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The Role of Ets-1 in Mast Cell Granulocyte-Macrophage Colony-Stimulating Factor Expression and Activation

Leigh H. McKinlay,* Martin J. Tyms,* Ross S. Thomas,* Arun Seth,† Suzanne Hasthorpe,‡ Paul J. Hertzog,* and Ismail Kola²* "Ets-1 is a transcription factor with restricted expression in lymphocytes, and it has been implicated in the regulation of T cell genes such as TCRα, TCRβ, CD4, IL-2, and TNF-α. We show in this study that Ets-1 is also expressed in some mast cells constitutively and can be induced in primary mast cells with stimuli that activate mast cells. We also show that Ets-1 plays a role in the regulation of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine expressed by activated mast cells. We have characterized a murine growth factor-dependent mast cell line, FMP6,—, derived from a factor-dependent cell line, FMP1.6. FMP6— has acquired a distinct connective tissue mast cell-like phenotype, as characterized by the expression of mast cell proteases MMCP-4 and MMCP-6, expression of IL-12, and the down-regulation of IL-4. The parental FMP1.6 cell line displays a mucosal mast cell-like phenotype. FMP6— cells have increased Ets-1 expression and achieve growth-factor independence by the autocrine production of GM-CSF and IL-3. Transient transfection of an Ets-1 expression construct in FMP6— cells results in transactivation of a GM-CSF reporter, while a point mutation in the consensus Ets binding site in the conserved lymphokine element, CLE0, abolishes Ets-1 transactivation. Importantly, antisense Ets-1 demonstrates an ability to repress the activity of the GM-CSF reporter. These data suggest a role for Ets-1 in mast cell growth regulation and activation, and because of the central role of mast cells in inflammatory processes, such as asthma and rheumatoid arthritis, they identify Ets-1 as potentially contributing to the pathophysiology of such diseases. The Journal of Immunology, 1998, 161: 4098–4105.

Activated mast cells play a central role in inflammatory and IgE-mediated immediate hypersensitivity reactions due to the production of molecules including histamine, heparin, proteases, leukotrienes, prostaglandins, and, importantly, cytokines that have inflammatory and vasoactive properties (1–4). Mast cell cytokines play a pivotal role in IgE-dependent reactions by affecting cell types present in the tissue (e.g., stromal cells, vascular endothelial cells, lymphocytes, and eosinophils) that amplify the inflammatory reaction by recruiting leukocytes to the region. The local expansion of mast cell populations occurs via autocrine factor production or as a result of cytokines secreted by local and recruited cell types. Mast cells originate from hematopoietic stem cells in the bone marrow. Murine mast cell-committed precursors, termed promastocytes, are Thy-1low c-kithigh cells containing cytoplasmic granules. Furthermore, they do not express the high affinity IgE receptor, but express mast cell proteases MMCP-2 and MMCP-4 (5). Following migration from the bone marrow and circulation in the bloodstream, promastocytes complete their differentiation in peripheral sites (6, 7). Mature murine mast cells are able to assume one of two phenotypes, as determined by cytokines present in the local microenvironment (8, 9). Mucosal mast cells (MMC), which are prevalent in the mucosa of the gastrointestinal tract and lung, are dependent upon T cell-derived cytokines IL-3 and IL-4 (10, 11). Connective tissue mast cells (CTMC), which are ubiquitous within connective tissue (i.e., skin, peritoneal cavity, and musculature), are independent of T cell cytokines, but depend on fibroblast-derived stem cell factor (12, 13). CTMC characteristically produce IL-12 and contain histamine and heparin in their granules. In contrast, MMC produce IL-4, store low quantities of histamine, and produce chondroitin sulfate E, rather than heparin (11). Differential expression of one or more mast cell proteases additionally may be used to distinguish between MMC, CTMC, and immature mast cell populations. CTMC are known to express MMCP-4 and MMCP-6, while neither of these is expressed in MMC (14). Immature bone marrow-derived mast cells (BMMC) express MMCP-6, but not MMCP-4, which is a late transcribed protease not present in immature progenitor populations (15).

