Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Inflammatory Stimuli Up-Regulate Secretion of the Soluble GM-CSF Receptor in Human Monocytes: Evidence for Ectodomain Shedding of the Cell Surface GM-CSF Receptor α Subunit


J Immunol 2002; 169:5679-5688; doi: 10.4049/jimmunol.169.10.5679
http://www.jimmunol.org/content/169/10/5679

References
This article cites 39 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/169/10/5679.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Inflammatory Stimuli Up-Regulate Secretion of the Soluble GM-CSF Receptor in Human Monocytes: Evidence for Ectodomain Shedding of the Cell Surface GM-CSF Receptor α Subunit

Jay M. Prevost, Jennifer L. Pelley, Wei bin Zhu, Gianni E. D'Egidio, Paul P. Beaudry, Carin Pihl, Graham G. Neely, Emmanuel Claret, John Wijdenes, and Christopher B. Brown

Soluble GM-CSF receptor α subunit (sGMαR) is a soluble isoform of the GMαR that is believed to arise exclusively through alternative splicing of the GMαR gene product. The sGMαR mRNA is expressed in a variety of tissues, but it is not clear which cells are capable of secreting the protein. We show here that normal human monocytes, but not lymphocytes, constitutively secrete sGMαR. Stimulation of monocytes with GM-CSF, LPS, PMA, or A23187 rapidly up-regulates the secretion of sGMαR in a dose-dependent manner, demonstrating that secretion is also regulated. To determine whether sGMαR arose exclusively through alternative splicing of the GMαR gene product, or whether it could also be generated through ectodomain shedding of GMαR, we engineered a murine pro-B cell line (Ba/F3) to express exclusively the cDNA for cell surface GMαR (Ba/F3.GMαR). The Ba/F3.GMαR cell line, but not the parental Ba/F3 cell line, constitutively shed a sGMαR-like protein that bound specifically to GM-CSF, was equivalent in size to recombinantly alternatively spliced sGMαR (60 kDa), and was recognized specifically by a mAb raised against the ectodomain of GMαR. Furthermore, a broad-spectrum metalloprotease inhibitor (BB94) reduced constitutive and PMA-, A23187-, and LPS-induced secretion of sGMαR by monocytes, suggesting that shedding of GMαR by monocytes may be mediated in part through the activity of metalloproteases. Taken together, these observations demonstrate that sGMαR is constitutively secreted by monocytes, that GM-CSF and inflammatory mediators up-regulate sGMαR secretion, and that sGMαR arises not only through alternative splicing but also through ectodomain shedding of cell surface GMαR.

leukemic cell lines (5, 6, 11), bone marrow progenitors, monocyte/macrophages, and synovial fibroblasts (6). Less is known about the expression of the alternatively spliced sGMRα protein. A soluble GM-CSF binding protein was identified in medium conditioned by a human chorioncarticoma cell line (JEG-3) (12), but no immunological or biochemical data were presented as to whether this protein was equivalent to alternatively spliced sGMRα. However, a 60-kDa soluble GM-CSF binding protein was recently identified in medium conditioned by human myeloid leukemic cell lines (U937, THP-1, and HL-60), by normal human granulocytes, and in normal human plasma (13). This protein migrated as a 60-kDa band by SDS-PAGE, was recognized by a mAb that was raised against the ectodomain of GMRα, and bound GM-CSF with the same affinity as recombinantly alternatively spliced sGMRα, suggesting that these two proteins were equivalent.

We were interested in determining whether normal human monocytes, which express both cell surface GMRα and the mRNA for sGMRα, could also secrete the alternatively spliced sGMRα protein. We were also interested in determining whether the secretion of alternatively spliced sGMRα by monocytes could be regulated by GM-CSF and other stimuli. Using an ELISA that recognized all soluble isoforms of GMRα, we found that monocytes but not lymphocytes could constitutively secrete a sGMRα-like protein. We also found that stimulation of monocytes with GM-CSF and mediators such as LPS, PMA, and A23187 (Calbiochem, La Jolla, CA) were solubilized in DMSO (Sigma-Aldrich, St. Louis, MO) was reconstituted in PBS. PMA (Sigma-Aldrich) and contaminating granulocytes accounted for expression of the alternatively spliced sGMRα isoform that shares the common ectodomain of GMRα. Linear regression analysis of ELISA data was performed using Graph Pad (Piris, San Diego, CA). All cultures were performed in duplicate and were measured again in duplicate by ELISA. Experiments were repeated with cells from an individual donor or from different donors, as indicated.

