I κ B Kinase-Dependent Chronic Activation of NF- κ B Is Necessary for p21^{WAF1/Cip1} Inhibition of Differentiation-Induced Apoptosis of Monocytes

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The molecular mechanisms regulating monocyte differentiation to macrophages remain unknown. Although the transcription factor NF- κ B participates in multiple cell functions, its role in cell differentiation is ill defined. Since differentiated macrophages, in contrast to cycling monocytes, contain significant levels of NF- κ B in the nuclei, we questioned whether this transcription factor is involved in macrophage differentiation. Phorbol 12-myristate 13-acetate (PMA)-induced differentiation of the promonocytic cell line U937 leads to persistent NF- κ B nuclear translocation. We demonstrate here that an increased and persistent IKK activity correlates with monocyte differentiation leading to persistent NF- κ B activation secondary to increased I κ B α degradation via the I κ B signal response domain (SRD). Promonocytic cells stably overexpressing an I κ B α transgene containing SRD mutations fail to activate NF- κ B and subsequently fail to survive the PMA-induced macrophage differentiation program. The differentiation-induced apoptosis was found to be dependent on tumor necrosis factor alpha. The protective effect of NF- κ B is mediated through p21^{WAF1/Cip1}, since this protein was found to be regulated in an NF- κ B-dependent manner and to confer survival features during macrophage differentiation. Therefore, NF- κ B plays a key role in cell differentiation by conferring cell survival that in the case of macrophages is mediated through p21^{WAF1/Cip1}.

The molecular mechanisms regulating cell differentiation involve a fine and complex balance of proteins and signal transduction pathways that modulate the progression through the cell cycle and control of cell survival (11, 48). Human macrophages are differentiated noncycling cells arrested at the G₁ checkpoint that derive from peripheral blood cycling human monocytes. These cells are present in multiple body tissues and play a key role in various immune functions and thus are relevant to a number of human diseases (26, 45). Unlike the well-characterized process of B-lymphocyte differentiation, little is known regarding the molecular mechanisms governing the monocyte differentiation to macrophage. Promonocytic human cell lines, like human primary monocytes, can be triggered to differentiate to human macrophages by defined stimuli (45). Agents such as phorbol esters (phorbol 12-myristate 13-acetate [PMA]) induce their exit from the cell cycle at G₁, leading to their differentiation, which is characterized by the appearance of macrophage-like features such as cell surface integrins, adherence to plastic, and the production of reactive oxygen intermediates (4, 8, 21).

A number of transcription factors such as PU.1 have been implicated in monocyte differentiation, as have other proteins, including p21^{WAF1/Cip1} (21, 32, 43, 44, 48, 60). p21^{WAF1/Cip1} is a nuclear protein of the family of cyclin-dependent kinase (CDK) inhibitors (17) which functions to inhibit CDK1, -2, -4, and -6 (9) and, hence, its induction by a number of stimuli causes cell cycle arrest at the G₁/S boundary (8, 20, 37, 51, 57), allowing the cell to exit the cell cycle and differentiate. Interestingly in differentiated human macrophages, p21^{WAF1/Cip1} is

predominantly located in the cytosol where it exerts an anti-apoptotic function by means of inhibiting the proapoptotic kinase Ask1 (1). Whether the critical role that p21^{WAF1/Cip1} plays in macrophage differentiation is dependent on its ability to inhibit the cell cycle or protect the cell from death while it is undergoing differentiation is unclear.