The cytokine GM-CSF is expressed by activated mast cells and has been implicated in the autocrine regulation of mouse mast cell proliferation (1, 2, 3, 16). Regulation of GM-CSF expression has been studied extensively in T cells, and a number of elements in the proximal promoter have been identified that are important for promoter activity. One such element is the conserved lymphokine element 0, or CLE0, which is also found in the proximal promoters of other cytokine genes, such as IL-4, IL-5, and granulocyte CSF (17, 18). This element contains consensus binding sites for transcription factors of the Ets and activator protein-1 families, and also a weak binding site for nuclear factor of activated T cells. Recent studies have implicated Ets-1 in the regulation of GM-CSF in T cells through the Ets site in CLE0 (19–21). Only one report exists in the literature regarding the transcriptional regulation of GM-CSF in mast cells in which a region between −108/−72 of...
the GM-CSF promoter was found to be responsive to FceRI-induced mast cell activation (22). Given that expression studies have shown that Ets-1 is highly expressed in all T and B cells and some, but not other mast cells (23), we have investigated the functional significance of Ets-1 expression, its relationship to mast cell characterization, and its role in the regulation of GM-CSF transcription in mast cells.

We have compared a factor-dependent mast cell line FMP1.6 with a spontaneous factor-independent derivative FMP6– and found these to have MMC- and CTMC-like phenotypes, respectively. We show that FMP6– has become factor independent through the up-regulation of cytokine genes including GM-CSF. We also show that Ets-1 is highly expressed in FMP6– compared with FMP1.6 cells, and that Ets-1 can transactivate a GM-CSF reporter in FMP6– cells, while an antisense Ets-1 construct represses reporter activity. Furthermore, Ets-1 expression is increased by stimulation in FMP6– and in primary cultures of BMMC, suggesting a role for Ets-1 in the activation process.

Materials and Methods

Cell lines

The murine mast cell line FMP1.6 was isolated from the FMP1.1 clone derived from a male DBA-2 mouse that was injected i.p. with cell-free supernatant from Friend virus producing erythroleukemia cells (24). Factor-dependent FMP1.6 cells were maintained in IMDM media supplemented with 20% FCS, 2% PWM (24), plus 100 U/ml penicillin, and 100 µg/ml streptomycin. Murine mast cell line FMP6– was derived as a spontaneous factor-independent variant from a culture of FMP1.6 cells that was observed to hyperproliferate. FMP6– cells were cultured in IMDM medium containing 10% FCS and antibiotics. Factor-dependent 32Cl2 cells were cultured in IMDM medium containing 20% PWM, 10% FCS, and antibiotics. Cell lines were cultured at 37°C in a humidified atmosphere with 5% CO2. Mast cells and lines were activated by incubation with 20 ng/ml PMA and 1 µM ionomycin for 3 h.

Production of BMMC

Primary BMMC populations were prepared essentially as previously described (25) using bone marrow cells flushed from the femurs and tibias of 6- to 10-wk BALB/c mice. RPMI 1640 containing 4 mM l-glutamine, 5 × 10–5 M β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 mM nonessential amino acids was used to collect bone marrow cells. Cells were washed twice in this media and resuspended at 2 × 105 cells/ml in RPMI media supplemented with 10% FCS and 5% X63 IL-3-conditioned medium produced serum-free. The X63 cell line is a murine mammary tumor cell line expressing a transfected murine IL-3 gene (26). The bone marrow cultures were incubated in a 5% CO2 incubator at 37°C. Every 3 to 4 days, the adherent cells were removed and the population of suspension cells was given one-half volume fresh medium. By 21 days, more than 95% of the cells were identified as mast cells, and this was confirmed by electron microscopy, staining with acidic toluidine blue, and by FACS analysis using IgE and c-kit Abs.

Histochemistry

Formalin-fixed paraffin-embedded sections of FMP6– and FMP1.6 cells were stained with Alcian blue/safranin, as described (27). After staining, sections were dehydrated before mounting in DePex (Poole, U.K.).

Ab neutralization experiments

Cells were seeded at 8 × 104 cells/ml in 96-well round-bottom microtiter plates and titrated in quadruplicate with neutralizing Abs for GM-CSF or IL-3 (Genzyme, Cambridge, MA) at a final concentration of 1 µg/ml. mAbs of the same isotype were used as a control. Each day for 3 days, viable cells were stained using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and absorbance was read at 540 nm (28).