**Materials and Methods**

**Cytokines, reagents, and cell culture**

Recombinant human GM-CSF (a gift of Cangene, Mississauga, ON, Canada) and G-CSF (Amgen Canada, Mississauga, ON, Canada) were reconstituted in sterile PBS. LPS (Escherichia coli serotype 0111:B4; Sigma-Aldrich, St. Louis, MO) was reconstituted in PBS. PMA (Sigma-Aldrich) and A23187 (Calbiochem, La Jolla, CA) was solubilized in DMSO (Sigma-Aldrich, Oxford, U.K.) was solubilized in DMSO and used directly. Recombinant alternatively spliced sGMRα was purified from medium conditioned by a baby hamster kidney fibroblast cell line engineered to express the alternatively spliced sGMRα (BHK-sGMRα) as previously described (7). Similarly, a recombinant isoform of sGMRα that was missing the unique 16-aa C-terminal domain of alternatively spliced sGMRα was purified from medium conditioned by the BHK.ECDα cell line (9, 14). Unless otherwise indicated, experiments were performed in sterile 1.5-ml Eppendorf tubes in HEPES-modified RPMI 1640 (Sigma-Aldrich) containing 1% penicillin/streptomycin (Life Technologies, Rockville, MD) and supplemented with 10% heat-inactivated FBS (Sigma-Aldrich) (complete medium) in a 37°C incubator containing 5% CO2.

**Cell isolation**

Blood was collected from healthy volunteers by venipuncture after informed consent. Blood was collected into vacuum containers containing sodium heparin (Vacutainer; BD Biosciences, Mountain View, CA). PBMCs were isolated by density centrifugation of diluted blood over a cushion of Ficollic-Paque Plus; Pharmacia, Peapack, NJ) followed by osmotic lysis of residual erythrocytes. PBMC viability was typically 98% and contaminating granulocytes accounted for <3% of cells as assessed by light scatter and flow cytometry. For lymphocyte isolation, PBMCs were depleted of monocytes by plating them on polystyrene petri dishes. The nonadherent cells were decanted and the procedure was repeated a total of three times. The lymphocyte preparations contained <1% CD14+ cells as assessed by flow cytometry. Monocytes were isolated from whole blood using a negative selection procedure (RosetteSep Monocyte Isolation Kit; StemCell Technologies, Vancouver, British Columbia, Canada) and density centrifugation over Ficoll. The enriched mononuclear cell layer typically consisted of 75–85% CD14+ monocytes.

**Flow cytometry**

The percentage of monocytes in either the PBMC preparations or the monocyte-enriched/depleted preparations was determined by CD14 staining and FACS. Briefly, 105 cells were stained for 15 min on ice with 0.5 μg of a FITC-labeled mouse anti-human CD14 mAb (BD Pharmingen, San Diego, CA). The cells were washed and fixed in PBS containing 1% PBS-buffered formalin. Data were acquired using a FACStation flow cytometer (BD Biosciences) and were analyzed using Flowjo (Treestar, San Carlos, CA). The expression of GMRα on the cell surface was determined in a similar manner using 1 μg of a FITC-labeled mouse anti-human CD14 mAb or an IgG2a-FITC isotype control mAb (BD Pharmingen, San Diego, CA). The cells were washed and fixed in PBS containing 1% PBS-buffered formalin. Data were acquired using a FACStation flow cytometer (BD Biosciences) and were analyzed using Flowjo (Treestar, San Carlos, CA). The expression of GMRα on the cell surface was determined in a similar manner using 1 μg of a FITC-labeled mouse anti-human CD14 mAb or an IgG2a-FITC isotype control mAb (BD Pharmingen, San Diego, CA).

**Detection of total sGMRα protein by ELISA**

Supernatants from cell culture experiments were screened by ELISA for the presence of all sGMRα protein (scD116 ELISA; Diaclone Research, Besancon, France). The scD116 ELISA uses a capture mAb raised against the ectodomain of GMRα (SC06), whereas the detection mAb (SC04) was also raised against the ectodomain of GMRα. Because of this, the scD116 ELISA is not specific for alternatively spliced sGMRα but instead recognizes any sGMRα isoform that shares the common ectodomain of GMRα. Linear regression analysis of ELISA data was performed using Graph Pad (Piris, San Diego, CA). All cultures were performed in duplicate and were measured again in duplicate by ELISA. Experiments were repeated with cells from an individual donor or from different donors, as indicated.

**Engineering of the pBabePuro3/GMRα retroviral construct**

The cDNA for GMRα was generated by PCR from the previously described λ-gt11/GMRα clone (7) with primers designed to amplify the entire coding sequence. The blunt-end PCR product was cloned into the pCR-Script Amp SK+EcoRI cloning vector (Strategene, La Jolla, CA). The full sequence of the cDNA was confirmed by sequencing and was subsequently subcloned into the XhoI–ClaI sites of the retrovirus expression vector pBabePuro3 (gift of Dr. S. Robbins).

**Retroviral infection of the Ba/F3 cell line**

The murine IL-3-dependent pro-B cell line Ba/F3 was kindly provided by Dr. K. Kaushansky (University of Washington, Seattle, WA) and was maintained in RPMI 1640 medium (Life Technologies) with 10% FBS supplemented with 10 ng/ml murine IL-3 (BD Pharmingen). TIG2, a tropic retroviral packaging cell line was maintained in DMEM (Life Technologies) plus 10% FBS. Retroviral infection was performed using stably transfected ϕ2 packaging cells as follows: ϕ2 cells were transfected with pBabePuro3/GMRα using Effectene Reagent (Qiagen, Valencia, CA) and stable transfectants were selected with 3 μg/ml puromycin. These retrovirus-producer ϕ2 cells were grown to subconfluent and used to transfect ϕ2 cells with 0.4 μg/ml polyethyleneimine (PEI)-mediated transfection. The retrovirus-producer ϕ2 cells were grown to subconfluent and used to transfect ϕ2 cells with 0.4 μg/ml polyethyleneimine (PEI)-mediated transfection. The infected cells were grown for 48 h and then were selected in medium containing 4 μg/ml puromycin. Cell surface expression of GMRα on the Ba/F3.GMRα cell line, or lack thereof on the Ba/F3 ceil line, was confirmed by flow cytometry.

