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Alteration of nuclear factor-κB (NF-κB) expression in bone marrow stromal cells treated with etoposide

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Abstract

Bone marrow stromal cells are an essential regulatory component in the hematopoietic microenvironment. Regulation of hematopoietic cell development is mediated, in part, through interaction of progenitor cells with stromal cell vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 expression has been shown to be driven primarily by binding of nuclear factor-κB (NF-κB) to two consensus binding sites in the promoter region. In this study, we show that down-regulation of VCAM-1 by the chemotherapeutic agent etoposide (VP-16) is associated with altered cellular localization of NF-κB. We demonstrated that VCAM-1 was diminished at the transcriptional level following treatment of stromal cells with VP-16, without alteration of VCAM-1 stability. Culture of bone marrow stromal cells in VP-16 resulted in reduced nuclear RelA (p65), a modest increase in nuclear NF-κB1 (p50), and reduced NF-κB binding to its DNA consensus sequence. Total levels of the NF-κB inhibitor Iκ-Bα were reduced during exposure to VP-16. Following removal of VP-16 from the culture, p65 and p50 nuclear profiles approximated those of untreated stromal cells, and VCAM-1 protein expression was restored. The current study indicates that NF-κB is a target molecule that is responsive to VP-16-induced damage in bone marrow stromal cells. As the primary transcription factor that promotes VCAM-1 expression, the observed changes in p65 and p50 cellular localization during treatment have a direct consequence for stromal cell function. The myriad of genes regulated by NF-κB, including both adhesion molecules and cytokines that contribute to stromal cell function, make chemotherapy-induced disruption of NF-κB biologically significant. Alterations in NF-κB activity may provide one measure by which the effects of aggressive treatment strategies on the bone marrow microenvironment can be evaluated.

Keywords: Stromal cell; NF-κB; VP-16; Hematopoiesis; VCAM-1; Chemotherapy

1. Introduction

The bone marrow microenvironment is the primary site of normal postnatal hematopoiesis and the location of hematopoietic recovery following chemotherapy or irradiation-induced injury to the immune system. Bone marrow stromal cells are critical components of the marrow microenvironment that regulate hematopoietic cell survival, proliferation, and differentiation [1–6]. As such, they ensure appropriate development of mature lymphoid and myeloid cells. Therefore, appropriate stromal cell function is a central regulatory component of hematopoiesis.

As treatments for many malignancies become increasingly aggressive, it has become clear that targets for damage include not only malignant cells, and frequently their normal counterparts, but also the supportive bone marrow stroma [1–6]. Stromal cell damage is implicated by delays in hematopoietic recovery, even when healthy progenitor cells are provided, as in the bone marrow transplantation setting [7–11]. While delays in reconstitution of the hematopoietic system are an expected consequence of aggressive chemotherapy, the extent to which a damaged microenvironment contributes to such delays requires further investi-

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Abbreviations: VP-16, etoposide; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4; NF-κB, nuclear factor-κB; IRF-1, interferon response factor-1; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte macrophage-colony stimulating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; poly(dI-dC), polydeoxyinosinic-deoxycytidylic acid; and HRP, horseradish peroxidase.

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gation. We have reported previously, using an *in vitro* model, that bone marrow stromal cells treated with VP-16 have diminished capability to support proliferation and survival of lymphoid and myeloid progenitor cells [7]. This altered support capacity is associated with reduced VCAM-1 protein expression [12,13]. Altered capacity to support hematopoietic cells was not due to initiation of stromal cell apoptosis. No laddering of DNA was detected following treatment, and stromal cells proliferated following removal of VP-16 [7]. Other investigators also have noted reduced VCAM-1 expression on bone marrow stromal cells derived from patients treated with a variety of chemotherapeutic agents [12]. Interestingly, previous reports have indicated a specific decrease in VCAM-1 expression in human umbilical vein endothelial cells following exposure to topoisomerase II inhibitors, including VP-16 [13]. These previous observations, combined with the frequent use of VP-16 in several treatment regimens [14,15], make it a clinically relevant drug for further investigation.

Several studies have demonstrated that physical interaction between stromal cells and hematopoietic progenitors is essential during hematopoiesis [16–21]. Co-culture of human hematopoietic progenitors with stromal cells separated by transwell membranes resulted in reduced viability of progenitor cells compared with those in direct co-culture [4]. VCAM-1 has been identified as a critical molecule that mediates adhesion of progenitors to stromal cells in both murine and human *in vitro* models [20,22–27]. Murine *in vivo* models have indicated that [22,23] interaction of VLA-4 on immature progenitor cells with stromal cell VCAM-1 engages signaling pathways essential to hematopoietic cell development [28]. Stromal cell cultures established from patients treated with a variety of chemotherapeutic regimens have diminished adhesion of immature B lineage progenitor cells, reduced capacity to support expansion of this same population, and lower levels of VCAM-1 than stromal cells from untreated control patients [12]. These combined observations suggest that disrupted stromal cell VCAM-1 during chemotherapy may contribute to delayed hematopoietic recovery following cessation of treatment.

It is well documented that transcription of VCAM-1 is regulated, in part, by binding of NF-κB to consensus binding sites in the VCAM-1 promoter [29–31]. In some cell types, binding of homodimeric p65 has also been shown to enhance VCAM-1 transcription [29,30]. While the contribution of NF-κB and/or p65 homodimers appears largely cell type specific, a consistent model prevails in which p65 activates VCAM-1 transcription [30,32]. Binding of the trans-activating factors Sp-1 and IRF-1 to their recognition sequences in the VCAM-1 promoter further enhances NF-κB gene transcription [31,33]. In contrast to p65, dramatic increases in the level of p50, favoring the generation of p50 homodimers, have been reported to repress VCAM-1 expression [30,31].