RT-PCR

Total RNA was extracted using guanidinium isothiocyanate, as previously described (29). Reverse transcription was conducted by annealing 300 ng of total RNA with 20 pmol of each of the 3’ oligonucleotides below. To this was added 1× reverse-transcriptase buffer, 5 mM MgCl2, 1 mM each dNTP, 0.5 U RNasin, and 3 U avian myeloblastosis virus (AMV) reverse transcriptase. Following a 60-min incubation at 42°C, samples were chilled on ice. Each 50-µl PCR contained 1× Taq DNA polymerase buffer, 125 µM dNTPs, 1.5 mM MgCl2, 50 pmol of each primer, and 5 U Taq DNA polymerase. PCR primer sequences were: IL-12p35, 5’-ACCAGCACCATTGAAGACTG and GACTGCTAGCCTCGAT; IL-4, 5’-AGGTCACAAGGAAAGGC and CAAGACGAGGTCTTCCG; β-actin, 5’-GGGCTGAGAAGGCTCTCTCTG and GGAACACTGTGTCAAT; MMCP-4, 5’-TCTGTGAAATGTTCTCCG and TTGTGACTCTCCGCTCC; and MMCp-6, 5’-TTATGTCCTGATCATTGCTG and GGACTCAAGGACGACT. Primer sequences for IL-12, IL-4, and β-actin were taken from Smith et al. (30), and PCR conditions used were 40 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s. Primers for MMCP-4 spanned regions 353–375 and 638–654 of the mRNA sequence and PCR conditions were 35 cycles of 94°C for 10 s, 56°C for 30 s, and 20 s for 20 s. Primer sequences for MMCp-6 were taken from Piao and Bernstein (31), and PCR conditions used were the same as for MMCP-4. RT-PCR was performed with appropriate controls.

RNase protection assay

RNase protections were performed as described elsewhere (32). GM-CSF mRNA was detected using 30 µg of total RNA from cycloheximide (10 µg/ml)-treated cells. IL-3 mRNA was detected using 20 µg of total RNA from ionomycin (1 µM)-treated cells. Cycloheximide and ionomycin were required to stabilize otherwise unstable and rapidly degraded mRNA (33, 34). Quantitation of signals in protected fragments was performed with a Fuji BAS1000 Phosphorimager (Berthold, Bundoora, Australia). Pilot experiments established that the amounts of probe used were in sufficient excess to allow quantitative measurement of input RNA.

RNase protection probes

The murine Ets-1 probe was derived from the cDNA clone pAB1 (35), linearized using EcoRI, and transcribed from the SP6 promoter to generate an antisense transcript of 302 bp. Digestion with ribonuclease yielded a specific 235-bp protected fragment. A 379-bp HindIII/EcoRV fragment from the 5’ end of the GM-CSF gene from clone E1-11 (36) was subcloned into pGEM5Zf+ and used as the template for the GM-CSF probe. Linearization using Ddel and transcription from the T7 promoter generated a probe of 432 bp and a 342-bp protected fragment. A 588-bp EcoRI fragment of murine IL-3 cloned into pGEM1 was linearized using Bsu36I, and transcription from the SP6 promoter generated a probe of 331 bp and gave a 279-bp protected fragment. Control protection experiments were performed using β2-microglobulin (obtained from Dr. R. Ramsay, Peter MacCallum Cancer Institute, Melbourne, Australia). Linearization using SnaBl and transcription from the T7 promoter yielded a 187-bp full-length probe and a 117-bp protected fragment.

Electrophoretic mobility shift assay

rEts-1 protein was produced in Escherichia coli using the pGEX expression system (Pharmacia, Uppsala, Sweden) as previously described (37). Oligonucleotides encoding Ets sites were labeled by the Klenow fill-in reaction using [α-32P]dATP, as described elsewhere (19). Oligonucleotides for EMSA were: GM2, 5’-GATCAGGCCAGGAAGTCCA-3’ and 5’-GATCTTTGACTTCTGGCC-5’; GM4, 5’-GATCAACTGTTGGAACTCTCTC-3’ and 5’-GATCAAGGATCTTCAGGT-5’; and GM5, 5’-GATCCACAGGGAAATGATT-3’ and 5’-GATCAACATTCTTCTCTGTTG-3’.