Members of the NF- κ B/Rel family of transcription factors are key regulators of a variety of genes involved in cell growth and survival (3, 4, 6). NF- κ B is generally found as an inactive dimer sequestered in the cytoplasm by inhibitor proteins termed I κ B. However, in a few exceptions, such as terminally differentiated B lymphocytes and plasma cells, as well as human macrophages, NF- κ B is constitutively present at very high levels in the nuclei of these cells (7, 35, 40). At least two potential mechanisms involving I κ B regulation can result in the nuclear translocation of NF- κ B. Acute stimuli such as interleukin-1 (IL-1) or tumor necrosis factor alpha (TNF- α) lead to the rapid proteolysis of I κ B molecules via a common, terminal transduction pathway leading to the rapid phosphorylation of I κ B by two highly related serine kinases, IKK1 and IKK2. These kinases phosphorylate I κ B on critical serine residues, such as Ser32 and Ser36, within the N-terminal signal response domain (SRD) of I κ B α (31, 61). In addition, I κ B molecules have a C-terminal PEST domain, which is a highly negative charged region conferring protein instability and hence favoring a slow but continuous level of I κ B degradation (55, 56). Cycling primary human monocytes have no NF- κ B in their nuclei. However, their differentiation to macrophages triggered by plastic adherence or phorbol esters leads to a progressive and significant degree of NF- κ B nuclear translocation, a hallmark of differentiated macrophages. The molecular mechanisms regulating NF- κ B translocation to the nucleus during macrophage differentiation and the role NF- κ B

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plays in the relevant cellular processes is unknown (24, 25, 34, 53).

The distinct changes in the cellular localization observed for p21^{WAF1/Cip1} and NF- κ B during macrophage differentiation and their potential influence in cell survival and cell cycle control raise the possibility that these key proteins may regulate each other, leading to a coordinated process of differentiation. Likewise, previous observations in a number of systems suggest that NF- κ B may regulate p21^{WAF1/Cip1} expression (5, 29, 49). The recent availability of genetic tools to inhibit the expression or function of such proteins has enabled us to address their role in macrophage differentiation.

MATERIALS AND METHODS

Cells, cell culture, and induction of differentiation. The human promonocytic cell line U937 was purchased from the American Type Culture Collection and grown in RPMI 1640 (BioWhittaker) plus 5% (vol/vol) heat-inactivated fetal bovine serum (FBS; Intergen), penicillin and streptomycin (100 U/ml; Gibco-BRL), and glutamine (0.3 mg/ml; Gibco-BRL). U937 clones expressing FLAG-I κ B α transgenes were previously described (2). Results shown herein were similar for each of three separate clones. U937 cells stably expressing either empty vector (pREP4) or p21(AS) constructs (F4 and B8) were previously described (60) and were maintained in RPMI 1640 supplemented with 10% FBS, penicillin-streptomycin, glutamine, minimal essential medium (MEM) nonessential amino acids (0.1 mM; Gibco-BRL), MEM sodium pyruvate (1 mM; Gibco-BRL), and 200 μ g of hygromycin B (Sigma) per ml. All cells were maintained at 37°C and 5% CO₂. Cells were seeded at 2×10^5 cells/ml and induced to differentiate with PMA (2 ng/ml; Sigma) or treated with vehicle control (dimethyl sulfoxide [DMSO]). TNF (10 ng/ml; Genzyme) was used at the indicated times as a positive control for I κ B α degradation and subsequent NF- κ B activation.

For the inhibition of differentiation-induced apoptosis, cells were seeded as described above and induced to differentiate with PMA. At time of culture initiation, TNF receptor (TNFR)-Fc (5.5 ng/ml; Immunex Corp., Seattle, Wash.) was added. An equivalent quantity of IL-4 receptor (IL4R)-Fc was added to parallel cultures as a control.

Evaluation of differentiation and survival. Cells were seeded and induced to differentiate with PMA as described above. At the indicated time points, cells were evaluated for differentiation markers. Cells were examined microscopically for differentiation morphology such as increased cell volume, granularity, and the appearance of irregularly shaped cellular processes. Adherence was determined by gently rocking the plates and by collecting nonadherent cells in the culture supernatant. Phosphate-buffered saline (PBS) was added back to the culture flasks, and adherent cells were lifted by gentle scraping. Viable cells were scored by trypan blue exclusion. Surface expression of CD11c was determined by staining with fluorescein isothiocyanate-conjugated CD11c antibody (BioSource) detected by fluorescence-activated cell sorter (FACS) and analyzed with CellQuest Software (Becton Dickinson). The cell cycle profile was determined by propidium iodide (PI) staining. In brief, cells were washed twice in ice-cold PBS and resuspended in approximately 100 μ l of PBS. Cells were fixed and permeabilized with ice-cold 70% ethanol at least overnight. Fixed cells were pelleted by cold centrifugation and resuspended in sample buffer containing 50 μ g of PI and 100 Kunitz U of RNase A per ml. Cells were analyzed by FACS and CellQuest Software. The sub-G₀ population was scored as apoptotic.

