained within cells after their activation (Supplemental Fig. 3C, 3D; note different scales). Thus, the defective cytokine production observed in the cSCF-BMMCs was largely a consequence of a decrease in cytokine generation.

Transformation to a hyporesponsive phenotype occurs in mature BMMCs after a 72-h exposure to SCF

To determine whether the switch to a hyporesponsive phenotype could be induced with reduced exposure times to SCF, newly seeded bone marrow cells were, after initial culturing in media with IL-3 alone, exposed to SCF at weekly intervals. Their capacity to respond to Ag was then examined as they approached maturity (4 wk). These experiments revealed that exposure to SCF for as little as 1 wk prior to reaching maturity (4 wk) diminished the ability of Ag to elicit degranulation (data not shown). To further
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We next determined whether the hyporesponsive phenotype was reversible upon removal of SCF. Because removal of SCF from mature BMMCs developed in the SCF-containing cultures leads to apoptosis (31), we attempted to analyze reversibility of the phenotype in BMMCs cultured in the presence of SCF for the initial 2 wk and then cultured in IL-3 alone for an additional 2 wk. Under such conditions, the majority of cells were still viable, as determined by Annexin V staining (data not shown). As shown in Fig. 4B, removal of SCF from cSCF-BMMCs allows at least a partial reversion of these cells from the SCF-generated hyporesponsive phenotype.

SCF-mediated downregulation of mediator release is concentration dependent

To examine whether the SCF-induced downregulation was “tunable,” bone marrow cells were cultured in the presence of IL-3 with increasing concentrations of SCF. As shown in Fig. 4C, the responsiveness to Ag was inversely proportional to increasing concentration of SCF, demonstrating that SCF can produce a graded MC hyporesponsiveness phenotype dictated by its concentration.

SCF-mediated downregulation of mediator release minimally affects signaling processes leading to calcium mobilization

FceRI-mediated activation of MCs requires a complex signaling cascade that leads to the activation of phospholipase (PL)Cγ and PI3K, ultimately resulting in an increase in cytosolic calcium concentrations, and protein kinase C (PKC) activation, which are obligatory signals for MC activation. In addition, such pathways, in association with those regulated by MAPKs, lead to cytokine production (1, 29, 32, 33). Therefore, we examined critical signaling events initiated by FceRI and SCF in the cSCF-BMMCs, following overnight cytokine deprivation, in an effort to identify the defect that would explain the hypoxic phenotype. We initially examined the phosphorylation of key molecules in the PLCγ-activation pathway (LAT, PLCγ1, PLCγ2, and Btk), PI3K-regulated signaling molecules (AKT and RSK), and MAPKs (ERK1/2, JNK, and p38). As can be seen, we observed very minor (albeit statistically significant) reductions in the phosphorylation of Btk, PLCγ1, PLCγ2, LAT, and JNK but no changes in other molecules examined (including AKT, as a surrogate marker for PI3K activation; RSK; ERK; and p38) (Fig. 5A, 5B). With the exception of Btk and PLCγ2, this reflected a similar minor reduction in protein level (Fig. 5C). To examine whether these changes were of sufficient magnitude to influence downstream signaling, we next examined whether the consequential calcium signal or PKC translocation/activation was attenuated in the cells cultured in SCF. As shown in Fig. 6A, culturing of BMMCs in SCF had no impact on the calcium signal in response to Ag in the absence or presence of acute SCF. Furthermore, there was no evidence of defects in Ag-mediated PKC translocation in the cSCF-BMMCs (Fig. 6B). Taken together, these data demonstrate that the hyporesponsive phenotype observed in the cSCF-BMMCs is not explained by underlying defects in major regulatory pathways responsible for the calcium signal following FceRI aggregation and, by inference, upstream signals regulating these processes. In addition, the lack of modifications of other major regulatory processes, including MAPKs and PKC, did not appear to be the contributory factors to the profound hyporesponsiveness of the cSCF-BMMCs.