Transient transfections of FMP6– cells, luciferase assays, and analysis

FMP6– cells (5 × 104) were electroporated in HEPES-buffered RPMI 1640 at 390 V, 960 µF in a Gene Pulser unit (Bio-Rad, Richmond, CA) together with a maximum of 20 µg of plasmid DNA. Cells were incubated in 15 ml of growth medium for 24 h and then lysed for 15 min in 300 µl 1× reporter lysis buffer (Promega, Madison, WI). Luciferase activity was then determined on a 20-µl aliquot of the supernatant in a Lumat LB 9501 luminometer (Berthold, Germany) using luciferase reagent (Promega). The mean of the various control replicates was normalized to one, and each individual result was normalized relative to the mean. All statistical analysis was performed using Student’s t test in the InStat statistical package (Jandel Scientific, San Rafael, CA).

Plasmid constructs for transfection

pMM1.6 luciferase contains 1.6 kb of sequence upstream of the TIS site of the mouse GM-CSF promoter linked to the luciferase reporter gene (obtained from Dr. P. Cockerill, Hans Centre, Adelaide, Australia). The murine GM-CSF promoter has been shown to be significantly more active than the 600-bp
human promoter, which also requires an upstream enhancer for maximal activity, indicating 1.6 kb of murine sequence contains all of the necessary regulatory elements (38). The construct pMGM1.6 luciferase-AGAA containing an A for G substitution in the GGAA core of GM5 was created by replacing a BstEII fragment with a specific PCR product containing the mutation. Oligo A (GTCTCTGTGGTACACATTAATCATTCCCTCTACTGTTG), spanning the BstEII site (bold) in the GM-CSF promoter and incorporating the mutation (underlined), and oligo B (CCACACCTTGGTTACCCAGTAGATCCAG), spanning the BstEII site (bold) within the luciferase gene, were used for PCR to generate a 760-bp product. This product was digested with BstEII and used to replace the fragment removed from pMGM1.6 luciferase also digested with this enzyme by standard procedures. The integrity of pMGM1.6 luciferase-AGAA was confirmed by DNA sequencing. The Ets-1 and antisense Ets-1 expression constructs were generated by blunt-end cloning the Ets-1 cDNA into a blunt-ended Xbal site within the pEF-BOS vector (39).

Results

Phenotypic characterization of mast cell lines

The mast cell line FMP6− is a spontaneous factor-independent clone that was derived from FMP1.6. To functionally characterize these lines, cytokine profiles were initially examined. RT-PCR analysis showed that IL-4, a cytokine characteristic of the MMC phenotype, was expressed by FMP1.6 cells (Fig. 1A), and this cell line produced no detectable IL-12, which is expressed by mast cells with a CTMC phenotype. FMP6−, in contrast, produced IL-12, but no IL-4 (Fig. 1A), indicating a CTMC-like phenotype.

Serine protease production by mast cells also permits their classification into specific subtypes. RT-PCR was used to examine the serine protease profiles of FMP6−, FMP1.6, and primary cultures of BMMC. FMP6−, like CTMC, expressed both MMCP-4 and (low levels of) MMCP-6 (Fig. 1C). On the other hand, no expression of either MMCP-4 or MMCP-6 could be detected in FMP1.6 cells, while BMMC expressed only MMCP-6 (Fig. 1C). The expression of MMCP-4 and MMCP-6 by FMP6−, together with IL-12 production and heparin storage, provides evidence for a CTMC-like classification for this cell line.

Autocrine GM-CSF and IL-3 are necessary for factor-independent growth of FMP6− cells

Proliferation of growth factor-dependent FMP1.6 cells requires PWCM that contains cytokines including GM-CSF and IL-3 (Fig. 2A). This PWCM mixture also supports the growth of IL-3-dependent mast cell line 32Dc23, which was employed as a positive control in these experiments (Fig. 2A) (40). In contrast, factor-independent FMP6− cells do not require conditioned medium for their continued proliferation in culture (Fig. 2A). To assess the possibility that FMP6− had become factor independent by autocrine production of growth factors, we conducted cell proliferation assays in the presence and absence of neutralizing mAbs to GM-CSF and IL-3. Neutralizing GM-CSF and IL-3 mAbs significantly reduced the rate of proliferation of the FMP6− cell line as compared with cells cultured in the presence of an IgG isotype control (Fig. 2B). These data demonstrate that GM-CSF and IL-3 at least are necessary for the factor-independent growth of FMP6− and suggest that these cytokines act in an autocrine or paracrine manner. This suggestion is further supported by