**GM-CSF ligand-affinity chromatography**

GM-CSF binding proteins were isolated from medium conditioned by the Ba/F3 cell line (240 ml) or the Ba/F3.GMRα cell line (120 ml) using affinity chromatography as previously described (7–9, 13–15). Briefly, cell-conditioned medium was passed over a Sepharose 4B column (Pharmacia) that was coupled to recombinant human GM-CSF. The column was washed extensively with PBS and the adsorbed proteins were eluted with a 0.1 M glycine buffer (pH 2.5). The eluted fractions were immediately neutralized with 1 M Tris buffer. The neutralized fractions were volume reduced to ~5 μl by centrifugal filtration (Ultrafree, 5 kDa; Millipore, Bedford, MA) and were analyzed by SDS-PAGE and Western blotting.

**SDS-PAGE and Western blotting**

The concentrated eluents were boiled in an equal volume of 2× SDS-PAGE loading buffer in the presence of the reducing agent DTT. The
solubilized and denatured proteins were electrophoresed on 8% SDS poly- 
acrylamide minigels and transferred to polyvinylidene fluoride mem- 
branes (BioTrace PVDF; Pall Corporation, Ann Arbor, MI). The blots 
were blocked in 5% skim milk in TBST and were probed with a mouse mAb 
raised against the ectodomain of GMRα (SD10; a gift of Dr. A. Lopez) 
followed by an HRP-conjugated rabbit anti-mouse IgG secondary Ab (Bio/ 
Can Scientific, Mississauga, Ontario, Canada). Proteins on the blots 
were visualized by ECL (Amersham Life Sciences, Oakville, Ontario, Canada) 
and exposure to x-ray film.

Preparation of rabbit splicing-specific antiserum against the 
16-aa C-terminal tail of alternatively spliced sGMRα

Peptide corresponding to the predicted 16-aa sequence of the alternatively 
spliced sGMRα C-terminal tail (LGYSGCSRQPHRSKTN) was synthe- 
sized and coupled to the carrier protein KLH by the Peptide Synthesis Core 
Facility of the University of Calgary. Female New Zealand white rabbits 
were immunized by bilateral i.m. injection with a total of 50 μg of KLH- 
conjugated peptide in CFA (200 μl total volume). Animals were boosted 
every 3–4 wk with an identical dose of peptide in IFA. A total of five 
injections were administered before the initial test bleed. An affinity col- 
umn was prepared by covalently attaching non-KLH-coupled 16-aa peptide 
to N-hydroxysuccinimide-activated Sepharose 4 Fast Flow resin (Pharma- 
cia), according to the manufacturer’s instructions. The rabbit antiserum 
was then affinity purified by passage first over a Sepharose 4B sham col-

Statistics

Data from representative and replicate experiments are presented as the 
mean ± SEM, whereas the significance of the differences between groups 
was determined by paired or unpaired t test, as applicable to the assay 
condition, with p < 0.05 being deemed a significant change.

Results

sGMRα is secreted constitutively by monocytes but not by 
lymphocytes

To test this hypothesis, we cultured PBMCs at 10^6/ml for 
0–96 h in complete medium. The con- 
ditioned medium was harvested and assayed for the presence of 
sGMRα by sCD116 ELISA (Diaclone Research). PBMCs from 16 
different donors secreted an average of 165 ± 26 pg/ml of sGMRα 
during the 24-h culture period (Fig. 1B). Monocytes were then 
isolated from PBMCs (~3 × 10^7/ml monocytes) and cultured for 
24 h. The monocyte-enriched population secreted 136 ± 58 pg/ml of 
sGMRα during the 24-h culture period, which is consistent with the 
concentration of sGMRα that was secreted by the unfrac- 
tionated PBMCs, suggesting that it was the monocytes and not the 
lymphocytes that were secreting sGMRα. To confirm this, PBMC 
preparations were depleted of monocytes by adherence to plastic 
and were cultured for 24 h. Lymphocytes cultured at 10^7/ml, a 
log-fold higher concentration than was used with the unfrac- 
tionated PBMCs, showed no detectable secretion of sGMRα, 

Secretion of sGMRα by monocytes in the absence of stimula-
tion suggested that monocytes could constitutively secrete 
sGMRα. To test this hypothesis, we cultured PBMCs at 10^6/ml for 
96 h in the absence of stimuli, and the cell supernatants were 
screened for the presence of sGMRα by sCD116 ELISA every 
24 h. PBMCs secreted increasing concentrations of sGMRα for up
to 96 h in culture with significant differences occurring between 0 and 24 h and between 24 and 48 h, suggesting that monocytes could constitutively secrete sGMα as well as constitutively secrete sGMα, because purified monocytes also constitutively secreted sGMα for up to 96 h (Fig. 1D). These observations demonstrate that monocytes constitutively secrete sGMα in the absence of external stimuli.