Nuclear and cytoplasmic extract preparation, immunoprecipitations, and in vitro kinase assays. Nuclear and cytosolic extracts were prepared by a modification of the method of Dignam et al. (14). Briefly, cells were washed twice in buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl). Cells were lysed two times for 5 min on ice in buffer A containing 0.1% NP-40; 0.5 mM dithiothreitol (DTT); 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 2 μ g each of aprotinin, leupeptin, and pepstatin per ml; and 1 mM sodium orthovanadate. After centrifugation, the cells were washed twice in buffer A, and the nuclei were collected by centrifugation. Pelleted nuclei were lysed in 10 to 15 μ l of buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA [pH 8.0], DTT, PMSF, aprotinin, leupeptin, pepstatin, and sodium orthovanadate) by rotation at 4°C for 30 min. After centrifugation, the supernatants were diluted in 2 $\times$ volumes of buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 50 mM KCl, 0.2 M EDTA, DTT, PMSF, aprotinin, leupeptin, pepstatin, and sodium orthovanadate).

For immunoprecipitations, cells were washed twice in 50 mM Tris-HC (pH 7.5) and 150 mM NaCl and then lysed for 5 min on ice in wash buffer plus 0.1%

Triton X-100; 2 μ g each of aprotinin, leupeptin, and pepstatin per ml; and 0.5 mM PMSF. Following clarification by centrifugation, cytoplasmic extracts (50 μ g) were rotated at 4°C for 1 to 2 h in the presence of antibodies to the N and C termini of RelA (Santa Cruz) and 10 μ l of protein A-conjugated agarose beads (Gibco-BRL). Precipitates were washed three times with lysis buffer and eluted with 2 $\times$ Laemmli sample buffer at 95°C. Subsequent immunoprecipitations on supernatants demonstrated complete precipitation of all RelA. Eluted proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel and transferred to Immobilon-P membranes (Millipore).

For in vitro kinase assays, whole-cell extracts were prepared by washing the cells twice in ice-cold PBS, followed by lysis for 5 min on ice with 40 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 0.1% NP-40, 6 mM EDTA, 6 mM EGTA, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate (P-NPP), 10 mM β -glycerophosphate, 0.3 mM sodium orthovanadate, DTT, PMSF, aprotinin, leupeptin, and pepstatin. Lysates were clarified by centrifugation. Then, 100 μ g of total cell extract was used in subsequent kinase assays. Lysates were immunoprecipitated with antibody to the IKK complex (IKK α or MKP1; Santa Cruz) for 1 h, after which protein A-agarose was added for 1 h. Beads were washed three times with lysis buffer, followed by one wash with buffer A. Beads were incubated in 15 μ l of kinase buffer (20 mM HEPES [pH 7.4]; 2 mM MgCl₂; 2 mM MnCl₂; 10 mM ATP; 10 mM NaF; 10 mM P-NPP; 10 mM β -glycerophosphate; 0.3 mM sodium orthovanadate; PMSF; 2 μ g each of aprotinin, leupeptin, and pepstatin per ml; 1 mM DTT) with 2 μ g of glutathione S-transferase (GST)-I κ B α ₁₋₅₃ and 0.1 μ Ci of [γ -³²P]ATP. The kinase reaction was carried out for 30 min at 30°C, and samples were resolved by SDS-PAGE.

