Identification of Upstream Signals Regulating Interleukin-6 Gene Expression During In Vitro Treatment of Human B Cells With Pokeweed Mitogen

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The present studies have characterized the regulation of interleukin-6 (IL-6) gene expression during pokeweed mitogen (PWM)-driven human B-cell differentiation. PWM induced an early and transient increase in the expression of immediate-early response genes of the jun/fos leucine zipper family (c-jun, jun B, c-fos, and fos B). The induction of c-jun mRNA by PWM was concentration dependent. Nuclear run-on assays showed that PWM treatment is associated with an increased rate of c-jun gene transcription. The induction of c-jun mRNA precedes the induction of IL-6 gene expression and IL-6 secretion by the B cells. c-Jun antisense, but not sense, oligodeoxynucleotide (ODN) significantly decreases PWM-related B-cell (1) proliferation; (2) IL-6 mRNA induction; (3) IL-6 secretion; and (4) nuclear extract binding to AP-1 in electrophoretic mobility shift assay. In contrast, c-Fos antisense ODN did not affect either IL-6 mRNA induction or IL-6 secretion triggered in B cells by PWM. The results further show activation of c-Raf-1 kinase in PWM-treated B cells. Raf-1 acts upstream to mitogen-activated protein (MAP) kinase; therefore, studies were performed to assay for MAP kinase activation in these cells. The results show an increase in phosphorylation of myelin basic protein (MBP) and c-Jun "Y" peptide in PWM-treated B cells. Taken together, these findings suggest that PWM is able to initiate an intracytoplasmic signaling cascade in normal human splenic B cells, which, at least in part, involves serine/threonine protein kinases. These results show transient induction of immediate-early response genes in B cells and support a potential role for the c-jun gene product in regulation of IL-6 transcription and secretion.

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We and others have previously developed systems to trigger human splenic and peripheral blood B cells to differentiate in vitro. These studies have permitted the definition of B-cell differentiation antigens (Ags) and delineation of the sequence of the Ag changes that accompany activation and differentiation to the plasma cell stage. Specifically, (1) pokeweed mitogen (PWM) and (2) anti-Ig or Staphylococcus Cowan A and T cells, T-cell supernatants or recombinant growth factors have been used as triggers of B-cell activation and differentiation in culture. Studies using dual fluorescence techniques to show coexpression of two cell surface Ags have not only permitted more precise definition of the acquisition and loss of cell surface Ags on B cells as they differentiate in vitro, but also confirmed the utility of these Ags for the identification of functionally distinct stages of B-cell differentiation in vivo. Interleukin-6 (IL-6) mediates differentiation of Ag or mitogen-activated normal B cells and is produced by activated monocytes, T cells, and B cells, as well as fibroblast, marrow stroma, and endothelial cells. Mitogen-driven B-cell differentiation in vitro is IL-6 mediated because IL-6 mRNA is induced in accessory (T cells and monocytes) and as well as B cells. Moreover, neutralizing monoclonal antibodies (MoAbs) against IL-6 can abrogate Ig secretion. IL-11 triggers accessory cell-dependent B-cell differentiation that is also, at least in part, IL-6 mediated. Therefore, IL-6 is the major growth factor regulating B-cell differentiation and Ig gene transcription.