**Induction of sGMα secretion by GM-CSF, LPS, PMA, and A23187**

GM-CSF, LPS, PMA, and A23187 alter the ability of monocytes and neutrophils to bind GM-CSF on their cell surface, suggesting a role for them in regulating the expression of cell surface GMα (19–23). Because sGMα arises via alternative splicing of the GMα transcript, it was possible that these stimuli might also regulate the secretion of sGMα. To test this hypothesis, we incubated PBMCs from individual donors with increasing concentrations of GM-CSF, LPS, PMA, or A23187 and analyzed the conditioned medium for the presence of sGMα. GM-CSF increased the secretion of sGMα in a dose-dependent manner, with half-maximal activity occurring at 262 pg/ml (Fig. 2A). LPS also up-regulated sGMα secretion in a dose-dependent manner with 1.5 ng/ml inducing a half-maximal response (Fig. 2B). PMA and A23187 also induced a dose-dependent increase in sGMα secretion with half-maximal induction occurring with 8 ng/ml PMA and 1 ng/ml A23187 (Fig. 2, C and D, respectively). DMSO, used at a maximal dilution of 1/1000 (0.1%) in the dose response experiments, had no effect on sGMα secretion by monocytes (data not shown). These results demonstrate that GM-CSF and other mediators increase the secretion of sGMα by monocytes in a dose-dependent manner.

To further investigate the kinetics of the up-regulation of sGMα secretion, we incubated PBMCs with 100 ng/ml PMA for 10, 20, or 30 min and analyzed the harvested cell-conditioned medium for the presence of sGMα using the Diaclone sCD116 ELISA. PMA up-regulated sGMα secretion by PBMCs within 10 min (Fig. 2E), demonstrating that induction of sGMα secretion occurs rapidly after cell stimulation.

**Assay development for the specific detection of alternatively spliced sGMα**

sGMα was believed to arise exclusively through alternative splicing of the GMα gene product (4–7). However, the rapid secretion of sGMα from monocytes in response to stimulation with PMA (Fig. 2E) suggested that sGMα may arise in part through a mechanism other than de novo translation of the alternatively spliced mRNA. Because available Abs were not specific for the alternatively spliced sGMα protein but instead recognized the shared ectodomain of GMα, we endeavored to produce an Ab that was specific for the alternatively spliced sGMα isoform. To this end, we immunized rabbits with a 16-aa peptide that corresponded to the unique C-terminal tail of the alternatively spliced sGMα protein. The rabbit antisera was purified by affinity chromatography using the 16-aa peptide as a ligand. The specificity of the purified antisera (splicing-specific antisera for alternatively spliced sGMα) was then tested by SDS-PAGE and immunoblotting. Both recombinant alternatively spliced sGMα protein, which contains the 16-aa C-terminal domain, and recombinant sGMα lacking the 16-aa C-terminal tail (non-alternatively spliced) were recognized by a mAb (8D10) that was raised against the shared ectodomain of GMα. Neither the alternatively spliced nor the non-alternatively spliced recombinant sGMα proteins were recognized using rabbit preimmune serum, and only the alternatively spliced sGMα protein was recognized with the splicing-specific antisera (Fig. 3A). Furthermore, preincubating the splicing-specific antisera with an excess of the 16-aa peptide Ag (splicing specific + peptide) completely inhibited the ability of the splicing-specific antisera to recognize alternatively spliced sGMα. Taken together, these results demonstrate that the rabbit splicing-specific antisera specifically recognizes only the alternatively spliced sGMα isoform, which contains the unique 16-aa C-terminal domain.

*Figures and equations*...
recognizes the extracellular domain of GMR onto a SCO6-coated ELISA plate. Total sGMR but importantly only the Diaclone sCD116 detected the non-alternatively spliced sGMR. ELISAs detected similar amounts of alternatively spliced protein, but importantly only the Diaclone sCD116 detected the non-alternatively spliced sGMR variant protein (Fig. 3B), which demonstrates the specificity of the splicing-specific ELISA for alternatively spliced sGMR. The splicing-specific ELISA also failed to detect a 1000-fold higher amount of the non-alternatively spliced sGMR variant protein (data not shown). Together, these results demonstrate the successful modification of the sCD116 ELISA using the splicing-specific antiserum to detect only the alternatively spliced sGMR protein. However, although the splicing-specific and sCD116 ELISAs are equally effective at detecting recombinant purified alternatively spliced sGMR, the splicing-specific ELISA presently has a background signal when evaluating alternatively spliced sGMR in PBMC-conditioned medium. Therefore, although our new splicing-specific ELISA is an excellent tool for determining the relative levels of alternatively spliced soluble receptor secreted by monocytes, it does not at present allow us to make any conclusions about the absolute amounts of alternatively spliced sGMR secreted by monocytes.