EMSA, Northern, and Western analyses. For electrophoretic mobility shift assays (EMSAs), 4 μ g of nuclear extract was incubated with a [γ -³²P]ATP-labeled double-stranded probe as previously described (2). Components of the probe binding complex were identified by preincubation of the extract with antibodies to specific components of NF- κ B (Santa Cruz) prior to probe exposure. DNA-protein complexes were resolved on a 6% polyacrylamide gel, dried, and visualized by autoradiography. Western blotting was performed as recommended by the ECL Kit (Amersham) package insert. Antibodies to p21^{WAF1/Cip1} (Transduction Laboratories), I κ B α (Santa Cruz), FLAG (Sigma), β -actin (Sigma), and IKK α and IKK β (Santa Cruz) were used. Where indicated, the intensities of the bands were quantified by densitometry and analyzed by using the Ambis software package. For Northern analysis, cells were treated with either DMSO, PMA, or TNF for 6 h. Total RNA was isolated by RNeasy (Qiagen) according to the manufacturer's instructions. RNA was resolved on an agarose-formamide gel and transferred to a HyBond-N⁺ membrane (Amersham) p21^{WAF1/Cip1} cDNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were prepared by using the Random Primed DNA Labeling Kit (Roche) as described in the package insert and then hybridized to membrane in RapidHyb Buffer (Amersham) and washed in sequentially more stringent SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) washes. Results were visualized by autoradiography and quantified by densitometry using the Ambis software package.

Adenoviral transduction. Peripheral blood lymphocytes were obtained from buffy coats using a Ficoll-Hypaque density gradient. Mononuclear cells were washed extensively and incubated with neuraminidase-treated sheep red blood cells to rosette T cells. CD14⁺ monocytes were then isolated from the resulting mononuclear cell population by negative depletion using the StemSep (StemCell) system according to the manufacturer's instructions. Enriched CD14⁺ cells were typically found to be 80 to 86% pure by FACS analysis. An aliquot of cells was lysed with whole-cell lysis buffer as above. CD14⁺ cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated human AB serum. Cells were allowed to plastic adhere for 30 h, and then washed gently with fresh media. Cells were fed with one-half conditioned medium plus fresh AB media. Five days after isolation, the cells were washed to remove nonadherent cells and transduced with adenovirus (100 PFU/cell) expressing HA-I κ B α Ser 32/36 Ala or alkaline phosphatase. The transduction efficiency was monitored by nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) staining (Roche) and typically was 90 to 95% efficient. Two days after harvest, cells were lysed in whole-cell lysis buffer as described above.

RESULTS

Persistent activation of NF- κ B correlates with cell differentiation and is dependent on the IKK complex kinase activity. To address the role of NF- κ B on monocyte differentiation, we first sought to characterize a model of monocyte differentiation

FIG. 1. U937 promonocytic cells differentiate into a macrophage-like phenotype following exposure to phorbol esters. (A) U937 cells grown in suspension become adherent following exposure to PMA (2 ng/ml) by 24 h with persistence of the phenotype for up to 3 days after the first appearance the PMA differentiation signal. FACS profiles of CD11c expression following treatment with PMA or DMSO vehicle are inset. (B) Differentiation leads to the nuclear translocation of prototypic NF- κ B heterodimers at 24 h post-PMA exposure. c-Rel and RelB antibodies had minimal effect on the mobilities of either NF- κ B complex (data not shown). TNF- α treatment lasted 8 min and served as a positive control. (C) I κ B α steady-state levels are markedly reduced at 24 h posttreatment with PMA and correlate with the presence of nuclear RelA. TNF- α treatment was for 8 min and served as a positive control.

in which NF- κ B activation is observed in the context of differentiation (24, 25) and which is amenable to genetic inhibition following identification of the mechanism regulating NF- κ B nuclear translocation. Using the human promonocytic U937 cell line, it is demonstrated that these cells become adherent to plastic and express CD11c integrin, a cell surface marker of differentiated macrophages, by 24 h following exposure to low-dose (2 ng/ml) PMA (Fig. 1A). As has been previously reported (24, 25), the differentiation program initiated by PMA correlates with the progressive nuclear localization of NF- κ B by 24 h posttreatment (compare lanes 1 and 2 in Fig. 1B with lanes 4 and 5 in Fig. 1C). EMSA analysis demonstrates that the nuclear complex of NF- κ B is composed mainly of RelA-p50 dimers (Fig. 1B, lanes 4 and 5). EMSA analysis shows that c-Rel or RelB antibodies fail to appreciably shift either NF- κ B

activity, while the lower activity can be completely shifted with p50 antibodies (data not shown). Nuclear localization of NF- κ B correlates with a marked reduction in I κ B α steady-state protein levels (Fig. 1C), as well as of I κ B β and p105 (data not shown). Therefore, PMA-induced differentiation of the U937 promonocytic cell line to a macrophage phenotype is accompanied by a progressive and persistent nuclear translocation of NF- κ B resulting from constitutively decreased I κ B protein.