The cellular events that ultimately direct the regulation of cellular proliferation and differentiation begin with a signal transduction mechanism in which an extracellular signal induces transcription of immediate-early response genes that encode DNA-binding transcription factors and includes: the leucine zipper family of proteins, the nuclear factor-xB (NF-xB) transcription factor family, and the zinc finger transcription factors. The IL-6 gene is regulated by a variety of stimuli in different cells, including glucocorticoids, IL-4, IL-1, IL-2, tumor necrosis factor (TNF), interferon, platelet-derived growth factor, and cyclic 3'-5' adenosine monophosphate (cAMP). Importantly, several transcriptional control elements within the IL-6 promoter mediate some of these effects: a glucocorticoid responsive element; multiple cytokine (IL-1, TNF, serum) and second messenger (cAMP, phorbol ester)-responsive enhancer regions (MRE I, MRE II); an NF-xB binding site; and a region highly analogous to the retinoblastoma control element. Trigerring of B cells through their surface Ig receptors results in activation of tyrosine-specific protein kinases and a polyphosphoinositide-specific phosphodiesterase leading to hydrolysis of membrane-associated phospholipid and generation of second messenger molecules that activate protein kinase C (PKC). PKC activation in turn results in expression of early response genes, including c-fos, EGR-1 and c-myc, some of which encode transcription factors that may also regulate IL-6 expression, i.e., via serum response element. Cross-linking slg receptor on a murine B-lymphoma line induces nuclear AP-1- and TRE-binding activity consisting of both Jun and Fos proteins, suggesting that AP-1 may function to couple the slgR to changes in nuclear gene expression in B cells. Treatment of human B cells with okadaic acid, an inhibitor of phosphatases 1 and 2A results in induction of AP-1, NF-xB, and TNF receptors mRNA, without inducing either B-cell proliferation or Ig secretion. Our recent studies in T cells have shown that either anti-CD3 or PWM induce c-jun gene expression by a transcriptional control element within the IL-6 promoter. The results further show activation of c-Raf-1 kinase in PWM-treated B cells. Raf-1 acts upstream to mitogen-activated protein (MAP) kinase; therefore, studies were performed to assay for MAP kinase activation in these cells. The results show an increase in phosphorylation of myelin basic protein (MBP) and c-Jun "Y" peptide in PWM-treated B cells. Taken together, these findings suggest that PWM is able to initiate an intracytoplasmic signaling cascade in normal human splenic B cells, which, at least in part, involves serine/threonine protein kinases. These results show transient induction of immediate-early response genes in B cells and support a potential role for the c-jun gene product in regulation of IL-6 transcription and secretion.
tional and posttranscriptional mechanism, and that AP-1 confers PWM inducibility of this gene. Importantly, induction of c-jun precedes induction of IL-6 in T cells, suggesting AP-1 involvement. However, to date, those early response gene products that regulate IL-6 gene expression in B cells during PWM-driven in vitro differentiation are not yet defined.

In the present report, we extend our preliminary studies to characterize the expression and regulation of IL-6 as well as selected early response genes (c-jun, junB, c-fos, fos-B) in B cells during in vitro B-cell activation and differentiation triggered by PWM. We show that PWM induces c-jun gene transcription, and that induction of c-jun precedes that of the IL-6 gene and secretion of IL-6 by B cells. To confirm the functional significance of c-jun in IL-6 regulation, we show that antisense oligodeoxynucleotide (ODN) to c-Jun significantly decreases PWM-triggered B-cell (1) proliferation, (2) induction of IL-6 mRNA, (3) IL-6 secretion, and (4) AP-1 binding of nuclear extracts in electrophoretic mobility shift assays (EMSA). In contrast, c-Fos antisense ODN did not block PWM-related IL-6 mRNA induction or IL-6 secretion. Triggering of human B cells in vitro with PWM also initiates upstream events including phosphorylation and activation of cytoplasmic c-Raf-1 and mitogen-activated protein (MAP) serine/threonine protein kinases. Therefore, our data support the view that triggering of B cells with PWM may result in sequential phosphorylation of Raf-1 and MAP kinases and an increased c-jun gene transcription; and that c-Jun, at least in part, regulates PWM-induced IL-6 expression in B cells.

EXPERIMENTAL PROCEDURES

Cells and cell culture. Normal spleen was obtained from operative specimens of patients not known to have any systemic or malignant disease. Single-cell suspensions were prepared by extrusion through sterile stainless steel mesh; mononuclear cells were isolated from sterile stainless steel mesh; mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density sedimentation. Cells were adhered for 1 hour at 37°C on plastic petri dishes to deplete monocytes; nonadherent cells were greater than 95% CD20+, as assessed by indirect immunofluorescence. The purified B cells were suspended in RPMI 1640 medium (Grand Island Biological Company, Grand Island, NY) with 10% fetal bovine serum and cultured for 3 to 4 hours at 37°C before stimulation with PWM (Sigma Chemical Co, St Louis, MO) and that induction of c-jun gene transcription; and that c-Jun, at least in part, regulates PWM-induced IL-6 expression in B cells.