NF-κB heterodimers can be retained in the cytoplasm by masking of the nuclear localization signal. This is mediated primarily through interaction with inhibitory proteins of the Iκ-B family [34,35]. Signals that result in degradation of Iκ-B molecules reveal the NF-κB nuclear localization signal, allowing transit to the nucleus and DNA binding. Inhibition of NF-κB activity can be mediated by sequestering the active heterodimer in the cytoplasm complexed with Iκ-B, or through displacement of p50/p65 heterodimers by repressive p50 homodimers [30,31].

NF-κB expression and cellular localization have been shown to respond to a variety of stimuli including chemotherapy, radiation, and cytokine stimulation [36–38]. However, the response of NF-κB to chemotherapy in bone marrow stromal cells has not been characterized. Data presented in the current study reveal altered NF-κB cellular localization following exposure of stromal cells to VP-16, consistent with reduced stromal cell VCAM-1. Diminished nuclear localization of NF-κB in bone marrow stromal cells following exposure to VP-16 may be central to the mechanism by which chemotherapy disrupts the bone marrow microenvironment.

2. Materials and methods

2.1. Establishment of fibroblastic bone marrow-derived stromal cell cultures

Stromal cell cultures were initiated from 200 μL of unfiltered human bone marrow from consenting donors, with approval by the WVU Institutional Review Board, as previously described [7]. Several stromal cell lines were used throughout the study that are functionally indistinguishable.

2.2. Chemotherapy treatment of bone marrow cells

VP-16 was obtained at a stock concentration of 20 mg/mL (Bristol-Myers Squibb) and diluted in culture medium to 100 μM prior to use to approximate serum levels reported for patients on high dose therapy [15]. Stromal cells were grown to confluence prior to treatment. For experiments that evaluated NF-κB recovery, cells were treated with 100 μM VP-16 for 3 hr, rinsed four times with fresh α-MEM, and cultured for 24–72 hr in fresh medium.

2.3. RNA isolation

Total RNA was isolated from stromal cells using the S.N.A.P. Total RNA Isolation kit and following the recommendations of the manufacturer (Invitrogen). Pelleted stromal cells were lysed by centrifugation through QIAshredder Spin Columns (QIAGEN Inc.). RNA was DNase treated and quantitated at 260 nm (Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer; PE Corp.).

2.4. PCR

To evaluate changes in VCAM-1 mRNA following VP-16 treatment, semi-quantitative PCR was performed. The linear ranges of amplification for actin and VCAM-1 were determined to be 25 and 30 cycles, respectively. Amplification cycles included denaturation at 94° for 30 sec, primer annealing at 55° for 1.5 min, and extension at 72° for 2 min and 15 sec (Perkin-Elmer GeneAmp PCR System 9600). cDNAs were generated by random priming of 1 μg of total RNA. Actin primer sets were: 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ (Stratagene) at 0.4 μg/reaction to generate an amplicon of 661 bp. VCAM-1 primers 5′-CATCCACAAAGCTGCAAGAA-3′ and 5′-GCCACCACTCATCTCGATTT-3′ [39] (0.5 μg/reaction) generated a 563 bp product. Controls that lacked reverse transcriptase or cDNA template were included in all experiments. VCAM-1:actin ratios were quantitated by EagleSight Version 3.21 (Stratagene) densitometric analysis.

2.5. RNase protection assay

To evaluate VCAM-1 mRNA, RNase protection assays were performed using the RPAIII kit according to the protocol of the manufacturer (Ambion). Confluent stromal cell layers were treated with 5 μg/mL of actinomycin D (Sigma Chemical Co.) with and without 100 μM VP-16. Ten micrograms of RNA from each sample was hybridized to ^{32}P-labeled VCAM-1 and GAPDH-specific complementary RNA probes. Anti-sense ^{32}P-RNA probes were generated using T7 RNA polymerase-directed synthesis from RiboQuant DNA templates (PharMingen). Nucleic acids were treated with RNase A and T1 to digest unhybridized sequences. RNAs corresponding to VCAM-1 (288 bp) and GAPDH (96 bp) were visualized by exposure to PhosphoImager cassettes (Molecular Dynamics). VCAM-1 band intensities were normalized to GAPDH controls in each treatment group.

2.6. Intracellular protein staining

Bone marrow stromal cells were cultured on coverslips (Corning Inc.) for 24 hr prior to treatment with 100 μM VP-16 for 3–6 hr. Following treatment, cells were fixed in methanol:acetone (1:1) for 20 min at room temperature and then were rinsed in autoclaved PBS. Non-specific antibody binding was blocked by incubation of stromal cells with PBS/5% BSA for 30 min at room temperature.

Cellular localization of p50 or p65 protein was evaluated by incubation of stromal cells with 10 μg of rabbit anti-human p50 or p65 (Santa Cruz Biotechnology, Inc.) for 1 hr at room temperature. Coverslips were then rinsed in autoclaved PBS and subsequently incubated with goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology). Fluoromount-G (Southern Biotechnology Associates, Inc.) was added, and coverslips were inverted onto slides for evaluation by fluorescent microscopy (Zeiss LSM 510).

2.7. Isolation of stromal cell nuclei

Isolation of nuclear proteins was completed as described by Andrews and Faller [40]. Briefly, 0.5×10^5 to 2.0×10^6 stromal cells were resuspended at 4° in 400 μL of Buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF]. Following incubation on ice for 10 min, samples were vortexed for 10 sec and centrifuged at 20,000 *g* for 30 sec at 4° to isolate nuclei. Nuclei were then resuspended in Buffer C [20 mM HEPES-KOH (pH 7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 20 min. Following centrifugation at 20,000 *g* for 2 min at 4° to remove debris, supernatants containing nuclear proteins were stored at −70° until used. Protein concentrations were determined using the BCA Protein Assay kit (Pierce).

