De novo synthesis of early growth response factor-1 is required for the full responsiveness of mast cells to produce TNF and IL-13 by IgE and antigen stimulation

Bo Li, Melanie R. Power, and Tong-Jun Lin

Early growth-response factor 1 (Egr-1) is a zinc-finger transcription factor that plays a regulatory role in the expression of many genes important for inflammation. Whether Egr-1 is involved in IgE-dependent mast-cell activation was investigated. We demonstrated that IgE and antigen (TNP) stimulation induced a rapid expression of Egr-1 mRNA in mouse bone marrow–derived mast cells (BMMCs). As early as 15 to 20 minutes after IgE + TNP stimulation, Egr-1 protein was detectable in the nucleus of BMMCs by immunofluorescence or electrophoretic mobility shift assay. To examine a role for Egr-1 in IgE-dependent cytokine production by mast cells, Egr-1-deficient (Egr-1−/−) BMMCs were developed from the bone marrow cells of Egr-1 knockout mice. Egr-1−/− BMMCs express similar levels of surface c-kit and IgE receptor as compared with those on Egr-1+/+ BMMCs. Importantly, IgE + TNP-induced TNF and IL-13 expression was significantly reduced at both mRNA and protein levels in Egr-1−/− BMMCs as compared with those in Egr-1+/+ BMMCs. Thus, our results suggest that de novo synthesis of Egr-1 represents a novel mechanism in FcεRI signaling and is required for the full responsiveness of IgE-dependent TNF and IL-13 production by mast cells. (Blood. 2006;107:2814-2820) © 2006 by The American Society of Hematology

Introduction

Mast cells are critical effector cells in allergic disorders through secretion of mast-cell mediators. Antigen-mediated aggregation of IgE bound to its high-affinity IgE receptor (FcεRI) on the mast-cell surface initiates a complex series of biochemical events, leading to the excytosis of granule-associated mediators and the generation of leukotrienes and cytokines. FcεRI aggregation induces rapid tyrosine phosphorylation of its β and γ subunits and activation of cytoplasmic protein tyrosine kinase Syk and Fyn. This initial event is followed by activation of multiple signaling pathways that consist of a complex network of enzymes and adaptor molecules. Mast-cell degranulation can occur in seconds after FcεRI aggregation, suggesting a rapid interaction between signaling molecules that control the excytosis machinery. Contrary to the excytosis of granule-associated mediators, IgE-dependent production of cytokines normally occurs in hours. Production of cytokines and chemokines requires activation of transcription factors. Although diverse cytokines and chemokines, including TNF and IL-13, that are important in inflammation have been described to be produced by mast cells, the number of the transcription factors that have been associated with IgE-dependent mast-cell activation is limited. Moreover, individual cytokines are likely regulated by different transcription factors. Transcription factors involved in TNF and IL-13 production by mast cells are not entirely clear.

The early growth response factor-1 (Egr-1), also known as zif268, krox-24, NGFI-A, TIS8, ETR103, and ZENK, is an 80- to 82-kDa nuclear protein consisting of 533 amino acids. The DNA-binding domain of Egr-1 consists of 3 zinc finger motifs, through which Egr-1 preferentially binds to GC-rich DNA sequences. Once bound to DNA, Egr-1 is capable of activating or repressing gene transcription. Cell type and specific stimulus determine whether Egr-1 acts to activate or inhibit promoter activity of target genes. This is likely due to the interaction between Egr-1 and tissue-specific factors and/or transcription factors that associate with proximal promoter elements such as Sp-1. The biologic activities of Egr-1 include activation or inhibition of gene expression and differentiation of macrophages. Egr-1 has been implicated in allergic responses because Egr-1−/− mice showed diminished TNF mRNA and protein in the lung in response to ovalbumin sensitization and airway challenge. Egr-1−/− mice had elevated IgE levels and were hyporesponsive to airway challenge with methacholine. Although Egr-1 has been reported to be expressed by mast cells, a role for Egr-1 in mast-cell development or in IgE-dependent cytokine production is unclear.