Preparation of RNA and Northern blot hybridization. Total cellular RNA was isolated by a modification of the guanidine-isothiocyanate technique, as previously described. Total cellular RNA (10 /g/lane) was subjected to electrophoresis in a 1% agarose/2 mol/L formaldehyde gel, transferred to nitrocellulose paper, and hybridized to one of the following 32P-labeled DNA probes: (1) the 1.8-kb BamHI/EcoRI insert of the human c-jun DNA probe containing a 1.0 kb cDNA and 0.8-kb 3' untranslated sequences purified from a plasmid transfected into a B cell line; (2) the 1.8-kb EcoRI fragment of the murine jun-B cDNA purified from the pc-jun-B plasmid; (3) the 0.9-kb SmaI/EcoRI fragment of the human c-jun DNA consisting of exons 3 and 4 purified from the pc-jun-B plasmid; (4) the 2.4-kb EcoRI insert of murine fos-B in plasmid pUC19; (5) the pAl plasmid containing a 2.0-kb Pst I insert of the chicken b actin gene; and (6) the Ban HI-Taq I fragment containing nucleotides 215 to 657 of a full-length IL-6 cDNA. The hybridizations were performed for 16 to 24 hours at 42°C in 50% (vol/vol) formamide, 2 × SSC (SSC: 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate), 1 × Denhardt's solution, 0.1% (w/vol) sodium dodecyl sulfate (SDS), and 200 /g/ml salmon sperm DNA. Filters were washed and exposed to Kodak X-Omat XAR film (Eastman-Kodak, Rochester, NY) using an intensifying screen. The autoradiograms were scanned using a laser densitometer.

Nuclear run-on assays. Normal human splenic B cells were treated as indicated, pelleted at 500g, and washed twice with ice-cold phosphate-buffered saline (PBS). Nuclear run-on assays were performed as previously described. In brief, the cells were resuspended in ice-cold lysis buffer and the nuclei were pelleted. The supernatant was removed and the nuclei resuspended in glycerol buffer. An equal volume of reaction buffer was added to the nuclei suspension and incubated at 20°C for 30 minutes with 250 /lCi (a-32P) UTP (800 Ci/mmol, New England Nuclear, Boston, MA). Transcription was terminated by the addition of 40 U DNase I, 10 mmol/L TRIS-HCl, pH 7.4, 100 mmol/L NaCl, 1 mmol/L Na2EDTA, 60 mg/ml yeast RNA, and 150 mmol/L Na3citrate. Proteinase K (750 /g/ml) and 1% (vol/vol) SDS were then added. Nuclear RNA was isolated by phenol/chloroform extractions and then ethanol precipitated; RNA was purified through a spin column.

Plasmid DNAs containing cloned inserts were digested with restriction endonucleases as follows: (1) the 2.0-kb BamHI fragment of the human c-jun DNA. The digested DNA was denatured by heating, separated in a 1% agarose gel, and transferred to nitrocellulose filters by the method of Southern. The filters were prehybridized and hybridization performed as previously described. The autoradiograms were scanned using a laser densitometer.

Subcellular fractionation. Subcellular fractionation was performed as described. B cells were washed twice with cold PBS and resuspended in 1 ml hypotonic lysis buffer. After swelling on ice for 30 minutes, the cells were disrupted by Dounce homogenization (25 strokes). The homogenate was layered onto 1 ml of 1 mol/L sucrose in lysis buffer and centrifuged at 16000g for 15 minutes to pellet nuclei. The supernatant above the sucrose cushion was collected and centrifuged at 150,000g for 30 minutes at 4°C to collect the soluble or cytoplasmic fraction.

MAP kinase assays. MAP kinase activity was assayed in B cells as described. Briefly, the cytosolic fraction was applied to Q-Sepharose fast flow columns (Pharmacia LKB) previously equilibrated with buffer containing 0.15 mol/L NaCl. After washing the columns with buffer A containing 0.15 mol/L NaCl, MAP kinase was eluted with buffer containing 0.55 mol/L NaCl. Each fraction was assayed for phosphorylation of the c-Jun Y peptide (amino acids 56 to 69, NSDLVSPDVGLLK) by incubating 5 to 25 ml of the fraction with 1 /g [Y-32P] adenosine triphosphate (ATP), 25 mmol/L TRIS-HCl, pH 7.5; 1, 1 mmol/L MgCl2, 50 /mol/L ATP and 2.5 /g of Y peptide in a reaction volume of 50 ml. After incubation at 30°C for 15 minutes, 25 /l of the reaction mixture was spotted onto a 1.5 × 1.5 cm p81 filter (Whatman, Maidstone, GB) and washed twice with 1% phosphoric acid and then water before quantitation of radioactivity by liquid scintillation counting. Protein concentrations of the cellular extracts were determined by the method of Bradford. Phosphorylation of myelin basic protein (MBP), also a substrate for MAP kinase, was studied as an additional measure of MAP kinase activity.