Because the presence of alternatively spliced sGMR mRNA in monocytes had previously been demonstrated (6), we anticipated that the sGMR protein being produced by monocytes would represent the alternatively spliced isoform. However, the rapid up-regulation of sGMR secretion by PBMCs in response to stimulation led us to hypothesize that monocytes might also secrete a sGMR variant generated through ectodomain shedding. With the development of the splicing-specific ELISA, we were now able to test this hypothesis. Because most known sheddases are metalloproteases (24), we decided to treat unstimulated and stimulated monocytes with the broad-spectrum metalloprotease inhibitor BB94 (batimastat) and to look at the effect of this inhibitor on the secretion of total and alternatively spliced sGMR by monocytes. To this end, we pretreated isolated PBMCs (from n = 7 individual donors) for 2 h at 37°C with 100 μM of either the metalloprotease inhibitor BB94 or the appropriate vehicle control (1% DMSO), pelleted the cells, and resuspended them in fresh medium containing either 1% DMSO or 100 μM BB94. Cell viability was assayed by trypan blue dye exclusion, and no significant difference between the viability of the DMSO- or BB94-treated cells was noted (data not shown). The PBMCs were then either left unstimulated or stimulated for an additional hour using concentrations of PMA, A23187, LPS, or GM-CSF, which were predicted to induce maximal sGMR secretion based on the dose response curves in Fig. 2. This conditioned medium was then collected and screened in parallel on three different ELISAs: the Diacalone sCD116 ELISA was used to quantitate total sGMR levels (Fig. 4A), the splicing-specific ELISA was used to look at levels of only the alternatively spliced sGMR variant (Fig. 4B), and the samples were also screened on an IL-8 ELISA. Importantly, treatment of PBMCs with 100 μM BB94 led to a significant decrease in the amount of total sGMR secreted constitutivelly (p = 0.02) and in response to PMA (p = 0.004), A23187 (p = 0.001), and LPS (p < 0.001), but it did not have a significant effect on the amounts of secreted alternatively spliced sGMR (Fig. 4B) or IL-8 (data not shown). The lack of effect on either alternatively spliced sGMR or IL-8 suggests that BB94 does not have a nonspecific effect on cell viability or protein secretion by PBMCs. This implies that a metalloprotease-mediated cleavage of GMR from the surface of human monocytes can lead to the generation of a shed sGMR variant.

Because we were concerned about the presence of 1% DMSO in all of these samples, in a separate experiment we looked at the effect of 1% DMSO on secretion of sGMR by PBMCs. The presence of 1% DMSO led to a statistically significant decrease in the

---

**FIGURE 3.** Production of rabbit antiserum against the 16-aa C-terminal domain of alternatively spliced sGMR. A. Recombinant sGMR protein containing the 16-aa C-terminal domain (A, alternatively spliced) or a recombinant version of sGMR that is missing the 16-aa epitope (NA, non-alternatively spliced) was fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunoblotting. The blots were probed with a mAb raised against the shared domain of cell surface GMR. B. 1 ng/ml purified recombinant alternatively spliced (A) or non-alternatively spliced (NA) sGMR protein was loaded onto a SCO6-coated ELISA plate. Total sGMR protein was detected using the biotinylated mAb SCO4, whereas alternatively spliced sGMR protein was detected using biotinylated splicing-specific antiserum.
Looking at the production of total sGMR either the alternatively spliced or shed sGMR (PMA, A23187, LPS, and GM-CSF) on the regulated secretion of –/H9262 (control) or in the presence of 100 ng/ml PMA, a concentration of 10^7/ml in fresh medium containing either DMSO or BB94. spliced sGMR/H9251 for that amount of total sGMR BB94 inhibits secretion of total sGMR FIGURE 4.

Expression of GMRα on the cell surface of the Ba/F3.GMRα cell line, but not on the surface of the parental Ba/F3 cell line, was confirmed by flow cytometry using the anti-GMRα mAb 8G6 or an IgG1 isotype control (Fig. 5A). The Ba/F3 and the Ba/F3.GMRα cell lines were then cultured at a density of 10^7/ml in complete medium for 2 h. The cell-conditioned medium was harvested and screened for the presence of total GMRα protein by sCD116 ELISA. There was no detectable sGMRα in the Ba/F3 conditioned medium, but the Ba/F3.GMRα cell line secreted 157 ± 13 pg/ml sGMRα (Fig. 5B), demonstrating that the ectodomain of GMRα could be shed from the cell surface. We were also interested in determining whether the ectodomain shedding of GMRα from the Ba/F3.GMRα cell line could also be induced using the stimuli shown previously to up-regulate sGMRα secretion by monocytes. The Ba/F3 cell line is not LPS-responsive (25) and lacks the β subunit of the human GM-CSF receptor. Therefore, we looked only at the effect of PMA and A23187 on the shedding of sGMRα from the Ba/F3.GMRα cell line. Neither stimulus appeared to have an effect on the shedding of sGMRα from the surface of these cells (data not shown).