The decreased steady-state protein level of I κ B α observed in PMA-differentiated U937 cells is secondary to a decrease in the protein's half-life (Fig. 2A). The decrease in I κ B α protein half-life could potentially arise from activation signals targeting the SRD. Alternately, the C-terminus PEST domain, previously shown to confer instability to a host of proteins, could

FIG. 2. I κ B α in differentiated U937 cells has a shorter half-life than in undifferentiated cells, and the half-life is dependent upon the SRD. (A) U937 cells differentiated by exposure to PMA (2 ng/ml) for 24 h were lysed at different points following treatment with cycloheximide and analyzed for I κ B α levels by Western blot. β -Actin was probed as a gel loading control. (B) To determine which region of I κ B α mediates reduced I κ B α steady-state levels, U937 clones stably expressing FLAG-tagged I κ B α transgenes were induced to differentiate by PMA (2 ng/ml) and lysed at 6 h into the differentiation program. WT, FLAG-I κ B α ; 32/36, FLAG-I κ B α 32/36^{Ser-to-Ala}; 4C, FLAG-I κ B α 283/288/293/291^{Ser/Thr-to-Ala}. I κ B α levels were determined by Western blot. Similar results were seen at 12 and 24 h postdifferentiation (data not shown). (C) In order to address the direct role of I κ B α in cytosolic retention of NF- κ B, clones expressing either the FLAG-tagged WT I κ B α or FLAG-I κ B α 32/36 were differentiated for 24 h and then treated with cycloheximide (50 μ g/ml). Treated cells were lysed and immunoprecipitated with anti-RelA. Immunoprecipitates were then analyzed for RelA-associated I κ B α levels by Western blot. Gels were analyzed by densitometry, and the half-life was plotted as a function of I κ B α levels over time. (D) Gel shift analysis demonstrates that mutation of the SRD of I κ B α abrogates differentiation-induced activation of NF- κ B by PMA. WT or 32/36 cells were treated with PMA for 24 h. TNF treatment was for 4 h and serves as a control. The identity of the upper band (filled circle) was confirmed by supershift analysis as RelA/p50. The lower band (arrow) was confirmed as p50 homodimers (data not shown). The results are representative of three independent clones.

FIG. 3. IKK activity is increased in differentiated U937 cells. The differentiation program of U937 cells was initiated by treatment with PMA (2 ng/ml). Cells were collected and lysed at 2, 24, or 48 h after PMA treatment. Lysates were immunoprecipitated with antibody to either PK-CK2 or the IKK complex. The resulting immunocomplex was used to radiolabel GST-I κ B α_{1-53} in an immunokinase assay. IKK β or PK-CK2 levels were detected by immunoblotting to ensure that there were equal levels of kinase in each sample. Membrane was Coomassie blue stained to ensure the presence of equal amounts of substrate in each reaction (data not shown). I κ B α levels at the indicated time points were detected by Western blot. β -Actin was probed as a gel loading control. TNF- α treatment was for 8 min and served as a positive control.

be targeted during differentiation in order to further decrease the I κ B α half-life. Using U937 cells stably expressing either a wild-type FLAG-tagged I κ B α (WT) or FLAG-tagged I κ B α in which either the SRD or the C-terminal PEST domain are mutated (32/36 or 4C, respectively), we observed that PMA-induced cell differentiation targets the SRD but not the PEST domain of I κ B α , as demonstrated by a decrease in the steady-state protein levels of FLAG-I κ B α WT and FLAG-I κ B α 4C (Fig. 2B, compare lanes 1 to 2 and lanes 5 to 6) but not of FLAG-I κ B α 32/36 (Fig. 2B, lanes 3 and 4). The functional relevance of this observation is demonstrated by the fact that RelA-associated I κ B is targeted by the PMA-induced differentiation process (Fig. 2C) and by the lack of nuclear translocation of NF- κ B in PMA-stimulated FLAG-tagged 32/36 I κ B α cells (Fig. 2D, compare lanes 2 and 5).