Hck is downregulated following prolonged exposure of MCs to SCF

In seeking to identify an alternative explanation for the reduced FceRI-mediated responses in the cSCF-BMMCs, we explored more global changes in expression of signaling proteins by gene-
expression microarray. BMMCs were developed from six litter
mate male mice and cultured in IL-3 in the absence or presence
of SCF for 4 wk, mRNA was extracted, and then relative gene ex-
pression was examined. As expected, a wide array of genes was
either upregulated or downregulated in the cells grown in IL-3+
SCF compared with the cells grown in IL-3 alone (Supplemental
Table I). Some of the more notable, but predictable, differences
were the marked increases in mRNA for the MC proteases,
tryptase \( \alpha/\beta \) 1 and MC protease 4, which would reflect the in-
creased granularity observed in the cSCF-BMMCs (Supplemental
Fig. I). Few, if any, of the genes downregulated by SCF would
be predicted to influence Fc\(\varepsilon\)RI-mediated degranulation, with the
exception of the Src kinase \( hck \) (Supplemental Table I, high-
lighted), which was one of the most highly downregulated genes
in the cSCF-BMMCs.

This downregulation of \( hck \) was of particular interest because
Hck-deficient BMMCs exhibit defective Ag-mediated degranula-
tion and cytokine generation without impairment in the calcium
signal (34), exactly as noted in this study for the cSCF-BMMCs.

To confirm that prolonged exposure to SCF downregulated Hck
expression in BMMCs, we assayed for Hck mRNA and protein in
the cSCF-treated cells. As shown in Fig. 7A, as observed in the
gene-expression array, there was a marked reduction in \( hck \) mRNA
in the cSCF-BMMCs when examined by real-time PCR. Fur-
thermore, there was a pronounced reduction in Hck protein in the
cSCF-BMMCs (Fig. 7B). Downregulation of Hck protein was also
observed at earlier times of SCF exposure (7 d) (data not shown).

To determine whether we could reverse the hypoactive pheno-

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cells are indicated. * respectively), and the differences between IL-3– and IL-3+SCF–cultured in (Ctrl) the mouse hck cDNA, and Hck mRNA expression was determined as SCF, transfected with retroviral vector pMX-puro with (HCK) or without BMMCs from two mice were cultured in IL-3 in the absence or presence of and Hck expression was analyzed by immunoblotting and evaluated. ( ) Determined by quantitative real-time PCR. ( ) The cells in (A) were lysed, and Hck expression was analyzed by immunoblotting and evaluated. (C) BMMCs from two mice were cultured in IL-3 in the absence or presence of SCF, transfected with retroviral vector pMX-puro with (HCK) or without (Ctrl) the mouse hck cDNA, and Hck mRNA expression was determined as in (A). Data in (A) and (B) represent mean and SEM (n = 3 and n = 4, respectively), and the differences between IL-3– and IL-3+SCF–cultured cells are indicated. *p < 0.05, t test.

Thus, although downregulation of hck correlates with, and may help to explain, the observed change to a hyporesponse phenotype, it is also possible that modification of other genes or reduction in the levels of critical signaling proteins following proteolytic digestion may contribute to the cSCF-BMMC hyporesponsiveness.

The hypoactive phenotype is associated with defective cytoskeletal reorganization

Both the calcium signal and Hck regulate cytoskeletal reorganization through processes that regulate actin polymerization/depolymerization, as well as formation of microtubules through tubulin polymerization. Both of these events are essential for MC mediator release (35–40). Thus, we examined whether an SCF-dependent reduction in cytoskeletal reorganization would provide a mechanistic explanation for the attenuated mediator release observed in the cSCF-BMMCs. As revealed by flow cytometry (Fig. 8A) and confirmed by immunofluorescence microscopy (Fig. 8B, 8C), FceRI-mediated actin depolymerization, which is required for degranulation, was substantially attenuated in the cSCF-BMMCs. Furthermore, immunofluorescence microscopy revealed that there was also a marked decrease in α-tubulin polymerization in the cSCF-BMMCs (Fig. 8D, 8E). Taken together, these data are consistent with the conclusion that the SCF-induced hyporesponse phenotype is a consequence of ineffective cytoskeletal reorganization required for mediator release, potentially as a consequence of downregulation of the Hck expression or other signaling proteins downstream of calcium mobilization.