2.8. Western blot analysis

Western blot analysis was performed following the method described by Laemmli [41]. For evaluation of p65 or p50, nuclear proteins (10 μg/lane) were separated by SDS–PAGE and transferred to PVDF-Plus nylon membranes (MSI). Membranes were probed with 0.5 μg/mL of a polyclonal rabbit anti-human p65 (Santa Cruz Biotechnology) or a 1:2000 dilution of a polyclonal rabbit anti-human p50 antibody (provided by Dr. Nancy Rice, National Cancer Institute, Frederick Cancer Research and Development Center). Membranes were incubated with a goat anti-rabbit-HRP antibody (Santa Cruz Biotechnology). Bands were visualized by ECL (Amersham International). Densitometric analysis using Eagle Eye II (Stratagene) or Optimus (Optimus Corp.) was completed for quantitation. Isotype-matched controls were included for all experiments. Protein from 5×10^4 stromal cells was evaluated by SDS–PAGE to determine total Iκ-Bα protein expression. Membranes were probed with 1.5 μg/mL of a monoclonal mouse anti-human Iκ-Bα antibody (Santa Cruz Biotechnology) followed by 1:2000 dilution of a goat anti-mouse-HRP antibody (Santa Cruz Biotechnology) for visualization.

Membranes were reprobed with a polyclonal rabbit anti-human TFIIF-RAP30 antibody (0.5 μg/mL, Santa Cruz Biotechnology) as an internal lane loading control. To establish ratios of p65 to p50 subunits, protein levels were compared within individual nuclear samples, and normalized to untreated controls set to 1.0. Statistical analysis was performed using Dunnett's test (SigmaStat Version 2.0 software, SPSS Inc.).

2.9. EMSA

To determine if VP-16 altered NF-κB binding to its consensus sequence, EMSAs were performed. NF-κB consensus oligonucleotides (Santa Cruz Biotechnology) were end labeled with [γ-^{32}P]ATP using "Ready·To·Go" T4 Polynucleotide Kinase (Pharmacia Biotech). Reactions included 2 μg of nuclear protein, 2 μg of poly(dI:dC), 1 mM spermidine, 1X Promega buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, and 0.05% NP-40]. Supershifts were completed by the addition of rabbit anti-human p50 or p65 antibodies (provided by Dr. Nancy Rice) to indicated samples. Following incubation on ice for 2 hr, 50,000 cpm (0.05 to 0.20 ng) of labeled NF-κB consensus probe was added to all reaction mixtures. A 30-fold excess (2.0 to 8.0 ng) of unlabeled specific (NF-κB) or non-specific (NF-AT) probe was added to indicated samples to evaluate binding specificity. Samples were then incubated at room temperature for 30 additional min.

Complexes were separated through 5% polyacrylamide/1X Tris-glycine-EDTA gels that were dried for 2.5 hr at 80° with a vacuum (Bio-Rad model 583 gel dryer) and exposed to BioMax MR-1 film overnight (Kodak). Relative band intensities were determined using Eagle Eye II (Stratagene).

2.10. (E)-Capsaicin treatment of stromal cells

To determine if reduced p65 in stromal cell nuclei was sufficient to reduce VCAM-1 expression, confluent stromal cells were treated for 4 or 8 hr (4-hr exposures on two consecutive days) with 200 μM (E)-Capsaicin (Alexis Biochemicals). (E)-Capsaicin has been shown previously to reduce nuclear p65 levels [42]. Stromal cell VCAM-1 protein was evaluated 24 and 48 hr later by ELISA as described below.

2.11. VCAM-1 ELISA

To determine whether stromal cell VCAM-1 expression is restored following removal of VP-16 from culture, confluent stromal cells were treated with 100 μM VP-16 for 24–72 hr. Following treatment, cells were trypsinized, rinsed three times in medium, counted, and replated into 96-well plates (10,000 cells/well) to allow recovery for up to 72 hr following the 24-hr VP-16 exposure, or up to 5 days following 72-hr chemotherapy treatment. Mouse anti-human VCAM-1 antibody (2 μg/mL; Santa Cruz Biotechnology) and a matched isotype IgG_1 control (Southern Biotechnology Associates) were incubated on stromal cell layers at 37° for 1 hr. Following incubation, layers were rinsed five times (PBS/0.5% Tween 20). A sheep anti-mouse HRP antibody (1 μg/mL; Amersham Life Science) was then incubated on stromal cell layers at 37° for 1 hr and rinsed as above. The TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories) was used for quantitation of VCAM-1 expression. Isotype-matched controls were included for all treatment groups to evaluate non-specific antibody binding.