In this study, we demonstrated that aggregation of FcεRI by antigen stimulation induced rapid Egr-1 expression at mRNA (15 minutes) and protein (30-60 minutes) levels. IgE-mediated Egr-1 expression precedes TNF and IL-13 production at both mRNA and protein levels. Using bone marrow–derived Egr-1−/− mast cells, we showed that IgE-dependent TNF and IL-13 production at mRNA and protein levels were both reduced because of Egr-1 deficiency as compared with wild-type mast cells. Thus, Egr-1 is...
required for the full responsiveness of mast cells to produce TNF and IL-13 by IgE and antigen stimulation. The need of de novo synthesis of Egr-1 in IgE-mediated mast-cell activation likely contributes to the delayed cytokine response and represents a novel mechanism in FcεRI-mediated TNF and IL-13 production.

Materials and methods

Animals

Egr-1−/− deficient mice and control C57BL/6NTac mice were purchased from Taconic Farms (Germantown, NY). The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

Antibodies

Antibodies to Egr-1 (sc-189 for immunofluorescence study and sc-189X for the blockade of DNA–protein complex formation), Egr-2 (sc-190X), Egr-3 (sc-22801X), and Egr-4 (sc-19868X) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 594–conjugated goat anti–rabbit (sc-22801X), and Egr-4 (sc-19868X), respectively, for 2 hours on ice before the addition of the radiolabeled probe.

Real-time quantitative PCR

Total RNA was isolated from BMMCs using TRIZOL Reagent (catalog no. 15596-026; Invitrogen, Carlsbad, CA) and reverse transcribed using SuperScript II RNase H-Reverse Transcriptase (catalog no. 18064-014; Invitrogen) according to the manufacturer’s instruction. Real-time quantitative polymerase chain reaction (PCR) was performed using a 7000 Sequence Detector (PE Applied Biosystems, Foster City, CA). Specific quantitative assays for Egr-1, TNF, and IL-13 were performed using Assays-on-Demand reagents containing 6-FAM dye-labeled TaqMan MGB probes (Applied Biosystems) according to the manufacturer’s protocol.26 GAPDH was used as an endogenous reference. Data were analyzed using relative standard curve method according to the manufacturer’s protocol.

In addition, PCR products of Egr-1 and GAPDH were separated on 2% agarose gel and stained with ethidium bromide.

Cytokine assays

The levels of TNF and IL-13 were measured by enzyme-linked immunosorbent assay (ELISA) using antibody (Ab) pairs from R&D Systems (Minneapolis, MN) according to the manufacturer’s protocol.

Immunofluorescence study

BMMCs (3 × 10⁶ cells/sample) were treated with TNP-BSA (10 ng/mL) for various times. After stimulation, BMMCs were fixed and permeabilized using Cytofix/Cytoperm solution (catalog no. 554714; BD Biosciences Pharmingen, San Diego, CA) for 20 minutes at 4°C. Cells were then blocked with 5% normal goat serum (catalog no. CL1200; Cedara Laboratories.) for 1 hour at 4°C. Then, cells were incubated with rabbit polyclonal anti–Egr-1 antibody (catalog no. sc-189; Santa Cruz Biotechnology) for 3 hours at 4°C. Cells were then stained for 2 hours with Alexa Fluor-594–conjugated goat anti–rabbit IgG (F(ab)₂) (Molecular Probes). Fluorescence-labeled mast cells were cytocrystified (Cytospin 3, Shandon, United Kingdom) onto slides at 45 rpm (200 rpm) for 5 minutes. To visualize cell nuclei, slides were mounted with DAPI, a fluorescent groove-binding probe for DNA, before cover slip attachment. Cells were examined using a fluorescence microscope (Nikon Eclipse E600; Nikon, Tokyo, Japan).

Histology

Tongue, skin, and trachea tissues from Egr-1−/− and Egr-1+/− mice were fixed in Carnoy fluid overnight and then in 100% ethanol for paraffin embedding and sectioning. Sections were deparaffinized with Citrisolv (Fisher, Fair Lawn, NJ) and rehydrated through decreasing concentrations of ethanol. Slides were stained with Alcian blue to visualize mast cells.