![FIGURE 1](http://www.jimmunol.org/)

**A** Phenotypic characterization of mast cell lines. A, RT-PCR analysis of IL-4, IL-12 (p35 subunit), and control β-actin transcripts in FMP6− and FMP1.6. PCR products were run on a 2% agarose gel together with low m.w. standards. Primers were designed across introns (see Materials and Methods for primer sequences), and products generated were of specific sizes for RNA. Expected sizes were 170 bp for IL-4, 260 bp for IL-12, and 100 bp for β-actin. Negative controls, as indicated (RT−). B, Paraffin-embedded sections of FMP6− and FMP1.6 cells were stained as described in Materials and Methods. Staining of FMP6− shows histamine-containing cells stained with Alcian blue and some granules containing heparin, which stain red with safranin (arrow). Granules within FMP1.6 cells show a lack of staining of heparin by safranin, although Alcian blue staining may be detected. C, RT-PCR analysis of MMCP-4, MMCP-6, and control β-actin transcripts in FMP6−, FMP1.6, and BMCC. PCR products were run on a 2% agarose gel together with low m.w. standards. Primers were designed across introns, and products generated were the expected size: MMCP-4 was 300 bp, MMCP-6 was 171 bp, and positive control β-actin transcripts were 100 bp. Negative controls, as indicated (RT−).
the finding of significantly elevated levels of GM-CSF and IL-3 mRNA in FMP6− cells compared with FMP1.6 cells in RNase protection assays (Fig. 2C).

Correlation of GM-CSF and Ets-1 expression in mast cells
We next decided to examine whether GM-CSF expression correlated with that of Ets-1 in these mast cell lines, given that Ets-1 is involved in the regulation of GM-CSF expression in stimulated Jurkat T cells (19, 20). RNase protection analysis from FMP1.6 and FMP6− cells showed that CTMC-like FMP6− cells expressed significantly higher levels of Ets-1 mRNA than that found in FMP1.6 cells (Fig. 3A). This correlates with the finding of higher levels of GM-CSF mRNA in FMP6− than FMP1.6 (Fig. 2C). To further explore the relationship between Ets-1 and GM-CSF, specifically during mast cell activation, we assayed the levels of Ets-1 and GM-CSF mRNA in both FMP6− and BMMC activated with PMA and ionomycin (PMA/I). PMA/I have previously been shown to mimic cellular activation in T cells and mast cells. PMA/I treatment of FMP6− and BMMC for 3 h resulted in a significant elevation of both Ets-1 and GM-CSF mRNA levels (Fig. 3, B and C), suggesting increased Ets-1 expression is associated with a concomitant increase in GM-CSF expression in primary BMMC cultures and in the FMP6− cell line during mast cell activation.

We then tested whether PMA/I treatment of FMP1.6 cells resulted in up-regulation of Ets-1 and mast cell cytokines expressed by activated cells such as GM-CSF. Ets-1 was not up-regulated in FMP1.6 cells upon PMA/I stimulation, and neither were cytokines such as GM-CSF and TNF-α (data not shown). Indeed it appeared that FMP1.6 cells could not be activated by PMA/I, but require additional factors for Ets-1 and GM-CSF up-regulation and mast cell activation. Nevertheless, the data provide further evidence of a correlation between Ets-1 and GM-CSF expression.

The GM-CSF promoter contains Ets-1 binding sites
The above data suggested the possibility that the expression of the Ets-1 transcription factor may participate in the transactivation of GM-CSF in mast cells. Previous studies in our laboratory have shown that the human GM-CSF promoter contains five putative Ets-1 binding sites, termed GM1-GM5 (19, 20). The murine GM-CSF promoter contains only three of the five Ets sites identified in the human promoter, namely sites GM2 (−295/−302), GM4 (−97/−104), and GM5 (−39/−46). EMSA were performed using oligonucleotides containing these putative Ets-1 binding sites and recombinant human Ets-1 protein to determine which of these sites may be functionally important. An MSV-LTR oligonucleotide sequence, which had previously been shown to bind Ets-1 strongly, bound the reEts-1 protein (Fig. 4, lane 1) and could be supershifted using an Ets-1-specific mAb, but not by an Ets-2 Ab (lanes 2 and 3). This Ets-1 complex was able to be competed using an excess of unlabeled MSV-LTR oligonucleotide (lane 4), but not by an oligonucleotide containing a mutation in the MSV-LTR sequence (lane 5). EMSA involving direct binding using reEts-1 protein showed that sites GM2 and GM5 from the GM-CSF promoter bound Ets-1 strongly (lanes 6 and 10). These Ets-1 complexes were able to be competed with an excess of unlabeled MSV-LTR oligonucleotide (lanes 7 and 11). In competition assays using labeled MSV oligonucleotides and an excess of unlabeled oligonucleotides to the putative Ets-binding sequences, GM2 and GM5 effectively competed for binding, with GM5 being the most effective (lanes 12–14). As GM5 bound Ets-1 particularly strongly, this suggested it might be a functional Ets site, and hence, subsequent studies focused on this cis-binding element.