Discussion

The results of this study demonstrate that MCs chronically exposed to SCF have a significantly reduced capacity to degranulate and to generate cytokines in response to Ag. This phenotype was linked to ineffective cytoskeletal reorganization potentially associated with downregulation of Hck. Thus, these findings provide evidence for a novel mechanism by which the extent of MC activation may be programmed through the influence of cytokines present within the immediate microenvironment.

Based on the relative lack of ability of long-term SCF exposure to reduce the capacity of PMA/ionomycin, adenosine, and PGE2 to enhance Ag-mediated degranulation and cytokine production, the hyporesponse phenotype induced by SCF appears to be restricted to MC responses mediated by receptors that signal through tyrosine kinases rather than G proteins. Together with the extended time period (>72 h) required for the onset of inhibition by SCF and the lack of effects on FceRI and KIT expression, we conclude that SCF induced the hyporesponse phenotype through changes in the expression of specific critical signaling protein(s) rather than as a consequence of short-term posttranslational modification (e.g., through protein phosphorylation). The lack of marked defects in inducible critical early and intermediary signaling events, including regulators of calcium mobilization, PI3K, PKC, and MAPKs, in the hyporesponse phenotype, further demonstrated that such downregulation was a consequence of a restricted targeting and not merely a generalized phenomenon.

The lack of any marked defects in these early signaling events additionally indicated that the activation phenotype observed in the cSCF-BMMCs likely reflected downregulation of gene products that control critical downstream processes. Although the expression array revealed that a multitude of genes is upregulated or downregulated in the cells grown in SCF and IL-3 compared with the cells grown in IL-3 alone, based on the current state of knowledge of MC-signaling processes (2, 29), the vast majority of the identified genes appeared to have little relevance to the regulation of MC degranulation and/or cytokine production and release. Nevertheless, a notable exception was the substantial reduction in the expression of the Src kinase family member hck. Indeed, real-time PCR and Western blot analysis confirmed a marked attenuation of the expression of Hck mRNA and protein in the cSCF-BMMCs.

By using hck−/− mice and MCs derived from the bone marrow of these mice, it was shown that Hck deficiency results in an MC phenotype characterized by defective Ag-mediated degranulation and reduced expression of TNF-α and IL-6 (34). The corollary is that upregulation of Hck activity was shown to produce a hyperresponsive MC phenotype (41). Of particular note was the observation that defective degranulation and cytokine production in the hck−/− BMMCs were not associated with a concomitant decrease in calcium flux (34), exactly as observed in the cSCF-BMMCs. However, it should be noted that not all observations in the cSCF-BMMCs recapitulated those observed in the hck−/− BMMCs. In the hck−/− BMMCs, for example, there was an increase in the phosphorylation of Lyn substrates, such as Dok2, as
a consequence of increased Lyn activity. However, we observed that the phosphorylation of Dok2 was slightly reduced in the cSCF-BMMCs (data not shown), suggesting that Lyn activity was not unregulated in these cells.

Hck binds to and/or tyrosine phosphorylates a number of ligands/substrates including WASP, WIP, ELMO1, and α-tubulin (42); with regard to Hck, both WASP (43) and WIP (35) were demonstrated to be essential for MC function. Hck, WASP, and WIP in MCs and other cell types (44, 45) are known to regulate cytoskeletal and/or microtubule reorganization (34, 46, 47) through the control of actin and tubulin polymerization/depolymerization, respectively. Certainly, both of these processes are recognized to be essential for MC degranulation (35–40) and, indeed, it is believed that Hck, WASP, and WIP may exert their influence on MC activation through the regulation of such processes. Thus, our findings that Ag-induced actin polymerization was attenuated in the cSCF-BMMCs support the conclusion that the downregulation of Hck and subsequent defective cytoskeletal reorganization may account for the attenuated degranulation in the cSCF-BMMCs. Nevertheless, it is possible that downregulation of other signaling molecules contributes to this phenotype.

The mode by which cytoskeletal reorganization would prevent FcεRI/KIT-mediated cytokine production is uncertain; however, because of the reduced total cytokine protein levels observed, this is unlikely to be solely due to defective trafficking to the cell surface. A potential explanation is inadequate access of signaling molecules to the nucleus in the cSCF-BMMCs. The process by which SCF leads to downregulation of Hck expression in MCs is also unknown. Thus, both of these issues warrant further investigation.