Fluorescence-activated cell sorting (FACS) analysis

For analysis of c-kit expression, BMMCs were stained with the FITC-conjugated rat anti–mouse CD117 (c-kit) monoclonal Ab (mAb) (IgG2a) for 1 hour at 4°C. FITC-rat IgG2a was used as control. For analysis of FcεRI expression, BMMCs were sensitized with IgE and then stained with FITC-conjugated rat anti–mouse
Results

Transient and early expression of Egr-1 by mast cells after IgE + TNP stimulation

To determine whether Egr-1 is expressed in mast cells after Ag stimulation, bone BMMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA (10 ng/mL) for various times (15, 30, 60, 180 minutes). Egr-1 mRNA expression was measured by real-time quantitative PCR. Egr-1 level was normalized to GAPDH in each sample. Egr-1 levels in the unstimulated mast cells were undetectable. An average value of Egr-1 after GAPDH normalization at the time point of 15 minutes (the highest Egr-1 expression level) was used as a calibrator to determine the relative levels of Egr-1 expression at various conditions (Figure 1A). TNP stimulation induced a transient and rapid Egr-1 expression. A strong Egr-1 expression can be seen at 15 minutes after TNP stimulation. Subsequently, the level of Egr-1 began to return toward basal conditions. After 180 minutes of TNP stimulation, little Egr-1 mRNA can be detected (Figure 1A). PCR-amplified Egr-1 products were also separated in agarose gel and visualized by ethidium bromide staining. A representative gel was presented in Figure 1B. A rapid and strong Egr-1 expression can be seen at 15 minutes after TNP stimulation. Egr-1 levels decreased after this time point.

Nuclear localization of TNP-induced Egr-1 protein in mast cells

To examine TNP-induced Egr-1 expression at protein level and its cellular localization, mouse BMMCs were treated with TNP for various times (15-360 minutes), permeabilized, and stained with anti-Egr-1 Ab. Nucleus was visualized by DAPI staining. Expression of Egr-1 protein can be seen as early as 15 minutes after TNP stimulation. Strong Egr-1 expression was observed at 30 minutes to 1 hour after TNP stimulation.

Interestingly, Egr-1 proteins were exclusively found in the nuclei of TNP-stimulated mast cells (Figure 2).

To evaluate the time course relation between Egr-1 expression and TNF and IL-13 production, BMMCs were treated with TNP for various times (15 minutes to 24 hours), and cell-free supernatants were used to determine TNF and IL-13 production by ELISA. Unlike Egr-1, which is detectable at 15 minutes and reaches its peak expression at 1 hour after TNP stimulation (Figure 2), production of TNF and IL-13 was undetectable at 30 minutes (data not shown). TNP-induced TNF and IL-13 production peaked at 3 to 6 hours (TNF: 3 hours at 799.3 ± 114.4 pg/mL; TNF: 6 hours at 787.1 ± 134.9 pg/mL; IL-13: 3 hours at 490.1 ± 63.5 pg/mL; IL-13: 6 hours at 417.6 ± 55.5 pg/mL). Thus, TNP-induced Egr-1 expression precedes TNF and IL-13 production.

Egr-1 activation induced by TNP stimulation in mast cells

To determine the specific DNA binding activity of TNP-induced Egr-1 in mast cells, nuclear proteins from TNP-stimulated BMMCs were isolated and examined by EMSA using a DNA probe that can be recognized by the Egr family. A strong DNA binding activity of nuclear proteins from mast cells treated with TNP can be observed (Figure 3A). Consistent with the immunofluorescence experiment, Egr binding was not observed in unstimulated mast cells and became apparent as early as 5 to 20 minutes after TNP stimulation. The binding specificity of nuclear proteins to Egr DNA sequence was verified through the competitive binding by nonradioisotope-labeled Egr probes (Figure 3A-B) but not by AP-1 (Figure 3A, last lane) or Sp-1 probes (Figure 3B). Interestingly, although Sp-1 has been shown to be closely associated with Egr-1 function because of similarity in their DNA binding sequences, no Sp-1 activation can be observed in TNP-stimulated mast cells (Figure 3B). As a control, the specific binding of Sp-1 was shown using nuclear extracts from HeLa cells (Figure 3C).