To verify that the sGMRα produced by the Ba/F3.GMRα cell line was in fact being derived by enzymatic cleavage rather than by nonspecific shearing from the cell surface, we pretreated Ba/F3.GMRα cells with BB94 (100 μM) or with a 1% DMSO vehicle control for 2 h, then pelleted the cells, resuspended them in fresh medium, and allowed them to condition medium for an additional hour. The cell-conditioned medium was then screened using the sGMRα sCD116 ELISA. Treatment of cells with BB94 completely inhibited the production of sGMRα by these cells (Fig. 5C), further supporting the hypothesis that a sGMRα variant can arise through metalloprotease-mediated ectodomain shedding. There was no significant difference in the viability of the DMSO vehicle control or BB94-treated Ba/F3.GMRα cells after 3 h, as measured by trypan blue dye exclusion (data not shown).

To determine the molecular mass of the constitutively shed sGMRα protein, we attempted to purify the sGMRα-like protein out of medium conditioned by the Ba/F3.GMRα cell line. To this end, Ba/F3 or Ba/F3.GMRα conditioned medium was passed over a GM-CSF-Sepharose 4B ligand affinity column and the adsorbed
the concentration of 10^7/ml in complete medium supplemented with murine IL-3 in the presence of 1% DMSO (vehicle control) or 100 μM BB94, and then the cells were pelleted and resuspended in fresh medium containing DMSO or BB94 and incubated in triplicate for an additional hour. The conditioned medium was then screened for the presence of the shed sGMRα variant using a GM-CSF ligand affinity column. The column was washed extensively with PBS and the adsorbed proteins were eluted with a 0.1 M glycine buffer (pH 2.5). The eluted fractions were pH neutralized, volume reduced, and fractionated by SDS-PAGE, along with recombinant alternatively spliced sGMRα. Proteins were analyzed by SDS-PAGE and Western blotting. No sGMRα-like proteins were purified from the Ba/F3 cell-conditioned medium; however, a distinct protein band was present in the Ba/F3.GMRα column eluent (Fig. 5D). This 60-kDa protein was the same size as recombinant alternatively spliced sGMRα and was specifically recognized by the anti-GMRα mAb (8D10) (Fig. 5D), but not by the anti-alternatively spliced sGMRα rabbit splicing-specific antiserum (Fig. 5E), demonstrating that the sGMRα protein released from the Ba/F3.GMRα cells arose via shedding of cell surface GMRα. Importantly, the fact that we were able to enrich this shed sGMRα variant using a GM-CSF ligand affinity column suggests that the shed sGMRα protein retained the ability to specifically bind to GM-CSF.

Discussion

The alternatively spliced sGMRα mRNA has been identified in many cell types that express GMRα, including human placental tissue (4, 7), myeloid leukemic cell lines (5, 11), monocytes, macrophages, bone marrow progenitors, and synovial fibroblasts (6). However, there is little information available about which primary human cells secrete the protein. In this paper, we demonstrate that monocytes constitutively secrete sGMRα but lymphocytes do not (Fig. 1). More importantly, we also demonstrate that secretion of sGMRα by monocytes can be rapidly up-regulated by GM-CSF, LPS, PMA, and A23187 (Fig. 2). We further describe the production of a rabbit antiserum that specifically recognizes the 16-aa tail of the predicted alternatively spliced sGMRα protein (Fig. 3), and using this new “splicing-specific” antiserum in an ELISA, we demonstrate for the first time that monocytes do indeed secrete the predicted alternatively spliced sGMRα protein (Fig. 4B). We also demonstrate that the secretion of total sGMRα (Fig. 4A), but not of alternatively spliced sGMRα (Fig. 4B) or IL-8 (data not shown), can be inhibited using the broad-spectrum metalloprotease inhibitor BB94. Together, these results show for the first time that monocytes secrete sGMRα protein that represents a mixed population of alternatively spliced and proteolytically cleaved species.

Monocytes but not lymphocytes express GMRα on their cell surface (Fig. 1A), suggesting that monocytes might also secrete sGMRα. PBMCs and an equivalent number of purified monocytes secreted similar concentrations of sGMRα in vitro, whereas lymphocytes secreted none (Fig. 1B). Secretion of sGMRα by human monocytes is consistent with previous findings that demonstrated expression of the alternatively spliced sGMRα mRNA in human monocytes (6) and secretion of a sGMRα protein by myeloid leukemic cell lines (13). The inability of lymphocytes to secrete GMRα is consistent with the lack of sGMRα mRNA in lymphocytes (11) and the absence of GMRα expression on the cell surface of normal human lymphocytes (Fig. 1A). Together, these results show that normal human monocytes but not lymphocytes can secrete a sGMRα protein.

In the absence of stimulation, human monocytes secreted sGMRα for up to 4 days in culture (Fig. 1, C and D), suggesting...
that monocytes constitutively secrete sGMRα. Other soluble cytokine receptors, such as sTNFR-p55/p75 (25) and sIL-6R, are also constitutively secreted in vitro by normal human monocytes and monocyte-like cell lines (27, 28). Furthermore, sIL-1RII (29, 30), along with sTNFR-p55 (31), sIL-6R (32), and sGMRα (13), are also constitutively present in normal human plasma, suggesting that sGMRα may also be constitutively secreted in vivo. The reason for the constitutive secretion of sGMRα is unclear. It is possible that sGMRα is constitutively secreted to modulate the activity of GM-CSF during homeostasis. However, GM-CSF, like most other cytokines, is secreted in a tightly regulated manner and does not normally circulate in human plasma in the absence of inflammation or disease. Elucidation of the role of sGMRα during homeostasis will have to await further investigation.