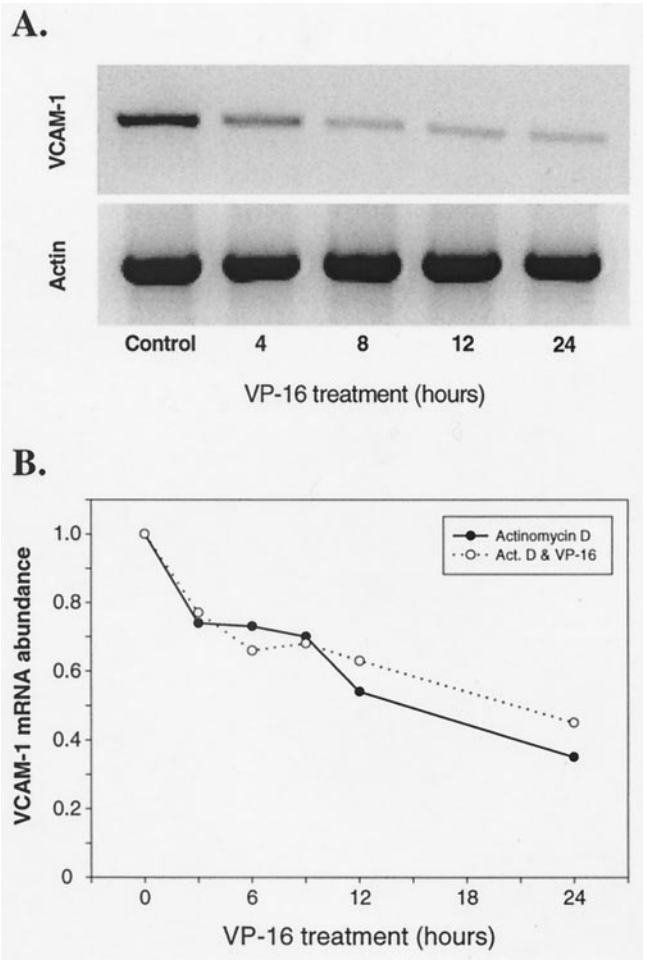

Fig. 1. VCAM-1 transcripts in bone marrow stromal cells treated with VP-16. (A) To determine if VCAM-1 mRNA expression was altered by VP-16 treatment, semi-quantitative PCR was performed as described in "Materials and methods." Actin and VCAM-1 specific sequences were amplified from RNA isolated from untreated control stromal cells and stroma treated for 4–24 hr with 100 μM VP-16, as indicated. Data shown are representative of four experiments. (B) To evaluate VCAM-1 message stability, stromal cells were treated with 100 μM VP-16 in the presence of actinomycin D as described in "Materials and methods." RNase protection was completed to quantitate VCAM-1 specific RNA in untreated control stromal cell layers in the presence of actinomycin D, compared with those treated for up to 24 hr with both VP-16 and actinomycin D. Values shown are relative to control values set to 1.0. Data shown are representative of two independent experiments.

3. Results

3.1. VCAM-1 transcripts in stromal cells treated with VP-16

To determine whether VP-16 reduced the amount of VCAM-1 transcripts in bone marrow stromal cells, their relative abundance was evaluated following 4–24 hr of 100 μM VP-16 treatment. At 4 hr, VCAM-1 message levels were approximately 65% of untreated control values. Message levels then progressively decreased at 8, 12, and 24 hr of exposure (Fig. 1A). Controls that lacked template or reverse transcriptase did not yield any product (data not shown).

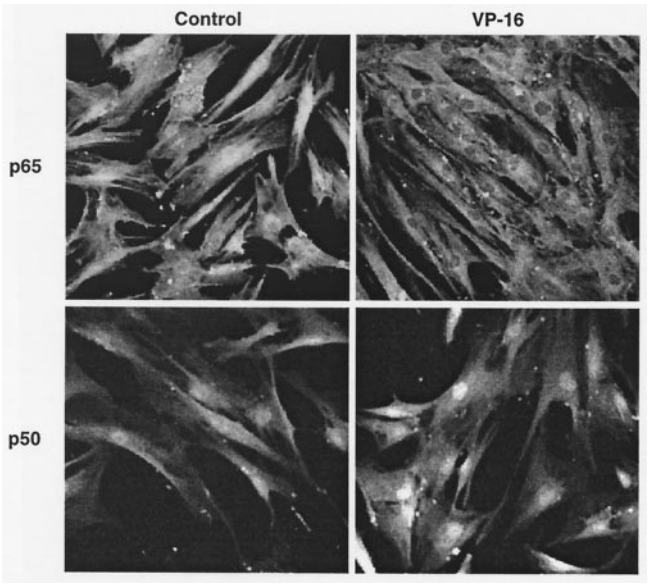

Fig. 2. Cellular localization of NF-κB p65 and p50 subunits following treatment of bone marrow stromal cells with VP-16. The relative amount and cellular localization of p50 or p65 in stromal cells treated with VP-16 for 3 hr (p65) or 6 hr (p50) were determined by fluorescent microscopy as described in "Materials and methods." Following treatment, stromal cells were fixed in methanol:acetone, and p65 or p50 was detected by incubation with 200 μg/mL of rabbit anti-human p50 or p65 antibodies. Data shown are representative of three stromal cell lines evaluated.

3.2. VCAM-1 message stability

To determine if the decrease in VCAM-1 message following treatment of bone marrow stromal cells with VP-16 was a chemotherapy-induced alteration in stability, confluent stromal cells were treated with actinomycin D or both actinomycin D and VP-16 as described. Following 3–24 hr of treatment, no significant differences were observed between the half-lives of VCAM-1 specific transcripts in the presence and in the absence of VP-16. The half-life of VCAM-1 transcripts in both treatment groups was approximately 12 hr (Fig. 1B).

3.3. NF-κB subunit cellular localization in stromal cells exposed to VP-16

Intracellular localization of p65 and p50 in treated and control stromal cells was evaluated by fluorescent microscopy as described. Untreated stromal cells expressed a significant level of nuclear p65, whereas nuclei of VP-16-treated bone marrow stromal cells had diminished to undetectable levels of nuclear p65 (Fig. 2). In contrast, modestly increased nuclear expression of p50 was observed following VP-16 treatment (Fig. 2).

