Analysis of Egr-1 Protein Induction in Murine Peritoneal Macrophages Treated with Granulocyte-Macrophage Colony-Stimulating Factor

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Transcription of early growth response gene-1 (Egr-1) is required for macrophage differentiation. Since granulocyte-macrophage colony-stimulating factor (GM-CSF) induces macrophage proliferation as well as the transcription of Egr-1 mRNA, we characterized the induction of Egr-1 protein in murine peritoneal macrophages following treatment with GM-CSF. As determined by Western blot analysis, the concentration of GM-CSF required to induce detectable Egr-1 protein in macrophages was 50 units/ml, and the inducible Egr-1 protein species was 80 kDa. Following stimulation of macrophages with GM-CSF, Egr-1 protein was detected within 25 min and reached maximum level at 70 min. The concentration of GM-CSF that was required to induce Egr-1 protein in macrophages was similar to that required to induce macrophage proliferation. A similar concentration has been detected previously in mouse serum exposed to bacterial endotoxin in vivo, suggesting that the 80 kDa Egr-1 protein may be induced in macrophages under the physiologic circumstances of pathogen invasion. Thus, our data support further study on the role of Egr-1 protein in mediating GM-CSF induction of macrophage proliferation.

INTRODUCTION

Early growth response gene-1 (Egr-1)\textsuperscript{c}, which belongs to the immediate early gene family, encodes a transcription factor with three zinc finger domains that bind to the DNA consensus sequence CGCCCGGC (for review see Ref. 1). Expression of Egr-1 (also known as Zif268, Krox24, TIS8, NGF1-A and d-2), is coregulated with c-fos and does not require de novo protein synthesis \cite{1, 2}. Transcription of Egr-1 is rapidly induced following mitogenic stimulation in many cell types \cite{1-12}. Egr-1 is also induced by several non-mitogenic stimuli in post-mitotic cells \cite{1}. The Egr-1 gene product is a nuclear phosphoprotein with a molecular weight of 75-88 kDa, depending on the cell type examined \cite{1, 3, 13, 14}.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23 kDa glycoprotein that regulates the growth and differentiation of several hematopoietic cell lineages \cite{15-17}. In particular, GM-CSF induces differentiation and proliferation of macrophages in response to tissue inflammation \cite{15}. Although GM-CSF plays an important role in the inflammatory response, the subcellular mechanism of its action in macrophages is not fully characterized. One possible pathway that links the action of GM-CSF with

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\textsuperscript{c}Abbreviations: Egr-1, early growth response gene-1; DMEM, Dulbecco's Modified Eagle medium; EDTA, ethylenediamine tetraacetic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; FCS, fetal calf serum; mrGM-CSF, murine recombinant GM-CSF; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
macrophage proliferation is through the induction of a subgroup of immediate early response genes. These genes encode transcriptional regulatory proteins, which include the jun family [18], the fos family [19], a member of the steroid hormone receptor superfamily [20] and the Egr-1 family [21]. Induction of these genes occurs within minutes of cell stimulation, and their transcription does not require de novo protein synthesis [1, 2]. These findings support the hypothesis that the immediate early response gene products (such as Egr-1 protein), which are rapidly synthesized following binding of intercellular second messengers (such as GM-CSF) to the cell surface, serve as nuclear mediators in signal transduction that lead to a cascade of gene expression in activated cells and ultimately to cell proliferation. Evidence suggesting that the Egr-1 gene product plays a role in cell proliferation comes from the recently described Wilms’ tumor suppressor gene, which encodes a zinc finger-containing protein that binds to the Egr-1 consensus sequence and represses transcription [22]. Additionally, Liu et al. report that in murine peritoneal macrophages a similar low concentration of murine recombinant GM-CSF (mrGM-CSF) induces both Egr-1 transcription and macrophage proliferation within minutes of stimulation [23]. Like GM-CSF, fetal calf serum (FCS) also induces rapid transient Egr-1 mRNA and protein synthesis in macrophages as well as cell proliferation [24]. By employing Egr-1 antisense oligonucleotides, Nguyen et al. further demonstrated that Egr-1 is required for differentiation of myeloblasts into macrophages [25]. Taken together, expression of an immediate early response gene, such as Egr-1, may be the initial and the essential step in macrophage differentiation and proliferation. Therefore, we studied the correlation of Egr-1 protein synthesis and macrophage proliferation induced by GM-CSF.