Stimulation of monocytes with GM-CSF, LPS, PMA, and A23187 up-regulated the secretion of sGMRα in a dose-dependent manner (Fig. 2, A–D), demonstrating that sGMRα secretion could be regulated by its cognate ligand, proinflammatory stimuli (LPS), and heterogeneous chemical stimuli (PMA and A23187). DMSO, used at a maximum dilution of 1/1000 (0.1%) in the dose response experiments, had no effect on sGMRα secretion (data not shown).

Together, these results demonstrate that stimulation of monocytes can increase the secretion of sGMRα.

The regulation of secretion of a soluble cytokine receptor by its cognate ligand has been demonstrated in vitro and in vivo for TNF-α (33–35), where stimulation of cells with TNF-α or i.v. injection of recombinant TNF-α into mice leads to the rapid up-regulation of sTNFR-p55/p75 secretion. Although the signals-transduction pathways responsible for ligand-induced secretion of soluble receptors are unclear, it is likely that secretion is initiated after ligand-induced phosphorylation of the cognate surface receptor complex. This suggests that soluble cytokine receptors such as sGMRα and sTNFR-p55/p75 may play a critical role in modulating the activity of their respective cytokines during inflammation and hematopoiesis.

It is interesting to note that recombinant sGMRα antagonizes GM-CSF activity in vitro, preventing GM-CSF-induced cell proliferation (5, 10), bone marrow colony formation (7), and neutrophil functional activity (J.M.P. and C.B.B., unpublished data), suggesting that sGMRα secretion in response to GM-CSF stimulation may indeed occur to moderate the activity of GM-CSF during inflammation and/or hematopoiesis.

Proinflammatory mediators such as LPS, along with chemical stimuli such as PMA and A23187 that work through divergent signaling pathways that do not necessarily result in phosphorylation of βc, also up-regulated monocyte secretion of sGMRα (Fig. 2), demonstrating a direct role for inflammatory mediators in regulating sGMRα secretion. LPS, PMA, and A23187 have also been shown to up-regulate the secretion of other soluble cytokine receptors such as sIL-1RII (29, 36), sIL-6R (27, 28), and sTNFR-p55/p75 (27), highlighting a common role for inflammatory mediators in regulating the secretion of not only proinflammatory cytokines, but also their respective soluble cytokine receptors.

The rapid release of sGMRα from monocytes in response to stimulation with PMA (Fig. 2E) made us wonder whether sGMRα may in fact arise through proteolytic cleavage of cell surface GMRα rather than, or in addition to, alternative splicing of the GMRα gene product. To this end, we generated rabbit antiserum that specifically recognized the 16-aa C terminus of alternatively spliced sGMRα, allowing us to differentiate between sGMRα that arose via alternative splicing and sGMRα that arose via another mechanism (Fig. 3A). Using this reagent, we developed a splicing-specific ELISA that allowed us to demonstrate conclusively for the first time that monocytes could secrete alternatively spliced sGMRα protein (Fig. 4B). However, it also became clear that there was an additional sGMRα variant being produced by monocytes. Because metalloprotease-mediated cleavage of cell surface receptors is a common mechanism through which other soluble receptors arise (24, 37, 38), we wondered whether our non-alternatively spliced sGMRα protein may also have arisen via metalloprotease-mediated ectodomain shedding of cell surface GMRα. Using a broad-spectrum metalloprotease inhibitor (BB94), we demonstrated that the amount of total sGMRα protein released by monocytes was reduced in the presence of BB94 (vs a 1% DMSO vehicle control) (Fig. 4A). Importantly, release of the alternatively spliced sGMRα protein (Fig. 4B) or of IL-8 (data not shown) from PBMC cultures was not inhibited by BB94, demonstrating that the observed inhibition of the non-alternatively spliced sGMRα protein production was not due to a nonspecific down-regulation of protein secretion. There was no difference in the viability of the 1% DMSO vehicle control and BB94-treated PBMCs after 2 h. However, the presence of 1% DMSO in PBMC cultures did significantly reduce the amount of sGMRα secreted by PBMCs as compared with PBMCs in the absence of DMSO.

We had expected that the sGMRα produced constitutively by monocytes would be the product of alternative splicing and that shedding would account for the additional sGMRα protein produced in response to stimulation, as has been previously demonstrated for sIL-6R in THP-1 cells (28). Using our new splicing-specific ELISA, we now have demonstrated that alternatively spliced sGMRα can be secreted constitutively by monocytes (Fig. 4B). However, both PMA and A23187 treatment led to a statistically significant up-regulation of the alternatively spliced sGMRα variant (Fig. 4B), which suggests that additional alternatively spliced sGMRα can be inducibly secreted by monocytes upon exposure to chemical stimuli. We conclude that alternatively spliced sGMRα can be produced by monocytes both constitutively and in response to stimulation.