To quantitate the changes in nuclear profiles of p65 and p50 in stromal cells treated with VP-16, western blot analysis was performed. Following treatment with VP-16 for 30 min to 3 hr, consistent decreases in nuclear p65 (Fig. 3A) and slight increases in p50 (Fig. 3B) were observed. Statistically significant decreases in nuclear p65 protein were observed after 1.5 and 3 hr of VP-16 exposure ($P < 0.05$).

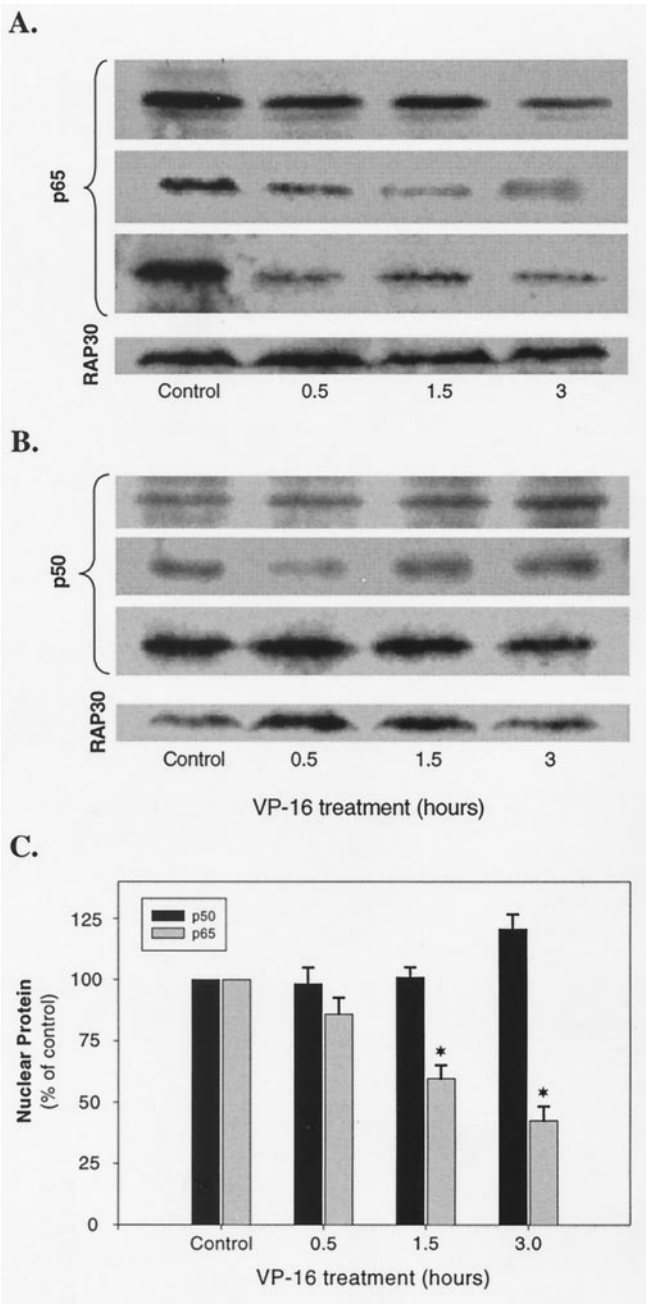

Fig. 3. Nuclear p65 and p50 in stromal cells following treatment with VP-16. To quantitate changes in nuclear levels of p65 and p50 in stromal cells exposed to VP-16, western blot analysis of nuclear proteins was completed as described in "Materials and methods." Stromal cells were treated with 100 μM VP-16 for 30 min to 3 hr as indicated, and nuclei were isolated as described previously for protein analysis. Nuclear p65 (panel A) and nuclear p50 (panel B) were evaluated by western blot analysis. A representative control (RAP 30) is shown for one experiment. Data from four independent experiments evaluating nuclear p65, and five independent experiments evaluating nuclear p50 expression, normalized to untreated control values are summarized in panel C. Data are shown with SEM. A significant difference relative to control values is indicated by an asterisk (Dunnett's test, $P < 0.05$).

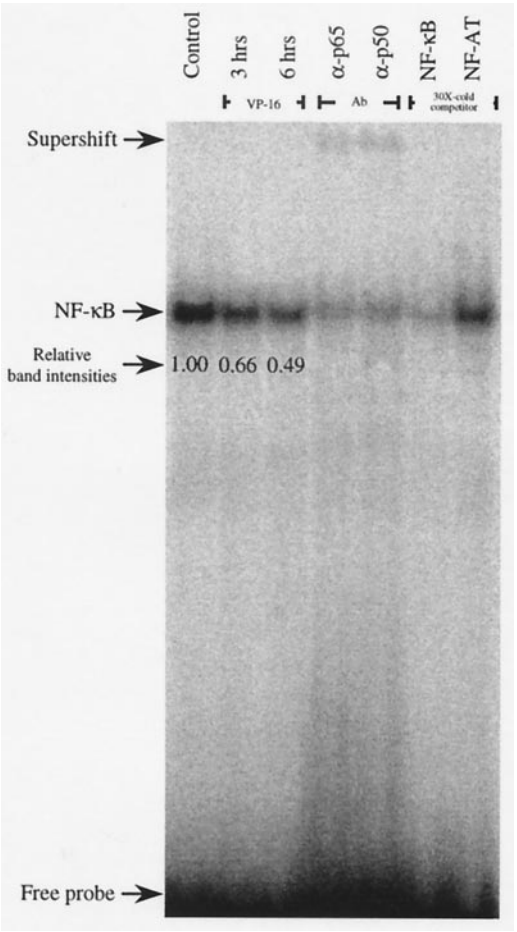

Fig. 4. NF-κB binding to its consensus sequence in stromal cells treated with VP-16. To determine if VP-16 exposure resulted in alterations of NF-κB binding to its consensus sequence, EMSAs were performed as described in "Materials and methods." Densitometric analysis was completed to quantitate relative band intensities.