Several attempts were made at comparing Ets-1 binding from nuclear lysates of FMP6− and FMP1.6 cells in EMSA. Although several Ets-related complexes bound the Ets binding site (GM5) in
lysates from FMP6− cells, we were unable to unequivocally demonstrate specific Ets-1 binding since we could not obtain any Abs that supershifted Ets-1 from murine nuclear lysates even in the control Ets-1-expressing cell line EL4 (data not shown).

Ets-1 transactivates GM-CSF in FMP6− cells via the GM5 site in CLE0

To test whether Ets-1 plays a role in GM-CSF regulation, and if so, whether this occurs via the GM5 site, FMP6− cells were transiently transfected with pMGM1.6 luciferase, a murine GM-CSF luciferase reporter construct containing 1.6 kb of promoter sequence, in the presence or absence of an Ets-1 expression construct. Ets-1 was able to significantly transactivate the GM-CSF reporter (Fig. 5A), indicating that Ets-1 was sufficient on its own in this mast cell line to increase the level of GM-CSF. The sensitivity of GM-CSF transactivation by Ets-1 would be expected to be greater in FMP1.6 cells compared with FMP6− cells, since FMP1.6 cells have low basal levels of Ets-1. However, FMP1.6 cells were nontransfectable by a variety of methods, and thus we performed and observed transactivation in FMP6− cells.

To examine whether Ets-1 mediated this transactivation via GM5, a mutation was made in the purine-rich Ets core of GM5, from GGAA to AGAA, to generate the reporter construct pMGM1.6lu-c AGAA, since a similar mutation in the human promoter has previously been shown to abolish binding of rEts-1 protein in EMSA (19). Transfection of this construct into FMP6− cells resulted in significantly lower levels of GM-CSF reporter activity (at or close to background levels), demonstrating the importance of the GM5 site for constitutive GM-CSF activity (Fig. 5A). In addition, pMGM1.6lu-c AGAA was unable to be transactivated by the Ets-1 expression construct, thereby demonstrating that Ets-1 requires this site to mediate its effects (Fig. 5A).

To test whether Ets-1 was the Ets family member that functioned in regulating constitutive GM-CSF synthesis seen in FMP6− cells, we performed transient transfections using an antisense Ets-1 expression vector together with the pGM1.6 luciferase reporter construct. Antisense Ets-1 was shown to significantly repress the activity of the GM-CSF reporter compared with the control of pEF-BOS vector without insert (indicated by BOS alone) (Fig. 5B), suggesting that Ets-1 plays a role in regulating the constitutive expression of GM-CSF observed in FMP6− cells.

Discussion

Several important findings have emanated from this study. First, we have identified and characterized FMP6− as a CTMC-like mast cell line. Second, we have demonstrated that this factor-independent cell line has dramatically up-regulated levels of GM-CSF expression, and neutralization of GM-CSF with specific Abs inhibits cellular proliferation. Third, we have shown that the levels of Ets-1 and GM-CSF mRNA correlate in FMP6− and FMP1.6 cells and in FMP6− and BMMC cells activated with PMA/I. Finally, we have shown that Ets-1 protein can bind sequences in the GM-CSF promoter, that an Ets binding site (GM5) is required for constitutive expression of GM-CSF (a mutation of this site to AGAA results in a loss of promoter activity), and that Ets-1 acts via this site in GM-CSF regulation since antisense Ets-1 represses constitutive GM-CSF expression. The significance of these findings is as follows.