Despite the importance of Egr-1 in macrophage differentiation, the molecular weight of immunoreactive Egr-1 protein and its pattern of induction in macrophages have not been characterized. Therefore, we employed Western blot analysis to characterize Egr-1 protein in murine thioglycollate-elicited peritoneal macrophages following stimulation with GM-CSF or FCS.

MATERIALS AND METHODS

Materials

Dulbecco’s Modified Eagle medium (DMEM) and FCS were purchased from BioWhittaker (Walkersville, MD) and HyClone Lab., Inc. (Logan, UT), respectively. Medium was supplemented with 1 mM glutamine, 25 μg/ml streptomycin, 25 units/ml penicillin, 25 mg/ml gentamicin and 0.05 mM 2-mercaptoethanol and was used to support the cultures of peritoneal macrophages. One unit/ml of murine recombinant GM-CSF (equivalent to 0.1 ng/ml; GIBCO BRL, Bethesda, MD), as defined by the supplier, stimulates 50 percent of murine bone marrow stem cells to differentiate (ED50). All other reagents were obtained from Sigma (St. Louis, MO), unless otherwise specified. Six- to eight-week old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments.

Isolation of macrophages and Western blot analysis

Murine thioglycollate-elicited peritoneal exudate cells were collected and purified as described previously [23]. Briefly, mouse peritoneal exudate cells were incubated at 37°C in five percent CO2 for 3 hr in 16-mm (2 x 106 cells) or 60-mm (5 x 106 cells) plastic tissue culture wells (Costar; Cambridge, MA) to permit adherence. Nonadherent cells were removed by washing twice with Hanks’ balanced salt solution (BioWhittaker), and the peritoneal macrophages were incubated overnight in DMEM plus two percent FCS for the stimulation experiments. Macrophages were maintained in serum-free DMEM for at least 4 hr and then stimulated with either GM-CSF or FCS as described previously [23, 26].
After stimulation, 2 x 10^6 macrophages in 16-mm wells were washed twice with cold phosphate-buffered saline (PBS) followed by adding 100 µl of Laemmlí sample buffer [27]. The tissue culture plates were then heated immediately for 10 min at 95°C, and cell lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [27]. In order to reduce the small molecular weight protein species that interact with anti-Egr-1 antisera, an alternative method was used. Stimulated or unstimulated macrophages (5 x 10^6 cells) in 60-mm wells were harvested in 1.5 ml TEN buffer (40 mM Tris; pH 7.5, 10 mM EDTA and 150 mM NaCl), centrifuged briefly at full speed in an Eppendorf microcentrifuge at room temperature and subsequently suspended in 25 µl of distilled water containing protease inhibitors [26]. Approximately 2.5 to 5 µl of each cell suspension was saved for β-galactosidase assay [28], and the remaining cell extract was mixed with sample buffer for SDS-PAGE. The β-galactosidase assay, used to determine the relative amount of protein, showed that all samples contained a similar amount of activity (± 10 percent; data not shown).

Transfer of fractionated proteins to nitrocellulose membranes (Bio-Rad, Melville, NY) was carried out with the Multiphor II NovaBlot system (LKB, Sweden) using transfer buffer and conditions specified by the manufacturer. Egr-1 proteins were detected with primary anti-Egr-1 rabbit antisera provided by V.P. Sukhatme [3], D. Chang (Amgen, Inc., Thousand Oaks, CA), or Santa Cruz Biotech., Inc. (Santa Cruz, CA), followed by incubation with secondary anti-rabbit IgG (Amersham Corp., Arlington, IL). The enhanced chemiluminescence detection system (Amersham) and Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) were used to visualize Egr-1 proteins.

Quantitation of induced Egr-1 protein bands on the Western autoradiograms was performed with a Macintosh Quadra 800 computer connected to a scanner (Hewlett Packard Scan Jet Plus). Computer programs Desk Scan II (Hewlett Packard, Palo Alto, CA) and Scan Analysis (BioSoft/Elsevier, The Netherlands) were used in this quantitative analysis.