Similarly, shedding of GMRα from the surface of monocytes also appears to occur constitutively, because BB94 treatment of unstimulated cells led to a decrease in secretion of total sGMRα. At present, because of a background signal observed when analyzing PBMC-conditioned medium on the splicing-specific ELISA, the sCD116 and splicing-specific ELISAs can be used to measure relative levels of total and alternatively spliced sGMRα, but cannot yet be used to reliably quantitate the amount of shed sGMRα being produced by monocytes. It is therefore somewhat more difficult to make conclusions about whether the shedding of GMRα from the surface of monocytes is also a regulated process. However, we would argue that because LPS up-regulates the secretion of total sGMRα in the absence of BB94 (p = 0.05) but not when BB94 is present, this implies that LPS might be specifically up-regulating the shedding of sGMRα. This conclusion is further supported by the observation that LPS did not significantly up-regulate the amount of alternatively spliced sGMRα produced by monocytes (Fig. 4B). Therefore, it appears that LPS may be acting to induce total sGMRα protein production by specifically up-regulating the proteolytic activity of the relevant sheddase. As mentioned previously, PMA and A23187 both led to the up-regulated secretion of both total and alternatively spliced sGMRα by monocytes. On the basis of the data presented in Fig. 4A, we would suggest that the degree of the up-regulated secretion in response to PMA and A23187 seems to be reduced by BB94 treatment. Thus, PMA and A23187 may up-regulate the shedding of GMRα from the surface of monocytes in addition to inducing the secretion of alternatively spliced sGMRα. However, we are cautious about making conclusions as to the effect of PMA and A23187 on shedding of sGMRα until we are able to specifically quantitate the amount of shed sGMRα being produced by monocytes or are able...
to directly measure the effect of PMA and A23187 on the activity of the relevant sheddase.

The ability of GMRα to be shed from the cell surface was confirmed using a murine pro-B cell line that we engineered to express cell surface GMRα but not alternatively spliced sGMRα (Ba/F3.GMRα; Fig. 5A). A sGMRα-like protein was detected by ELISA in medium conditioned by the Ba/F3.GMRα cell line but not from the parental Ba/F3 cells (Fig. 5B) or pBabe vector control cells (data not shown). Importantly, treatment of Ba/F3.GMRα cells with BB94, but not with the DMSO vehicle control, led to a complete inhibition of sGMRα by these cells (Fig. 5C), confirming that the sGMRα released by these cells is generated by proteolytic cleavage rather than by vesicle budding or nonspecific shearing of the receptor from the cell surface.

The sGMRα variant was then purified from Ba/F3.GMRα-conditioned medium using a GM-CSF-Sepharose affinity column, which indicates that this shed sGMRα protein retains the ability to bind specifically to GM-CSF. This was not surprising because we have previously demonstrated that purified recombinant GMRα extracellular domain binds to GM-CSF with an affinity comparable to the interaction between GM-CSF and alternatively spliced sGMRα (9, 14). Furthermore, the 60-kDa shed Ba/F3.GMRα variant was recognized specifically by a mAb raised against the ectodomain of GMRα (Fig. 5D), but not by our splicing-specific antisera (Fig. 5E). The similar electrophoretic mobility of the shed and alternatively spliced isoforms (Fig. 5D) is, at least in part, accounted for by the heterogeneous glycosylation of the extracellular domain of GMRα (7) that would blur the differences in the length of the primary sequence of the two isoforms. The similar mobility may also provide a hint as to the cleavage site of the shed GMRα. The apparent 60-kDa molecular mass of the shed GMRα suggests that the cleavage site lies very close to the membrane. If we assume that the WSSWS motif conserved among cytokine receptors and spanning residues 305–310 in GMRα (9, 14) is cleaved during the shedding of the soluble receptor, we can rapidly up-regulate its secretion. We have developed an Ab that specifically recognizes the predicted alternatively spliced GMRα protein and have used it in an ELISA to demonstrate that monocytess do in fact secrete alternatively spliced GMRα. Furthermore, we have demonstrated for the first time that the ectodomain of cell surface GMRα can be shed in vitro and that shedding is mediated in part through the activity of metalloproteases. This dual mechanism of soluble receptor production has also been seen for other cytokine receptors such as IL-6Rα, IL-4R, and the growth hormone receptor (reviewed in Ref. 39). It will be of interest to determine whether the alternatively spliced and shed GMRα proteins have similar or unique biological functions in mediating GM-CSF-induced inflammation and/or hematopoiesis.

Acknowledgments

We thank Laurie Robertson of the Alberta Cancer Board/University of Calgary Flow Cytometry Facility, the staff of the Alberta Cancer Board/University of Calgary Hybridoma Facility, and Diane Teoh for excellent technical assistance. We would also like to thank Dr. Angel Lopez for mAbs B6G and B6D10, Dr. Ken Kaushansky for the Ba/F3 cell line, and Dr. Stephen Robbins for thoughtful discussions during this project and in the preparation of this manuscript.

References