FMP6− is a unique and useful cell line, since only a few murine mast cell lines that resemble a CTMC-like phenotype have been established (41). The evidence for FMP6− being CTMC-like and parental FMP1.6 being more MMC-like is: 1) MMCP-4 and MMCP-6, proteases typically expressed by CTMC populations,

FIGURE 3. Correlation of Ets-1 and GM-CSF expression in mast cells. A, RNase protection analysis of Ets-1 mRNA levels in FMP6− and FMP1.6 cells. Antisense 32P-labeled riboprobes of 302 bp for murine Ets-1 and 187 bp for β2MG were used on 20 μg of total RNA. Protected fragments for Ets-1 and β2MG were 235 and 117 bp, respectively. B, Ets-1 RNase protections performed on 20 μg of total RNA extracted from control (−) and PMA/I-stimulated (+) FMP6− or BMMC cultures. C, GM-CSF RNase protections performed on 20 μg of total RNA extracted from control (−) and PMA/I-stimulated (+) FMP6− or BMMC cultures. The full-length 423-bp GM-CSF probe gave a 342-bp protected fragment.
FIGURE 4. The GM-CSF promoter contains Ets-1 binding sites. In vitro EMSA analysis of Ets-1 binding sites using rEts-1 protein as well as competition between the putative ETS binding sequences from the GM-CSF promoter and a MSV LTR-Ets-1 binding sequence. The gel shows binding of Ets-1 to MSV-LTR (lane 1); a supershift with Ets-1 Ab (SS, lane 2); absence of a supershift with an Ets-2 Ab (lane 3); competition in the presence of a 100× excess of unlabeled MSV-LTR (lane 4); and a lack of competition by a mutant oligonucleotide containing an AGAA substitution in the GGAA core binding site (MUT; lane 5). Lanes 6 to 11 show binding of Ets-1 to putative Ets binding sites from the GM-CSF promoter in the presence or absence of competition from a 100× excess of unlabeled MSV-LTR oligonucleotide. Lanes 12 to 14 show competition of MSV LTR-Ets-1 with oligonucleotides representing the potential Ets binding sites. GM2 and GM5 compete MSV LTR-Ets-1 binding.

FIGURE 5. Ets-1 transactivates GM-CSF via the GM5 site in CLEO. A, FMP6− cells (5 × 10⁵) were transiently transfected with 15 µg of pMGM1.6 luciferase or pMGM1.6 luc-AGAA (containing a GGAA→AGAA mutation in GM5) in addition to 5 µg of the Ets-1 expression construct (or 5 µg pEF-BOS vector alone) by electroporation. Luciferase data are represented as mean ± SEM of at least six replicates (θ, p = 0.0018; *, p = 0.012). B, FMP6− cells were transiently transfected with pMGM1.6 luciferase together with 20 µg of an antisense Ets-1 expression construct or 20 µg of pEF-BOS vector alone. Data are represented as mean ± SEM of three replicates (*, p = 0.004).

were expressed by FMP6−, but not by parental FMP1.6 cells; 2) FMP1.6 was found to express IL-4, a cytokine that defines MMC populations, while, in contrast, FMP6− cells expressed CTMC-defining IL-12 (30); and 3) both cell lines contained histamine granules, but only FMP6− cells stored heparin-containing granules, a property of CTMC. However, the number of granules containing heparin in FMP6− cells was quite low when compared with CTMC-like lines generated by Reynolds et al. (41), possibly indicating these cells may be intermediate in the pathway between immature CTMC (which stain Ab⁺, S⁻) and fully mature CTMC (which stain Ab⁺, S⁺) (42). Due to the limited availability of transformed mast cell lines with a CTMC-like phenotype, these cells could be a useful model to study the molecular mechanisms of mast cell function.