**Macrophage proliferation analysis**

Macrophage proliferation was determined by measuring the incorporation of [methyl-^3H]-thymidine (5 Ci/mmol, 18.5 GBq/mmol; Amersham Corp.) as described previously [23]. Briefly, approximately 2.5 x 10^4 peritoneal exudate cells resuspended in 100 µl DMEM were loaded in each well of a 96-well flat bottom tissue culture plate (Costar) as in the previous section. After a 3-hr incubation at 37°C in five percent CO₂, nonadherent cells were removed and macrophages were incubated in 100 µl DMEM with GM-CSF plus 0.5 percent FCS or 10 percent FCS for 48 hr. [^3H]-thymidine, 1 µCi/well, diluted in DMEM (10 µl final volume) was added to the macrophages and incubated for 24 hr. Cells were treated with 50 µl of 0.25 M NaOH and then collected on glass-fiber filter strips with an automated cell harvester (Cambridge Technologies Inc., Cambridge, MA). [^3H]-thymidine incorporation was measured in a liquid scintillation analyzer (Model Tri-Carb 1500, Packard, Downers Grove, IL).

**RESULTS**

**Dose response of GM-CSF on Egr-1 protein induction**

In order to determine the molecular weight of Egr-1 protein and to investigate the dose response of mrGM-CSF on Egr-1 induction, murine peritoneal macrophages were treated with different concentrations of mrGM-CSF. Cells were harvested and lysates were then subjected to SDS-PAGE followed by Western blot analysis with polyclonal anti-Egr-1 rabbit antiserum provided by Santa Cruz Biotech., Inc. Figure 1A shows that after 60 min of incubation with 50 units/ml of mrGM-CSF, 80 kDa Egr-1 protein was detected by Western blot analysis. As determined by densitometry (Figure 1B), the level
of Egr-1 further increased nearly linearly with increasing concentrations of mrGM-CSF above 100 U/ml (up to 500 U/ml). In addition to the 80 kDa form, other protein species in macrophages also reacted to anti-Egr-1 antibody (Figure 1A). However, these protein species were not consistently induced upon stimulation with mrGM-CSF. Multiple antigenically related protein species of Egr-1 have also been reported in other cell types [3, 13, 14].

**Time course of Egr-1 protein induction**

We then determined the pattern of Egr-1 induction in macrophages treated with mrGM-CSF or FCS for various time periods. As shown in Figure 2A, Egr-1 protein (80 kDa) was induced within 25 min of GM-CSF treatment and reached a maximal level at 70 min, as determined by densitometry of the protein band. FCS (10 percent), which served as the positive control, exhibited a similar pattern of Egr-1 protein induction except the amount of Egr-1 protein reached the maximum level at 45 min (Figure 2B). Similar results were obtained in at least two experiments for each stimulant.

**Correlation of the Egr-1 protein synthesis and macrophage proliferation**

The relationship between proliferation of macrophages and induction of Egr-1 following mrGM-CSF stimulation was investigated. As shown in Figure 3, [3H]-thymidine uptake by macrophages increased in response to increasing amounts of mrGM-CSF. In particular, the trend of macrophage proliferation induced by GM-CSF at the concentration of 25 U/ml or greater was similar to the Egr-1 protein expression pattern effected by GM-CSF above 50 U/ml (Figure 1B). The results suggest that similar concentrations of GM-CSF are required to induce Egr-1 protein and proliferation in murine peritoneal macrophages. As the positive control, we used FCS to stimulate macrophage proliferation.
Figure 2. Time course of Egr-1 protein induction in macrophages treated with GM-CSF or FCS. Murine peritoneal macrophages (Panel A, 2 x 10⁶ cells; Panel B, 5 x 10⁶ cells) were stimulated with 300 units/ml mrGM-CSF (A) or 10 percent FCS (B), and an aliquot of cells was removed at each time point for SDS-PAGE and Western blot analysis using polyclonal anti-Egr-1 rabbit antiserum from Santa Cruz Biotech, Inc. (A) or V.P. Sukhatme (B). The arrows indicate the inducible 80 kDa Egr-1 protein. The relative intensity of Egr-1 protein quantitated by densitometry is also shown. Similar results were obtained in at least two experiments for each stimulant.