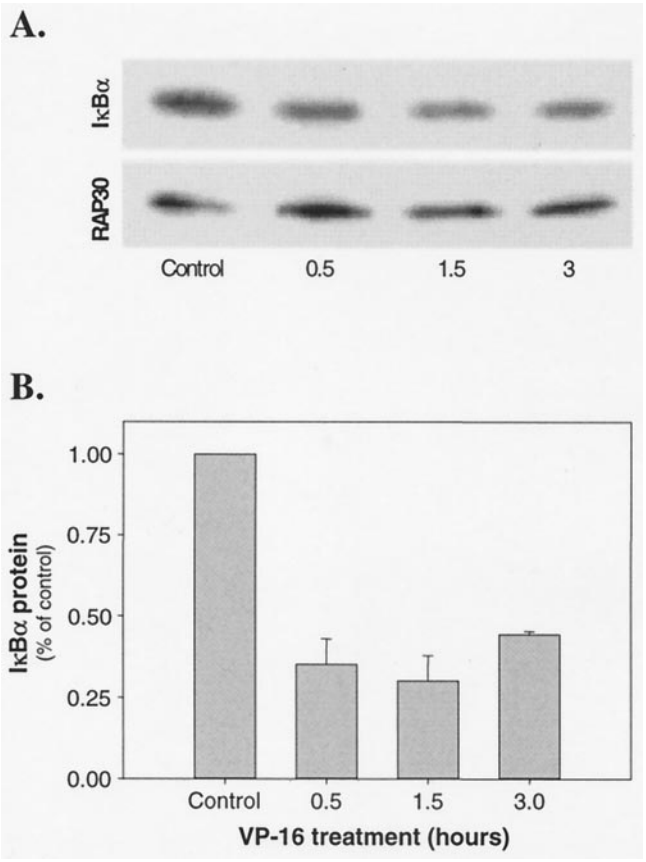

Fig. 5. Iκ-Bα protein expression in stromal cells exposed to VP-16. (A) To evaluate total Iκ-Bα protein levels in stromal cells following VP-16 exposure, confluent stromal cell layers were treated for 30 min to 3 hr, and western blot analysis was performed. (B) Densitometric analyses of three representative experiments are shown (mean ± SEM). Reduced Iκ-Bα protein was observed at all time points evaluated.

3.4. NF-κB binding to a κB consensus sequence

To investigate whether VP-16 altered binding of NF-κB to its consensus site, EMSAs were completed as described in "Materials and methods." DNA binding of NF-κB was reduced to approximately 50% after 6 hr of VP-16 exposure compared with untreated stromal cells (Fig. 4). A single complex was observed that could be shifted with antibody specific for p50 or p65. A 30-fold excess of unlabeled NF-κB oligonucleotide reduced the NF-κB binding by approximately 80%, with negligible reduction of binding observed by inclusion of 30-fold excess NF-AT oligonucleotide (Fig. 4).

3.5. Iκ-Bα expression in stromal cells treated with VP-16

To determine if elevated Iκ-Bα was a potential mechanism by which p65 was reduced in the nucleus of stromal cells treated with VP-16, total Iκ-Bα protein was evaluated in treated and control stromal cells. A consistent reduction of Iκ-Bα protein was observed in VP-16-treated stromal cells (Fig. 5).

3.6. VCAM-1 expression in stromal cells treated with (E)-Capsaicin

To determine whether reduced nuclear p65 was sufficient to down-regulate VCAM-1 expression, confluent stromal cell layers from two independent stromal cell lines, P154 and P155, were treated with (E)-Capsaicin, as described. VCAM-1 protein was consistently reduced following (E)-Capsaicin exposure (Fig. 6).

3.7. Nuclear p65:p50 ratios following cessation of short-term chemotherapy

To evaluate recovery of nuclear p65 and p50 following short-term exposure to VP-16, stromal cells were treated with VP-16 for 3 hr, and then allowed to recover for up to 72 hr (Fig. 7A). After 24 hr of recovery, nuclear p65:p50 ratios began to approximate the untreated control ratio, and

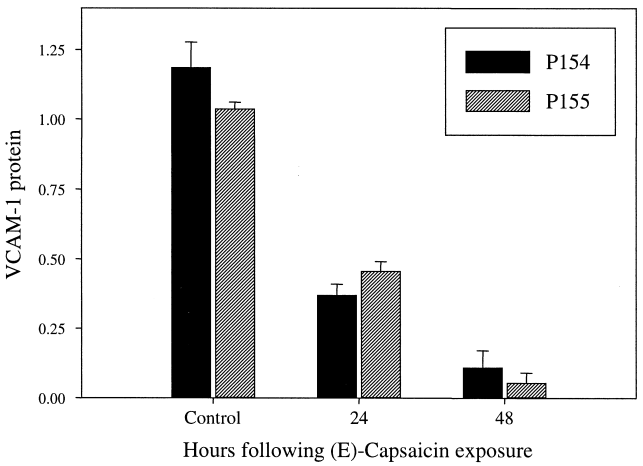

Fig. 6. VCAM-1 expression in (E)-Capsaicin-treated stromal cells. Confluent bone marrow stromal cells were exposed to a single 4-hr treatment of Capsaicin, or to 4-hr treatments on two consecutive days, and VCAM-1 ELISA was completed at 24 and 48 hr, respectively. Isotype-matched control binding indicative of non-specific background was subtracted from each sample. Data from two independent stromal cell lines, P154 (solid bar) and P155 (hatched bar), are shown with SEM. The data are representative of three independent experiments.

after 72 hr the p65:p50 ratio was significantly higher than that immediately post-treatment (Tukey test; $P < 0.05$).