Another feature that makes FMP6− a relatively unique cell line is related to its acquisition of factor independence via an autocrine or paracrine mechanism. Many mast cell lines that are IL-3 dependent can accomplish subsequent loss of growth-factor dependence by either an autocrine or a nonautocrine mechanism. For instance, retroviral transformation with AbMuLV generated IL-3-independent mast cell lines from murine fetal liver cultures via a nonautocrine mechanism due to an inability of these cells to enter pathways of terminal differentiation (43, 44). Several other mast cell lines have achieved growth-factor independence via a nonautocrine mechanism involving mutation of the c-kit receptor, resulting in constitutive signaling in the absence of the ligand (45–48). In contrast, abrogation of growth factor-dependent proliferation, as observed by FMP6− cells when compared with the parental FMP1.6 cell line, was accomplished by an autocrine/paracrine mechanism. FMP6− cells expressed mRNA for both GM-CSF and IL-3 and presumably secreted active protein that is necessary for proliferation, as neutralizing Abs to these two cytokines resulted in a loss of cellular proliferation. Hence, the factor independence of FMP6− cells appears to involve up-regulation of GM-CSF and IL-3 cytokine expression at least. Interestingly, FMP6− conditioned medium did not support the proliferation of FMP1.6 cells. This may be due to the relative instability of the cytokines in the FMP6− conditioned medium or due to GM-CSF acting in a paracrine or autocrine manner without ever reaching levels in conditioned medium sufficient to support cell growth upon transfer of the medium. Indeed, precedents for the latter possibility are widely published. For example, autocrine effects of IFN can be demonstrated despite the inability to detect levels in conditioned medium (49). A correlation between Ets-1 and GM-CSF expression occurred in FMP6− and FMP1.6 cells, and both genes were up-regulated by PMA/I-induced activation of FMP6− and BMMC, thus raising the possibility that Ets-1 may be involved in GM-CSF regulation. Further evidence for this is that Ets-1 protein binds GM-CSF promoter.
sequences, Ets-1 can transactivate a GM-CSF reporter, and a mutation that abolishes Ets binding in general destroys constitutive promoter activity. Evidence implicating Ets-1 as being the Ets family member involved in regulation of GM-CSF expression in FMP6—mast cells was provided by the antisense Ets-1 experiment, in which cotransfection with an antisense Ets-1 construct significantly reduced basal GM-CSF reporter activity. Interest-ingly, other investigators have demonstrated that Ets-1 can transactivate a GM-CSF reporter in Jurkat and MLA144 T cell lines (19–21), and thus the finding that it can also regulate GM-CSF in FMP6—mast cells suggests Ets-1 involvement in the regulation of this cytokine may also occur in other cell types.

Understanding the mechanisms involved in the regulation of mast cell cytokines is important to the understanding of mast cell activation and expansion that occurs in inflammatory diseases. Ets-1 can regulate GM-CSF in a mast cell line, and this study suggests a role for Ets-1 in inflammation. A number of genes encoding inflammatory proteins have functional Ets binding sites in their promoters, including TNF-α (50, 51), cox-2 (52), IL-2R (53), ICAM-1 (54), and GM-CSF (19). Although the identity of the specific Ets family member(s) involved in transactivation of these inflammatory proteins remains to be elucidated, the findings of this study have clearly demonstrated that Ets-1 has a functional role in transcriptional regulation of GM-CSF in mast cells. Furthermore, we have shown previously that a functional Ets binding site is required for the transcriptional activation of NxFB, a transcription factor involved in the regulation of a number of inflammatory genes, and that Ets-1 can bind to this site (55). We have also demonstrated that, in T cells, Ets-1 is the Ets gene involved in the regulation of GM-CSF (19), and that both NF-kB and AP-1 require this Ets-1 binding site for optimal regulation of GM-CSF transcription (20). Importantly, agents that trigger inflammation such as protein kinase C activation/intracellular calcium mobilization (PMAI, this study) and cytokines TNF-α and IL-1α up-regulate the expression of Ets-1 (56), supporting the idea that these stimuli signal through Ets-1, which in turn transcriptionally induces the expression of effector inflammatory proteins.

The findings of this study implicate Ets-1 in mast cell function/activation. Previous studies have suggested that the Ets transcription factor plays a role in angiogenesis in chicken and human embryos (57, 58), in organogenesis and tissue remodeling of mouse embryos, and in hemopoiesis in chicken and mouse embryos (23, 59–61). Mast cells have themselves been implicated in biologic processes such as angiogenesis (62, 63). Furthermore, injection of tumor cells into mast cell-deficient mice resulted in cancer much later than in control mice, and the rate of metastases was reduced, demonstrating enhancement of angiogenesis by mast cells (64). Thus, a further correlation exists between the proposed functions of mast cells, mast cell cytokines, and roles of Ets-1.

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References


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