Raf-1 immunoblot analysis. Assays of Raf-1 phosphorylation were performed on the cytosolic fraction as described. Proteins (50 /g) were separated in 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose paper by the dry transfer method (BioRad, Richmond, CA). The residual binding sites were blocked by incubating the filters in 5% dry milk in PBST (PBS/0.05% Tween-20) for 1 hour at room temperature. The blots were subsequently incubated.
with anti–Raf-1 (anti-SP63, 1:1000 dilution; kindly provided by U. Kapp, National Cancer Institute, Frederick, MD) antisera in 5% milk/PBS for 18 hours at 4°C. After washing, the blots were incubated for 1 hour on temperature with antirabbit IgG (whole molecule) peroxidase conjugate (Sigma) in 5% milk/PBST. Blots were washed and antigen-antibody complexes visualized by the Enhanced Chemiluminescence detection system (Amersham, Arlington Heights, IL).

**Immune complex Raf-1 kinase assays.** Assays for Raf-1 kinase activity were performed using immune complexes as described. In brief, cells were washed three times with ice-cold PBS and resuspended in lysis buffer. Cell lysates were preclarified by incubation with preimmune rabbit serum and protein A sepharose and the supernatant fraction then incubated with anti–Raf-1 antisera (SP63) and protein A sepharose. The immunoprecipitates were washed and the immune complexes suspended in 20 μL of reaction buffer (20 mmol/L TRIS-HCl, pH 7.3, 10 mmol/L MgCl₂). The reaction was started by adding 50 mmol/L ATP, 20 μCi (γ³²P) ATP and 10 to 12 μg H1 histone, and allowed to proceed at 30°C for 30 minutes. An equal volume of 2X SDS polyacrylamide gel electrophoresis (PAGE) buffer was then added, and the samples were incubated at 90°C for 5 minutes. The proteins were separated by electrophoresis in SDS-12.5% polyacrylamide gel. The gel was stained with Coomasie blue and then subjected to autoradiography.

**Preparation of nuclear extracts and electrophoretic mobility shift analysis.** Nuclear extracts were prepared by hypotonic lysis and extraction of nuclei with high salt buffer as described. Protein concentrations were determined using the Bradford assay, and equivalent amounts of extracts were used for in vitro binding assays. Binding assays were performed in a final volume of 10 μL containing 6 mmol/L HEPES, pH 7.9, 60 mmol/L NaCl, 0.6% glycerol for 15 minutes using indicated probes. Ap-1 probe used was generated by annealing synthetic ODNs containing the AP-1 site from the metallothioneine gene promoter. The probe was labeled with polynucleotide kinase and γ³²P-ATP. Binding reactions were analyzed by electrophoresis through 4% polyacrylamide gels in 0.5× TRIS-borate buffer.

**Measurement of IL-6.** IL-6 levels in the supernatants obtained from 72-hour cultures of B cells were measured in a bioassay by using IL-6-dependent B-9 cells, as previously described. IL-6 was also measured using enzyme-linked immunosorbent assay (ELISA). Serial dilutions (100 μL) of B-cell culture supernatants were added in duplicate to 96-well plates (Costar, Cambridge, MA) coated with IgG1 anti–IL-6 antibody (murine IgG1, TORAY, Osaka, Japan). Bio-tnylated detector anti–IL-6 MoAb (TORAY) was next added and developed with streptavidin (Amersham). IL-6 levels in each supernatant were determined by comparison with a standard curve. Level of detection was 1 ng/mL.

**c-Jun and c-Fos sense or antisense ODN treatment of splenic B cells.** B cells were cultured at a concentration of 1 × 10⁷/mL in 24-well plates (Costar) in media alone or were pretreated for 8 hours with either c-Jun or c-Fos sense or antisense ODN (40 μg/mL). The B cells were then stimulated with PWM (2.5 μg/mL) for 24, 72, and 129 hours. To measure in vitro DNA synthesis, B cells were pulsed with [³H]TdR (0.2 mCi/well) for the last 14 hours of culture, harvested with the aid of an automatic cell harvester (Cambridge Technology, Cambridge, MA), and counted on a liquid scintillation counter (Packard Tri-Carb 4530, Downers Grover, IL). B cells were also cultured with PWM and either c-Jun sense or antisense ODN and/or c-Fos sense or antisense ODN, and effect on induction of IL-6 mRNA and IL-6 secretion determined by Northern blotting and IL-6 assays, respectively, as described above. The sequence of c-Jun sense ODN (codon 1-6) was 5'-ATGACTGCAAAGATGGAA-3'; the sequence of c-Jun antisense ODN (complementary to codon 1-6) was 5'-TCCATCTTTGCCATGCG-3'. The sequence of the c-Fos antisense ODN was 5'-AAACCGGAAACATCAT-3'; and the sequence of c-Fos sense ODN was 5'-ATGATGTTTCTCGGTTT-3'.