3.8. VCAM-1 protein expression recovery following VP-16 treatment

To determine if VCAM-1 protein recovered following treatment with VP-16, stromal cell cultures were treated for 24–72 hr followed by 1–5 days of recovery in fresh medium. Following a short (24-hr) VP-16 exposure, VCAM-1 protein levels increased at 24 hr, and reached control levels after 48 hr of recovery (Fig. 7B). In contrast, when stromal cells were treated for 72 hr with VP-16, reduced VCAM-1 protein expression was observed for up to 5 days (Fig. 7C).

4. Discussion

We previously reported functional alteration of bone marrow stromal cells treated with VP-16 [7]. Specifically, treated stromal cells exposed to VP-16 had reduced capacity to support survival and proliferation of lymphoid and myeloid progenitor cells, and reduced levels of VCAM-1 protein expression [7]. Because interaction of hematopoietic progenitor cells with VCAM-1 on stromal cells has been shown to be important for the development of progenitor cells [21–23,28], we investigated a potential mechanism by which VP-16 exposure results in dysregulated VCAM-1 expression.

Consistent with our previous report of reduced VCAM-1 protein in stromal cells treated with VP-16 [7], VCAM-1 mRNA was reduced following VP-16 treatment (Fig. 1A).

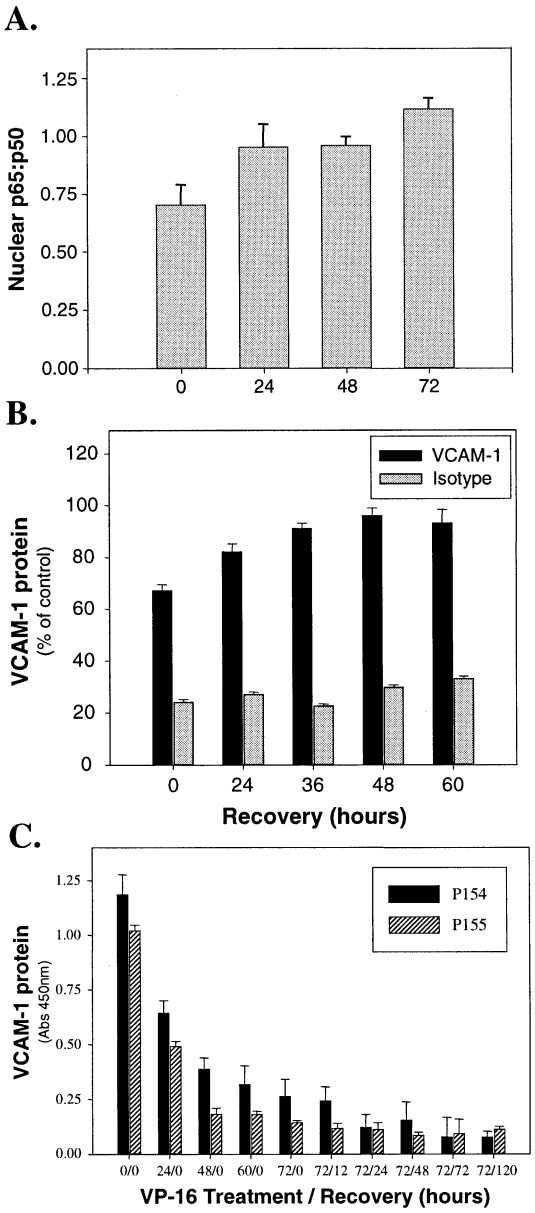

Fig. 7. Return of nuclear p65 and p50, and VCAM-1 protein expression, to baseline levels following cessation of chemotherapy. To determine if nuclear profiles of p65 and p50 returned to baseline following termination of VP-16 exposure, confluent stromal cell cultures were treated for 3 hr with 100 μM VP-16, then thoroughly rinsed with medium, and allowed to recover for up to 72 hr as described in "Materials and methods." Subsequently, nuclear p65 and p50 were evaluated by western blot analysis, and densitometry was completed (panel A). Values are shown with SEM, and are representative of three experiments. (B) Stromal cell VCAM-1 protein following removal of VP-16 from cultures was evaluated by ELISA as described in "Materials and methods." Confluent layers were treated for 24 hr, and allowed to recover for up to 60 hr in fresh medium. Isotype-matched controls are shown for each sample. Values shown with SEM are representative of two independent experiments evaluated in triplicate. (C) VCAM-1 protein was evaluated by ELISA following up to 72 hr of VP-16 exposure, followed by 1–5 days recovery in fresh medium as described in "Materials and methods." Values from two different human stromal cell lines, P154 (solid bar) and P155 (hatched bar), are shown with SEM. Isotype-matched control antibody binding was subtracted from each value. Data shown are representative of three experiments that evaluated several combinations of exposure and recovery times.

This observation suggests that VP-16 either reduced the stability of VCAM-1 mRNA, or resulted in diminished transcription of VCAM-1 in stromal cells. The reduction in VCAM-1 RNA in the presence of VP-16 was not due to altered stability (Fig. 1B). This observation prompted investigation of transcription factors that potentially mediate diminished expression.

We have reported previously that while VCAM-1 expression is reduced by treatment of stromal cells with VP-16, fibronectin is not altered [7], suggesting VP-16 modulation of transcription factors that bind to the VCAM-1 promoter and not fibronectin. Two NF-κB consensus binding sites have been identified in the VCAM-1 promoter region [43], while fibronectin is activated primarily by Sp-1 binding [44,45]. NF-κB or p65 homodimeric molecules have been reported to enhance transcriptional activity of the VCAM-1 gene in a variety of systems through binding to these sites [29,43,46,47].