The SRD of I κ B α is targeted by a number of kinases, including the IKK complex, pp90^{rsk}, and PK-CK2 (23, 31, 52). While IKK is well characterized to mediate I κ B α phosphorylation by punctual and rapid stimuli such as TNF or IL-1, we questioned whether it could also be involved in targeting the I κ B α SRD as a result of monocyte differentiation. The kinase activity of the IKK complex was analyzed at different time points along the cell differentiation program. As seen in Fig. 3, an increase in IKK activity toward the SRD of the I κ B α substrate is detected as early as 2 h following PMA, with further

increases at 24 and 48 h. The persistent and heightened IKK activity further correlates with reduced steady-state levels of I κ B α (Fig. 3, compare panels 1 and 5). In contrast, the kinase activity of PK-CK2, previously shown to directly phosphorylate the PEST (39) and SRD domain of I κ B α (52), is not observed during the same time points. Therefore, the persistent decrease in I κ B α levels observed during monocyte differentiation correlates with persistent and increased activity of the IKK complex. This is notable in demonstrating that the IKK complex is not only punctually activated by defined inflammatory stimuli such as TNF or IL-1 but also in a persistent manner by processes such as cell differentiation.

Activation of NF- κ B is necessary for surviving the monocyte differentiation program. Although it is well established that NF- κ B is activated during monocyte differentiation, little is known about the functional relevance of this activation. Identification of the IKK complex and the SRD domain of I κ B α as targets of signaling pathways triggered by PMA-induced monocyte differentiation enables us to test the role of NF- κ B in this process by inhibiting its activation using genetic approaches. U937 clones stably expressing FLAG-tagged I κ B α containing mutations of the key SRD serines (32/36) or empty vector (SFFV) were treated with PMA and evaluated thereafter for differentiation markers as in Fig. 1. As early as 24 h and as late as 72 h post-PMA treatment, a marked reduction in the num-

FIG. 4. Activation of NF- κ B is necessary for surviving the differentiation program in U937 cells. (A) U937 cells induced to differentiate with PMA (2 ng/ml) were scored for adherence at 24 or 72 h post-PMA treatment, and values are expressed as in Fig. 1. (B) Vector control (SFFV) or cells stably expressing an SRD mutant I κ B α (32/36) were treated with either PMA or DMSO and harvested at 2, 6, 12, 24, or 72 h posttreatment. Only viable cells were scored. No differences were seen in vehicle control SFFV or 32/36 cells. Only PMA-treated points are shown (*, $P < 0.05$; ‡ $P < 0.10$; Student's t test). (C) SFFV and 32/36 cells treated for 0, 24, or 72 h with PMA were collected, fixed, and DNA stained with PI. The cell cycle distribution is shown. The apoptotic population was scored as the sub-G₁ DNA fraction by FACS analysis.

ber of U937 cells expressing the SRD I κ B α mutant (32/36) which become plastic adherent is observed. Following the initiation of the differentiation program, both SFFV and 32/36 cells can complete at least one turn of the cell cycle, as evidenced by the cell cycle profile in Fig. 4C and the increase in cell numbers for SFFV cells (Fig. 4B). However, there is a marked decrease in viable 32/36 cells following PMA-induced monocyte differentiation compared to SFFV control cells (Fig. 4B). That the decreased cell survival observed in the PMA-treated 32/36 U937 cells is apoptotic in nature is demonstrated by the increase of subdiploid DNA in the PI cell cycle profile

shown in Fig. 4C. From these data it is inferred that the persistent nuclear translocation of NF- κ B confers a protection to promonocytic cells undergoing the differentiation process.