**Solid-phase ELISA for IgG determination.** The quantitative ELISA used to measure the IgG secreted into the culture supernatants was done in microtiter plates, as previously described. (1) wells were coated with goat-antihuman IgG antibody diluted at 1/1,000 in 200 μL of PBS for 16 hours at room temperature; (2) wells were saturated with 1% bovine serum albumin (BSA)-PBS for 1 hour; (3) IgG standards (sample of 200 μL of 11% BSA-PBS) or 200 μL of samples to be assayed were incubated for 2 hours; and (4) 200 μL of peroxidase-conjugated goat-antihuman IgG antibodies diluted in 1% BSA-PBS at 1/10,000 were added for 2 hours and washed with phenylendiamine. The IgG standard curve was linear in range of 0.05 to 15 ng/mL.

**RESULTS**

PWM induces c-jun, jun-B, c-fos, and fos-B mRNA in B cells. The effect of PWM on the expression of early response genes of the jun and fos families was studied in human splenic B cells. The B cells, isolated by adherence to plastic and double E rosetting to deplete monocytes and T cells, respectively, expressed low to undetectable levels of these genes after culture for 3 to 4 hours in media (Fig 1A). Triggering of B cells with PWM resulted in 20- to 30-fold increases in c-jun- and jun-B transcripts by 15 minutes. Longer periods of incubation were associated with downregulation of these transcripts. c-fos and fos-B mRNA were also increased 20- to 30-fold by 15 minutes, but showed sustained expression until 1 hour after PWM treatment. Probing for β-actin confirmed equal loading of the gel. These findings show that multiple genes coding for leucine zipper transcription factors are induced by PWM in normal splenic B cells.

PWM induces increased c-jun transcription. Run-on assays were performed to determine whether the effects of PWM on c-jun expression in B cells are related to increases in the rate of transcription of this gene. Nuclear RNA was isolated from cells treated with 2.5 μg/mL of PWM for 5 minutes and hybridized to actin and c-jun cDNA probes. The actin gene was constitutively transcribed in B cells and the rate of actin gene transcription was unaffected by PWM treatment (Fig 1B). Low-level transcription of the c-jun gene was also detectable in untreated B cells. However, the rate of c-jun transcription was increased up to 12-fold (as determined by densitometric scanning) in PWM-treated B cells (Fig 1B). These findings show that PWM induces c-jun expression, at least in part, by a transcriptional mechanism.

Activation of MAP kinase in PWM-treated B cells. Previous studies have shown that MAP kinase is activated by cross-linking slg on B-cell lymphoma lines and that MAP kinase contributes to the transcriptional activation of the c-jun gene. Given the above demonstrated induction of c-jun in B cells by PWM, we next examined the MAP kinase activity. The cytosolic extracts of PWM-treated and control B cells were first subjected to in vitro phosphorylation assays using MBP, a known substrate for activated MAP kinase. Exposure to PWM for 15 minutes induced a marked increase in MAP kinase activity in B cells, as shown by a threefold to fourfold increase in phosphorylation of MBP (Fig 2A). The activity decreased by 30 minutes and reached basal level by 60 minutes. The effect of PWM was dose dependent, and
Transcripts were hybridized to plasmids containing 2 pg of actin or c-jun mRNA expression. Decreased by 30 and 60 minutes (Fig 2A). The rapid increase in MAP kinase activity resulted in increased phosphorylation of c-Jun, a synthetic Y-peptide (amino acids 57 through 69) of the c-Jun was used as a substrate in the MAP kinase assay. PWM induced a rapid twofold to threefold increase in phosphorylation of c-Jun Y-peptide by 15 minutes that decreased by 30 and 60 minutes (Fig 2A). The rapid increase in MAP kinase activity corresponds to maximal induction of c-jun mRNA expression.

Activation of Raf-1 kinase in PWM-treated B cells. Because it is known that Raf-1 kinase can activate MAP kinases, we next determined whether Raf-1 kinase was activated by PWM in B cells. B cells from normal human spleen were treated with PWM (2.5 µg/mL). Equal amounts of protein were taken from B-cell lysates and analyzed on 7.5% SDS-PAGE. Raf-1 was detected by immunoblotting with anti-Raf-1 antisera. Treatment of B cells with PWM resulted in decreased electrophoretic mobility of Raf-1 kinase, corresponding to a shift from 72 to 74 kD at 10 to 30 minutes (Fig 2B), consistent with hyperphosphorylation and activation of Raf-1 kinase. To confirm these findings, Raf-1 kinase activity was also measured by assaying Raf-1 immune complexes for phosphorylation of histone. Immunoprecipitation with an anti-Raf-1 antibody showed low levels of Raf-1 activity in untreated cells, whereas treatment of B cells with PWM for 10 minutes was associated with an increase in Raf-1 activity with increased phosphorylation of histone phosphorylation (Fig 2C). This phosphorylation of Raf-1 kinase corresponds temporally with both the activation of MAP kinase and the increased transcription of c-jun mRNA induced in B cells by PWM.