Figure 3. Proliferation of macrophages upon induction with GM-CSF. Approximately 2.5 x 10⁴ macrophages were used in each well of a 96-well plate. Macrophages were activated with concentrations of mrGM-CSF ranging from 0 to 300 units/ml plus 0.5 percent FCS for 24 hr before 1 μCi of [³H]-thymidine was added to the cells. The effect of GM-CSF (the horizontal axis) on macrophage [³H]-thymidine uptake (the vertical axis) is plotted. 10 percent FCS was added to macrophages as a positive control. The cpm for cells treated with 10 percent FCS was 6033.5 ± 740.1. The bars shown in this figure represent average measurements of eight samples (n = 8) ± standard error.
DISCUSSION

As shown in Figure 1, Western blot analysis using polyclonal anti-Egr-1 antibody raised against an Egr-1 protein revealed a predominant inducible immunoreactive species with a molecular weight of 80 kDa. Other protein species with various molecular weights (58 kDa, 41 kDa, 40 kDa and 33 kDa), however, were also detected. These proteins were either not inducible (58 kDa, 41 kDa and 40 kDa species) or were inconsistently induced upon stimulation (33 kDa species) in multiple experiments (data not shown). In order to determine the relationship between these protein species detected in the Western analysis and Egr-1 protein, we used two other polyclonal rabbit antisera raised against Egr-1 protein (from D. Chang and V. P. Sukhatme). All these protein species, in addition to the 80 kDa protein, were simultaneously detected by each of these antisera (data not shown). These results suggest that they were antigenically related to the Egr-1 gene product. Since the lower molecular weight forms recognized by anti-Egr-1 antibodies may represent proteolytic products of Egr-1 protein produced during extraction of cellular proteins, we included a mixture of protease inhibitors (final concentrations: leupeptin, 0.5 μg/ml; EDTA, 1 mM; pepstatin, 1 μg/ml; phenylmethylsulfonyl fluoride, 0.5 mM) during cell extraction. The use of protease inhibitors, however, did not reduce the quantity of lower molecular weight proteins detected with the anti-Egr-1 antibodies (data not shown). The molecular weight of the 80 kDa Egr-1 protein consistently identified in murine peritoneal macrophages was similar to the form identified in human [3] and murine [24] fibroblasts. Three inducible phosphorylated proteins, with molecular weights of 135 kDa, 100 kDa and 54 kDa, that are immunoprecipitated from mouse BALB/c 3T3 fibroblasts were not found in murine peritoneal macrophages [3]. Furthermore, other proteins recognized by anti-Egr-1 antibody such as the 73-75 kDa proteins found in rat fibroblasts and PC12 cells [14], were not detected in macrophages. These findings raise the possibility that different forms of Egr-1 protein may be present in various cell types. Since the Egr-1 genome contains one intron [1], alternative splicing is unlikely to explain the heterogeneity of immunoreactive proteins identified in these studies. Conceivably, some of the heterogeneity may be due to variations in post-translational modifications. Recent data of Cao et al. [3] indicate that Egr-1 protein is phosphorylated and that a high molecular weight form (100 kDa) is inducible by inhibition of protein phosphatases.

The kinetics of synthesis of the 80 kDa Egr-1 protein (Figure 2) followed the pattern predicted from the induction of Egr-1 mRNA by GM-CSF [23]. For example, following GM-CSF treatment of macrophages, maximum Egr-1 protein levels lagged 45 min behind maximum mRNA levels [23]. In addition, the amount of Egr-1 protein increased in proportion to the concentration of GM-CSF (Figure 1), which was similar to the trend of Egr-1 mRNA induction determined previously [23]. These data suggest that following stimulation by GM-CSF, the transcription and translation of the Egr-1 are coordinately regulated in murine macrophages. Furthermore, in response to FCS (Figure 2B), the kinetics of Egr-1 induction in murine peritoneal macrophages were similar to those in BALB/c 3T3 cells [24] suggesting that the regulatory mechanism controlling the synthesis of Egr-1 protein may be similar in both types of cells.

Since Egr-1 is induced by all mitogenic stimuli [1, 2, 6] and is required for terminal differentiation of macrophages [25], we investigated the relationship between proliferation of macrophages and induction of Egr-1 protein following GM-CSF stimulation. These data show that Egr-1 protein (Figure 1) and macrophage proliferative response, as measured by [3H]-thymidine uptake (Figure 3) were induced by similar concentrations (25-50 U/ml) of mrGM-CSF. This concentration of GM-CSF is within the range observed in mouse serum exposed to endotoxin in vivo [15]. Conceivably, Egr-1 protein induced by

GM-CSF in vivo may act as an essential step in controlling the proliferation of macrophages.

In summary, mrGM-CSF induced Egr-1 protein of 80 kDa in murine peritoneal macrophages in a dose-dependent manner. The concentration of GM-CSF required for induction of Egr-1 protein in macrophages and for macrophage proliferation were similar, supporting further study on the role of Egr-1 protein in mediating GM-CSF induction of macrophage proliferation.

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