There are 4 members of Egr family members, including Egr-1, -2, -3, and -4. To further examine the binding specificity, nuclear proteins from TNP-treated BMMCs were preincubated with anti–Egr-1 Ab, anti–Egr-2 Ab, anti–Egr-3 Ab, or anti–Egr-4 Ab, respectively. Then Ab-treated samples were used for binding with 32P-labeled Egr probe. Treatment of nuclear proteins with anti–Egr-1 Ab, but not anti–Egr-3 or anti–Egr-4 Ab, reduced nuclear protein binding to Egr probe, suggesting the specificity of Egr-1 binding (Figure 3D). Interestingly, anti–Egr-2 Ab also reduced nuclear protein binding to 32P-labeled Egr probe, suggesting that TNP stimulation may also induce Egr-2 activation (Figure 3D).

Development of mast cells in the absence of Egr-1

A number of cytokines that are important in allergy, such as TNF, contain an Egr-1–binding sequence in their promoters. To determine whether mature mast cells can be developed from Egr-1–deficient mouse bone marrow progenitor cells.

Bone marrow cells from Egr-1–deficient and wild-type mice were cultured in conditioned medium for 4 to 6 weeks. Cells were analyzed by flow cytometry for c-kit and IgE receptor expression at the end of each week during the culturing process. No difference of c-kit and IgE receptor expression pattern between wild-type and
Egr-1–deficient cells was observed during mast-cell maturation (week 1 to week 6; data not shown). The c-kit and IgE receptor expression after 5 weeks of culture was presented in Figure 4A. Cells after 5 weeks of culture were also used for toluidine blue staining. Morphologically, no difference was observed between Egr-1–deficient and wild-type mast cells (Figure 4B). In addition, various tissues from Egr-1–deficient mice were used to examine the presence of mast cells. Similar number and morphology of mast cells were observed in tongue (Figure 4C), skin and trachea (data not shown), in both Egr-1–deficient and wild-type mice.

Reduced IgE-dependent TNF and IL-13 production resulting from Egr-1 deficiency

Egr-1–deficient mature mast cells have been observed during mast-cell maturation (week 1 to week 6; data not shown). The c-kit and IgE receptor expression after 5 weeks of culture was presented in Figure 4A. Cells after 5 weeks of culture were also used for toluidine blue staining. Morphologically, no difference was observed between Egr-1–deficient and wild-type mast cells (Figure 4B). In addition, various tissues from Egr-1–deficient mice were used to examine the presence of mast cells. Similar number and morphology of mast cells were observed in tongue (Figure 4C), skin and trachea (data not shown), in both Egr-1–deficient and wild-type mice.

Discussion

An earlier study showed that treatment of mast cells with cycloheximide (a protein synthesis inhibitor) inhibits IgE-dependent mediator release, suggesting a need of de novo synthesis of proteins in FcεRI-mediated mast-cell activation.30 In this study, we demonstrated a clear role for the newly synthesized immediate early gene Egr1 in FcεRI-induced TNF and IL-13 mRNA expression in Egr-1–deficient BMMCs as compared with that in wild-type BMMCs (Figure 5A-B).

Similarly, TNP-induced production of TNF and IL-13 at the protein levels by Egr-1–deficient BMMCs was significantly reduced compared with that by wild-type BMMCs (Figure 5C-D). Interestingly, Egr-1 deficiency only had a minor effect on TNP-induced IL-6 production by BMMCs (data not shown), suggesting that Egr-1 may selectively regulate specific genes in mast cells.

Figure 2. Immunofluorescence staining of Egr-1. After IgE sensitization, mouse BMMCs from wild-type mice or Egr-1–deficient mice were either left untreated (no treatment) or stimulated with TNP-BSA (10 ng/mL) for various times. Cells were fixed, permeabilized, and then stained with anti–Egr-1 Abs or control rabbit serum. Alexa 594–conjugated goat anti–rabbit IgG F(ab′)2 was used as a secondary Ab. DAPI staining was used to visualize the nucleus of the cells. TNP stimulation induced a transient expression of Egr-1, which is localized in the nucleus of the cells. As a control, no Egr-1 staining was observed in BMMCs from Egr-1–deficient mice. Original magnification, × 40. Immunolabeled specimens were mounted in DAPI containing Vectashield (Vector Laboratories, Burlingame, CA). Cells were examined using a fluorescence microscope (Nikon E600; Nikon, Tokyo, Japan) equipped with a DMX1200 camera and a CFI Plan-Fluor DDL 40 ×/0.75 objective lens. Images were processed using Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA).
protein levels in Egr-1–deficient mast cells when compared with that in wild-type mast cells. These data suggest that FceRI aggregation induces the new synthesis of Egr-1, which in turn amplifies Tnf and Il13 gene expression by mast cells. Thus, the de novo synthesis of Egr-1 represents an indirect mechanism through which FceRI aggregation induces the full strength of gene expression by mast cells.