Kinetics of PWM-related induction of c-jun mRNA and IL-6 mRNA in B cells. Previous studies have shown that IL-6 is a differentiation factor for B cells and that PWM can induce IL-6 mRNA in B and accessory cells. In addition, the IL-6 promoter has AP-1 binding sites (MREI and MREII) that may bind Jun-Jun homodimers or Jun/Fos heterodimers and serve as transcriptional regulators. Therefore, we examined the kinetics of c-jun and IL-6 induction in B cells triggered with PWM. A transient induction of IL-6 mRNA was observed that was maximal (12- to 14-fold increase by densitometric analysis) at 6 hours and then down-regulated by 12 and 24 hours (Fig 3A). The peak (20- to 25-fold) increase in c-jun mRNA occurred earlier, at 15 minutes (Fig 1A), and c-jun transcripts were no longer detectable when IL-6 mRNA was induced (Fig 3A). These effects of PWM were not associated with any alterations in the level of actin transcripts.

C-jun regulates PWM-related induction of IL-6 mRNA and IL-6 secretion by B cells. To examine whether c-jun plays a role in the induction of IL-6 by PWM, antisense ODN to c-jun was added to the B-cell cultures and effects on IL-6 gene expression were assayed (Fig 3B). A significant block (47% ± 8%, n = 4, P < 0.05) in IL-6 mRNA expression was observed in B cells cultured with PWM and c-jun antisense ODN, whereas culture of PWM-treated B cells with c-Jun sense ODN had little effect on the induction of IL-6 mRNA. Moreover, culture with c-Jun antisense ODN strongly inhibited PWM-related induction of c-jun mRNA in B cells.

Given that c-Jun antisense ODN significantly blocked the PWM-related IL-6 mRNA induction in B cells, we next examined its effect on IL-6 protein secretion. B cells were cultured with media, PWM, and PWM with or without c-Jun sense or antisense ODNs. Supernatants from these cultures were assayed for IL-6 in a bioassay using the B9 IL-6-dependent hybridoma cells or by ELISA. Importantly, B9 cells were not stimulated when cultured with PWM alone (data not shown). As shown in Fig 4A, low levels (<5 ng/mL) of IL-6 were secreted by B cells cultured in media alone.
Culture with PWM reproducibly increased IL-6 secretion by B cells (>300 μg/mL). Culture with c-Jun antisense ODN inhibited PWM-induced IL-6 secretion by B cells: 62% ± 8% inhibition (n = 4, P < .07) detected by B9 bioassay (Fig 4B) and 42% ± 8% inhibition (n = 3, P < .07) by ELISA (Fig 4C). In contrast, IL-6 secretion by B cells cultured with PWM and c-jun sense ODN was not significantly different from that triggered in B cells by PWM alone. Additional experiments were also performed in B cells cultured with media, PWM, and PWM with or without sense c-Fos ODN or antisense c-Fos ODN. Supernatants from these cultures were also assayed for IL-6. In contrast with antisense c-Jun ODN, antisense cFos ODN did not inhibit IL-6 secretion by PWM-treated B cells.

Electrophoretic mobility shift assay. Nuclear extracts from control and PWM-treated B cells were assayed for binding to synthetic polynucleotide Kinase and γ32-P-ATP-labeled AP-1 probe. As shown in Fig 5, three specific bands representing AP-1 complexes were detectable in EMSA. All of these bands disappeared upon preincubation of nuclear extracts with anti-c-Jun antibody, indicating the presence of c-Jun protein in the bands and binding specificity. The results further showed a low to undetectable level of AP-1 DNA binding activity in untreated control B cells (upper and lowermost bands, Fig 5). In contrast, the PWM-treated B cells showed induced AP-1 DNA binding activity. c-Jun antisense, but not sense, ODN significantly decreased the AP-1 DNA binding activity. The band indicated by the middle arrow (Fig 5) shows constitutive levels of AP-1 DNA binding activity that was induced upon PWM treatment of
B cells and was decreased to the baseline level by c-Jun antisense ODN treatment.