The identification of the MC phenotypic change induced by chronic exposure to SCF led us to conclude that the cytokine milieu to which MCs are exposed in vivo may exert not only homeostatic control of growth and survival but also of activation. The importance of such a mechanism for regulating MC function in a physiological setting may have evolved to temper inappropriate MC activation during SCF-mediated cell division, expansion, and development where the influence of SCF on MCs would be expected to be greatest. In addition, the plasticity of the phenotype in developing cells would allow reversal of the hyporesponsive phenotype to a more normal responsive phenotype when MCs are mature and resident in tissues. Of potential relevance to this argument was our observation that the degree of the phenotypic hyporesponsiveness correlated with the concentration of SCF to which the cells were exposed. Local concentrations of SCF also dictate the described phenotypic differences associated with serosal (connective tissue) compared with mucosal MCs. Thus, it is also possible that SCF similarly dictate the activation capacity of MCs, as a function of their compartmentalization within their resident tissues. Of interest is the report that connective tissue and mucosal MCs display differential gene expression that may impact Ag-mediated degranulation (48). Nevertheless, the gene profiles associated with these phenotypes appear to be different from those observed in our study. For example, although there was markedly reduced expression of FcγRIIB (Fcgr2b), Rab-GEF1 (Rabgef1), and CD81 (Cdb1) reported in connective tissue MCs associated

FIGURE 8. The effect of extended SCF exposure on actin and α-tubulin polymerization. (A) BMMCs cultured in IL-3 in the absence or presence of SCF were sensitized and then activated with Ag (10 ng/ml) for 0, 2, 5, 10, or 30 min. The cells were fixed/permeabilized, and the amount of F-actin was determined by phalloidin-FITC staining and flow cytometry. (B) The cells in (A) were activated with Ag (10 ng/ml) for 0, 2, or 10 min and fixed/permeabilized, F-actin was labeled with phalloidin-FITC (green), and the nuclei were labeled with DAPI (blue). (C) The fluorescence intensity of the visualized cells in (B) was evaluated. (D) The cells in (A) were activated with Ag (10 ng/ml) for 0, 2, or 10 min and fixed/permeabilized, α-tubulin was labeled with anti–α-tubulin/secondary–Alexa Fluor 568 Ab (red), and the nuclei were labeled with DAPI (blue). (E) The fluorescence intensity of the visualized cells in (D) was evaluated. Images in (B) and (D) are representative of four mice. Data in (A), (C), and (E) represent mean and SEM (n = 4), and differences between IL-3– and IL-3+SCF–cultured cells are indicated. *p < 0.05, t test.
with reduced Ag-mediated degranulation (48), we observed little change in the expression of these genes in the cSCF-BMCMCs (Supplemental Table I).

Thus, it is of interest whether SCF is unique in its ability to downregulate FcεRI-mediated MC activation in the manner reported in this study. It was reported, for example, that IL-10 (49) and TGF-β (50) downregulate MC activation in response to Ag. Although, the mechanism for the downregulation by TGF-β is unknown (50) or may be due to reduction in cell survival (51), the IL-10-mediated downregulation could be explained by downregulation of FcεRI expression (49). However, this is not the case with the hyporesponsive phenotype induced by SCF reported in this article. Given the role of MCs in allergic and autoimmune disorders (52), if these agents or the pathways that they regulate could be used to downregulate MC activation in a clinical setting, these processes would have considerable potential for the treatment of these conditions.

In summary, in this article we described that Ag-mediated MC degranulation and cytokine release are dependent on phenotypic programming in response to extended culture of the cells with SCF, conditions that are likely to exist in vivo. As such, these observations imply that the capacity of MCs to release inflammatory mediators in a physiological setting may be as much dictated by the cytokine microenvironment present during expansion and maturation of the MCs as by the extent of Ag-mediated FcεRI aggregation. Therefore, it is tempting to speculate that polymorphisms or mutations resulting in disruption to such a regulatory process could result in exaggerated mediator release, with the potential of leading to MC-driven pathology. Such observations may aid in the design of novel approaches to downregulate MC activation, providing potential therapeutic approaches for the treatment of allergic disorders.

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Disclosures

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References