Several studies using differentiation-inducible myeloid cell lines as well as normal bone marrow cells have demonstrated a role of Egr-1 in the monocytic differentiation of myeloid cells.19,31-33 Egr-1 stimulates development along the macrophage lineage at the expense of development along the granulocyte or erythroid lineages.32 In addition, Egr-1 also has a role in T-cell, B-cell, and neuronal-cell development.28,34,35 We used bone marrow cells from Egr-1–deficient mice as well as wild-type mice and examined their differentiation into mast cells. IgE receptor and c-kit were used as cell-surface markers. Levels of these 2 receptors were examined every week during the differentiation process. No difference of IgE receptor or c-kit was observed between Egr-1–deficient cells and wild-type cells during the entire course of differentiation. Metachromatic staining (toluidine blue) showed normal morphology and granulation of Egr-1–deficient mast cells. In addition, toluidine blue staining showed a similar number of mast cells in various tissues (skin, tongue, and trachea) between Egr-1–deficient mice and wild-type mice. Thus, Egr-1 appears to have no effects on mast-cell granulation and c-kit and IgE receptor expression during mast-cell development. This effect is distinct from that in macrophages, T cells, or B cells. This further supports the notion that the function of Egr-1 is cell-type specific.

TNF and IL-13 are important inflammatory mediators. The Tnf gene is activated in response to multiple signals of stress and inflammation. A distinct set of transcription factors are recruited to the Tnf promoter dependent on a specific stimulus.36-38 Although, it has been well characterized that FceRI aggregation induces TNF production by mast cells, the transcription factors involved in this process are less clear. Our finding of a role for Egr-1 in the regulation of IgE-dependent TNF and IL-13 production by mast cells is consistent with an in vivo study, demonstrating that Egr-1 modulates TNF and airway responsiveness in mice.20

The Tnf promoter contains an Egr-1 responsive element.27 Binding of Egr-1 to the Tnf promoter can either activate or inhibit TNF production.36,38 For example, LPS-induced Egr-1 binding activates TNF expression.39 In contrast, PGE2-induced Egr-1 binding inhibits TNF expression.17 This is likely due to the distinct transcription factors that may interact with Egr-1 under different stimulation conditions. Because Egr-1 shares similar consensus binding sites with transcription factor Sp-1 (−GGGCGG−)16 and forms a complex with Sp-1,40 we examined whether FceRI

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**Figure 3. Stimulation of mast cells by FceRI aggregation induces Egr-1 but not Sp-1 activation.**

(A) Mouse BMMCs were sensitized with anti-TNP IgE and stimulated with TNP-BSA (10 ng/mL) for various times. Nuclear proteins were isolated and subjected to EMSA analysis (see “Materials and methods”) using 32P-labeled Egr-1– or Sp-1–specific probes. BLK indicates blank, no nuclear proteins were added; NT, no treatment, nuclear proteins were isolated from BMMCs without TNP stimulation. Numbers indicated are minutes after TNP stimulation; nuclear proteins were isolated from BMMCs after the indicated times following TNP stimulation. Fifty times concentrated unlabeled Egr-1 oligonucleotide was used to compete with 32P-labeled Egr-1 oligonucleotide, whereas 50 × concentrated unlabeled AP-1 oligonucleotide was used as a nonspecific control probe. (B) Nuclear proteins from TNP-BSA (10 ng/mL, 60 minutes) treated BMMCs (TNP) or from untreated BMMCs (NT) were subjected to DNA probe competition experiment using unlabeled probes or 32P-labeled mutant probes to demonstrate specific Egr-1 binding. No Sp-1 binding was observed in TNP-stimulated BMMCs. (C) As a control, nuclear extracts from HeLa cells (Promega) were subjected to EMSA using 32P-labeled Sp-1 oligonucleotide. 32P-labeled Sp-1 binding was blocked by unlabeled Sp-1 probe, but not by unlabeled Egr-1 or AP-1 probes (50 ×). (D) Antibody blockade of the DNA-protein complex formation. Nuclear proteins from TNP-BSA (10 ng/mL, 60 minutes) treated BMMCs or from untreated BMMCs (NT) were incubated with or without specific antibodies to Egr-1, Egr-2, Egr-3, or Egr-4 for 2 hours on ice before EMSA experiment using 32P-labeled Egr-1 oligonucleotide.
aggregation also induces Sp-1 activation. Interestingly, no Sp-1 activation can be observed in TNP-stimulated mast cells by EMSA analysis. Interaction between Egr-1 and NF-κB family members has been described to be involved in TNF production. We reported that FcεRI aggregation-induced TNF production requires IKK-IκB-NF-κB pathway activation. Thus, it is likely that FcεRI aggregation-induced Egr-1 may interact with NF-κB family members in the regulation of TNF production.