In the same experiments, a portion of B cells were further incubated for 72 hours to measure IL-6 secretion in culture supernatants. The results showed decreased IL-6 secretion in PWM-treated B cells cultured with antisense c-Jun ODN (Fig 4, B and C). The data suggest that a correlation exists between the decreased AP-1 binding activity and decreased IL-6 secretion, and further supports a potential role for c-Jun gene product in regulating IL-6 gene expression and secretion.

c-jun regulates PWM-related induction of DNA synthesis in B cells. We next examined the functional significance of c-Jun sense and antisense ODN on DNA synthesis and Ig secretion by B cells cultured with PWM. B cells were cultured with PWM with or without c-Jun sense and antisense ODNs. B-cell proliferation was inhibited (44% ± 7%, n = 4; P < .05) in the c-Jun antisense ODN-treated cultures compared with either PWM alone or c-Jun sense ODN treated cultures (Fig 6). To determine whether this inhibition of DNA synthesis was correlated with c-jun transcript level, total RNA (10 μg) was extracted from each sample. Very low levels of c-jun mRNA were detectable in the PWM-treated cells cultured with c-Jun antisense ODN, but it was abundantly expressed in cells cultured with c-Jun sense ODN and in cells cultured with PWM alone (Fig 3B).

Supernatants from these B-cell cultures were also subjected to solid-phase ELISA to assay for IgG secretion. No IgG secretion was observed by purified B cells cultured in media with PWM alone, or with c-Jun sense or antisense ODNs. Because PWM is an accessory cell-dependent B-cell mitogen, the lack of IgG secretion confirms the homogeneity of B cells studied and the lack of contaminating accessory cells (T cells and monocytes) in all cultures.

Discussion

In the present studies, PWM induced an increase in c-jun, jun B, c-fos, and fos B transcripts in purified B cells that occurred early and was transient. The induction of c-jun mRNA was PWM concentration dependent. Nuclear run-on assays further showed that the induction of c-jun gene expression by PWM in B cells is controlled, at least in part, at the transcriptional level. Moreover, PWM induces IL-6 mRNA in B cells after maximal c-jun mRNA expression and at a time when c-jun mRNA expression is no longer detectable. Our previous studies in T cells have suggested that PWM, as well as anti-CD3, also induces early transient induction of c-jun transcripts. This induction of c-jun mRNA in T cells is related to both transcriptional and post-transcriptional mechanisms. As in the present studies in B cells, PWM also induces IL-6 mRNA in T cells after maximal c-jun mRNA expression and at a time when c-jun mRNA is no longer detectable. PWM mediates accessory cell-dependent IL-6–mediated B-cell differentiation; given that the IL-6 promoter has functionally important AP-1 binding sites (MREI and MREII), these studies suggest that c-Jun may play a role in triggering IL-6 in B cells, as well as accessory cells, by PWM. The current studies were performed with B cells purified by depletion of monocytes and T cells. Given the absolute dependence on accessory cells for PWM-triggered Ig secretion in vivo, the lack of Ig secretion in any cultures confirmed the absence of contaminating accessory cells (T cells and monocytes). Thus, the current observations are restricted to human B lymphocytes.

Previous studies have shown that activation of B cells by ligation of sIg can activate protein kinase C, which may result in induction of early response genes, ie, c-fos, and thereby potentially regulate IL-6 expression. In a murine B-lymphoma line, cross-linking of sIg induces both nuclear AP-1 and TRE-binding activity consisting of both Jun and Fos proteins. Our studies of human B cells suggest that both c-jun and c-fos are induced by PWM and support a regulatory role for c-jun in IL-6 gene induction. In particular, IL-6 has been shown to be the major differentiation factor in murine and human B cells, and PWM-driven B-cell differentiation in vitro is IL-6 mediated because antibodies to IL-6 can abrogate PWM-induced Ig secretion.
IL-6 REGULATION IN PWM-TREATED B CELLS

Fig 4. (A) Effect of PWM on IL-6 secretion by B cells. B cells were cultured for 72 hours with media, PWM, PWM plus antisense c-Jun ODN, or PWM plus sense c-Jun ODN. Supernatants from cultures were harvested, heat inactivated, filter sterilized and assayed for IL-6 bioactivity. Values represent the mean ± SD of three experiments. IL-6 secretion in cultures of B cells in media was normalized to one to accurately show the relative secretion of IL-6 by bioassay (B) and by ELISA (C) under indicated culture conditions.
Jun antisense ODN, but not c-Fos sense ODN, significantly blocked induction of IL-6 mRNA induced by PWM. Further support for a regulatory role for c-Jun is the demonstration that PWM-induced IL-6 secretion by purified B cells is also inhibited by c-Jun antisense ODN. Because the c-Jun antisense ODN-related block of IL-6 mRNA expression and of IL-6 secretion by PWM-treated B cells was not complete, there may be other early response genes, ie, the NF-κB or zinc-finger families, which may also be functionally important. Nonetheless, these studies support a role, at least in part, for c-Jun in triggering IL-6 expression and secretion by human B cells.