Evaluation of stromal cell nuclear profiles of p65 and p50 indicated reduced levels of nuclear p65 following treatment with VP-16 (Figs. 2 and 3A). This observation is consistent with reduced availability of p65 to maintain VCAM-1 transcription during VP-16 exposure. Ratios of p65:p50 were not significantly different in whole cell lysates of treated stromal cells compared with matched controls (data not shown). These data suggest that VP-16 primarily results in signals that alter cellular localization of NF-κB subunits, but does not impact on total expression levels of p65 or p50. One direct means by which VP-16 may alter nuclear translocation of p65 is through physical interaction that either masks the nuclear localization signal, or alters nuclear pore permeability. However, while VP-16 has been shown to directly bind various proteins [48,49], no binding of [^{3}H]VP-16 to immunoprecipitated stromal cell p65 was observed (data not shown). To address the possibility that VP-16 masked the protein sites necessary for antibody interaction, immunoprecipitations were completed with nuclear protein, unlabeled VP-16, and α-p65 antibody. Subsequent western blot analysis readily detected p65 (data not shown).

Iκ-Bα is the prototypic molecule reported to detain p65 in the cytoplasm, through either increased expression or stability [34,35]. However, Iκ-Bα did not play an obvious role in sequestering p65 in the cytoplasm of stromal cells following VP-16 exposure. Total Iκ-Bα protein levels were diminished following treatment (Fig. 5). The reduction of Iκ-Bα protein was consistent with previous reports of NF-κB binding sites in the Iκ-Bα promoter [35,50].

The observation that Iκ-Bα did not play a clear role in the retention of p65 in the cytoplasm following VP-16 treatment did not rule out the possibility that another inhibitory molecule may have enhanced expression following treatment with VP-16. To investigate this possibility, levels of p100 and p105, which can also detain p65 in the cytoplasm, were evaluated as potential regulatory molecules that may respond to chemotherapy exposure. No consistent increase in either protein was observed following VP-16 exposure in three stromal cell lines examined (data not shown). In combination, these observations suggest a mechanism, other than interaction of p65 with an obvious inhibitory protein, that accounts for reduced p65 levels in the nucleus following treatment. Alteration of the nuclear pore complex seems unlikely as p50 entrance to the nucleus was not disrupted in VP-16-treated stromal cells.

In contrast to p65, modest increases in nuclear p50 were noted in VP-16-treated stromal cells (Figs. 2 and 3B). Homodimers of p50 have been reported previously to repress VCAM-1 transcription in cell lines generated to overexpress p50 [30,31]. However, p50 homodimer complexes were not discerned by EMSA in our model (Fig. 4). Homodimeric p50 may have been present at a level below our sensitivity of detection. However, a modest increase in stromal cell nuclear p50, combined with a consistent decrease in nuclear p65 in cells treated with VP-16, results in a ratio less conducive to NF-κB-driven VCAM-1 expression.

Treatment of stromal cells layers with (E)-Capsaicin reduced VCAM-1 expression at 24 and 48 hr (Fig. 6). We previously determined that the half-life of stromal cell VCAM-1 is approximately 24 hr (unpublished data), providing the rationale for evaluation of VCAM-1 protein 24 and 48 hr after disruption of nuclear p65 by (E)-Capsaicin. This observation supports the premise that reduced nuclear p65 is sufficient to diminish VCAM-1 expression, and suggests that chemotherapeutic agents that modulate cellular localization of p65 potentially alter stromal cell VCAM-1 expression through this mechanism.

The recovery of p65 and p50 nuclear levels to baseline following removal of VP-16 after a 3-hr exposure suggests an absence of permanent damage to stromal cells following short-term exposure (Fig. 7A). In addition, restoration of VCAM-1 protein expression following removal of drug occurred after 24 hr of treatment (Fig. 7B), but not after 72 hr of VP-16 exposure (Fig. 7C). This observation suggests a potential link between duration of drug exposure and severity of damage that may be as critical as drug dose. We reported previously that while VP-16 disrupts the ability of confluent stromal cell layers to support hematopoiesis, it does not induce stromal cell apoptosis, confirmed by an absence of DNA laddering or propidium iodide staining [7]. The inability of VP-16 to initiate apoptosis in non-cycling confluent stromal cell cultures is in marked contrast to its ability to efficiently induce death in dividing cells. This suggests unique NF-κB-mediated signaling pathways in cycling versus quiescent cells.

The relevance of the current study may not be in designing strategies to prevent damage to the microenvironment, but rather in understanding the kinetics of stromal cell recovery following chemotherapy. Attempts at maintaining nuclear levels of p65 during treatment to sustain VCAM-1 expression on stromal cells would likely prove detrimental. NF-κB has been shown to be a critical component of the apoptotic pathway in various model systems [37]; therefore,

blocking translocation of p65 or p50 subunits in response to chemotherapy would likely diminish the efficacy of treatment in many circumstances.

The rationale for investigating the response of NF-κB to chemotherapeutic agents rests in its ability to regulate several stromal cell genes. In addition to altered VCAM-1 expression, we have observed reduced transcription of stromal cell GM-CSF (unpublished data) in VP-16-treated cells. Like VCAM-1, GM-CSF expression has been shown to be regulated, in part, by NF-κB binding [51]. In combination, the appropriate expression of stromal cell adhesion molecules and cytokines defines the ability of the microenvironment to support hematopoiesis. The response of stromal cell NF-κB to specific chemotherapeutic agents may provide insight into mechanisms that contribute to a disrupted bone marrow microenvironment, and the delayed hematopoietic recovery that is associated with specific chemotherapeutic agents.

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