The role of Egr-1 in the regulation of IL-13 production has not been reported previously. IL-13 plays a central role in allergic inflammation. Mast cells are ready to produce and release IL-13 protein on stimulation. Impaired IL-13 production by Egr-1−/− deficient mast cells following FcεRI aggregation suggests a role for Egr-1 in the regulation of this important cytokine in allergic response. Detailed analysis of the IL-13 promoter may shed new light onto the mechanisms involved.

It is noticeable that Egr-1 deficiency only partially inhibited FcεRI aggregation-induced TNF and IL-13 production by mast cells. Others also found that, although substantial evidence supports a role for Egr-1 in macrophage differentiation, little defect of macrophage lineage was observed in Egr-1−/− deficient mice. It is possible that other transcription factors such as Egr-2 may compensate for the effect of Egr-1 under the circumstance of Egr-1 deficiency. All 4 Egr family members, Egr-1, Egr-2, Egr-3, and Egr-4, share highly homologous DNA-binding domains, and all bind to the sequence GCGGGGGCG. Our EMSA experiment revealed that FcεRI aggregation-induced protein binding to the Egr recognition sequence was blocked by anti–Egr-1 or anti–Egr-2 Abs, but not by anti–Egr-3, or anti–Egr-4 Abs, suggesting that Egr-2 may also be induced in TNP-stimulated mast cells. Further studies are needed to clarify the role of Egr-2 in the regulation of mast-cell function.

In an effort to examine potential mechanisms involved in FcεRI-induced Egr-1 expression, we used various kinase and phosphatase inhibitors, including protein tyrosine kinase inhibitor PP2, PKC inhibitor Ro 31-8220, Erk1/2 inhibitor PD 98059, PI3K inhibitor wortmannin, p38 MAP kinase inhibitor SB 203580, mTOR inhibitor rapamycin, and PP2A inhibitor okadaic acid. Although, the MAPK pathway and MEK-Erk1/2 were reported to be required for Egr-1 activation and PD 98059 blocked Egr-1 activation in monocytes, no effects of PD 98059, SB 203580, wortmannin, or rapamycin were observed on TNP-induced Egr-1 activation using EMSA analysis (data not shown). Interestingly, tyrosine kinase inhibitor PP2 and PKC inhibitor Ro 31-8220 individually partially inhibited TNP-induced Egr-1 activation.
When these 2 inhibitors were combined, an additive inhibitory effect on Egr-1 activation was observed (data not shown). Thus, the early tyrosine kinase phosphorylation following FceRI aggregation and subsequent PKC activation are likely upstream signaling events required for Egr-1 activation. The mechanism involved in Egr-1 activation in mast cells is different from that in other cells and requires further study.

In summary, we demonstrated that FceRI aggregation induced a rapid and transient de novo synthesis of Egr-1 and subsequent DNA binding. Egr-1 deficiency resulted in impaired IgE-dependent TNF and IL-13 production. The identification of Egr-1 in this context represents a novel mechanism involved in FcεRI aggregation-induced TNF and IL-13 production by mast cells.

References

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