Previous studies have also examined activation of protein kinases related to triggering of B cells via the antigen receptor or mitogens. For example, P21^SOS^ is a substrate for tyrosine kinases activated by cross-linking slg on murine B cells. P21^SOS^ is expressed in human B Epstein-Barr virus-transformed IL-2 receptor-positive lymphoblasts, with impaired lg gene expression, consistent with the view that the ras oncogene is involved in B-cell activation and may block differentiation to the plasma cell stage. Cross-linking slg on B cells also stimulates phosphorylation of c-Raf-1 and MAP kinases. Raf-1 acts upstream of MAP kinase; MAP kinases specifically phosphorylate serine residues in the amino terminal domain of c-Jun. Our studies show that PWM also triggers phosphorylation of Raf-1 kinase, confirmed by immunoblotting and by Raf-1 immune complex assay using H1 histone as a substrate. In the present studies, MAP kinase activation was evidenced by phosphorylation of MBP and specifically, of the synthetic Y peptide derived from c-Jun. Activation of these kinases was associated with induction of c-jun transcription and preceded induction of IL-6 mRNA and secretion of IL-6 by PWM-triggered B cells. Therefore, our studies suggest that triggering in vitro

![Fig 5. Effect of antisense c-Jun ODN on DNA binding in electrophoretic mobility shift assays of PWM-treated B-cell nuclear extracts.](image)

PWM

Control B cells

PWM

sense c-Jun

sense c-Jun antisense

PWM

α-sense c-Jun

α-sense c-Jun antisera

Fig 5. Effect of antisense c-Jun ODN on DNA binding in electrophoretic mobility shift assays of PWM-treated B-cell nuclear extracts. Nuclear extracts from control and PWM-treated B cells were assayed for binding to synthetic polynucleotide and γ32-P-ATP-labeled AP-1 probe using polyacrylamide-gel electrophoresis. Nuclear extracts assayed were from cultures of B cells in media, with PWM, with PWM and c-Jun sense ODN, and with PWM and c-Jun antisense ODN. Nuclear extracts from PWM-treated B cells were also incubated with c-Jun antisera to confirm specificity of binding. AP-1 DNA binding activity was low to undetectable in untreated control B cells (upper and lowermost bands). In contrast, the PWM-treated B cells showed induced AP-1 DNA binding activity. c-Jun antisense ODN, but not sense ODN, significantly decreased the AP-1 DNA binding activity. The band indicated by the middle arrow shows constitutive levels of AP-1 DNA binding activity that was induced upon PWM treatment of B cells and was decreased to baseline level by c-Jun antisense ODN treatment.

![Fig 6. Antisense c-Jun ODN inhibits PWM-induced DNA synthesis in B cells. B cells (1 × 10^6/mL) cultured in 96-well plates were pretreated with 40 μg/mL sense or antisense c-Jun ODNs. The cells were then stimulated with 2.5 μg/mL of PWM for the indicated times. 3[H] TdR was added 14 hours before harvesting the cells, and 3[H] TdR uptake quantitated by liquid-scintillation counting. The results represent mean ± SD of five independent experiments in media (■), PWM (□), PWM plus antisense c-Jun ODN (●) and PWM plus sense c-Jun ODN (▲).](image)
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differentiation of B cells with PWM in sequence activates Raf-1 and MAP kinases and increases c-jun gene transcription and AP-1 DNA binding activity of nuclear extracts. c-Jun, as a homodimer, is known to bind to AP-1 sites, and therefore, may bind to the IL-6 promoter and thereby regulate its transcription. This pathway is also likely to be involved in regulating PWM-triggered DNA synthesis in purified B cells, because c-Jun antisense ODN also significantly decreases B-cell proliferation. Ongoing studies will delineate the precise relationship between IL-6 regulation and proliferation. Future studies will also define additional signal transduction pathways triggered in PWM-treated B cells to fully account for the regulation of IL-6.

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