Differentiation-induced cell death of monocytes is triggered by TNF. The novel observation that NF- κ B is necessary to survive the differentiation process led us to examine what NF- κ B opposed factors may be initiating differentiation-induced apoptosis. It is well established that NF- κ B protects a number of cells from TNF-induced apoptosis (3, 6, 53, 59). In addition, several studies have shown that TNF is involved in a number of hematopoietic cell differentiation models (16, 18,

FIG. 5. TNF- α -mediated differentiation-induced apoptosis. U937 cells stably transfected with either empty vector (SFFV) or the I κ B α SRD mutant (32/36) were induced to differentiate with PMA (2 ng/ml) for 24 h. DMSO served as a vehicle control. At the initiation of differentiation, cells were treated with TNFR-Fc or IL4R-Fc. Apoptotic cells were scored by FACS analysis as the sub-G $_1$ DNA fraction following PI staining as in Fig. 4. columns: $\square$, DMSO; ▨ , PMA; ▩ , PMA plus TNFR-Fc; ▤ , PMA plus IL4r-Fc.

38, 62). We asked, therefore, whether endogenous production of TNF is mediating the apoptosis seen during differentiation. U937 clones stably expressing mutant FLAG-tagged I κ B α (32/36) or empty vector (SFFV) were treated with PMA and evaluated for apoptosis using PI staining as described above. Selected wells were also treated with a TNFR-Fc protein to block binding of endogenous TNF to native TNF receptors. IL4R-Fc was used as a control. As seen in Fig. 5, a relatively low dose of TNFR-Fc blocked nearly half of the differentiation-induced cell death in 32/36 U937 cells, while the IL4R-Fc fusion protein had no effect. From these data, it can be inferred that endogenously produced TNF by differentiating monocytes triggers apoptosis. Whether this TNF elicited during differentiation (following PMA treatment) itself is a differentiation signal is unknown.

NF- κ B regulates p21^{WAF1/Cip1} expression which is required for monocyte survival during the differentiation process. The antiapoptotic characteristics of NF- κ B are presumed to be mediated by a number of known and yet-to-be-identified survival genes (3, 10, 12, 19). Several studies have previously reported a link between NF- κ B activation and p21 expression in a number of cell systems (see the introduction). Because of the p21^{WAF1/Cip1} upregulation observed during monocyte differentiation and the ability of this protein to confer resistance to apoptosis in cells of monocytic origin (1, 9, 29, 60), we hypothesized that the requirement of NF- κ B to protect the cell from death during monocyte differentiation may be through the upregulation of p21^{WAF1/Cip1}. To test this, we first analyzed whether there exists a direct correlation between levels of p21^{WAF1/Cip1} and nuclear translocation of NF- κ B during the process of macrophage differentiation. PMA-induced differentiation of SFFV vector control U937 cells correlated with the upregulation of cytosolic p21^{WAF1/Cip1} in the absence of cell

death. By contrast, PMA treatment of 32/36 U937 cells did not result in the upregulation of p21^{WAF1/Cip1} expression and, as expected, resulted in cell death in the absence of NF- κ B activation (Fig. 6A and B). Northern analysis of mRNA from SFFV vector control and 32/36 U937 cells verifies the role of NF- κ B in regulating p21^{WAF1/Cip1} transcription. Interestingly, TNF is unable to induce p21^{WAF1/Cip1} expression, despite being a potent inducer of NF- κ B activity, in direct contrast to observations in Ewing sarcoma cells (29). Therefore, the inability of 32/36 U937 cells to survive the differentiation program may be due to their inability to induce expression of p21^{WAF1/Cip1}, which we infer is an NF- κ B-dependent process. To formally address this issue, we utilized U937 cells stably expressing or not antisense constructs for p21^{WAF1/Cip1}. While PMA-induced differentiation of p21(AS) and control U937 cells induces NF- κ B nuclear translocation in both cells types, p21^{WAF1/Cip1} expression is, as expected, not observed in the p21(AS) U937 cells, indicating that NF- κ B is upstream of p21^{WAF1/Cip1}. Moreover, the fact that p21(AS) U937 cells die following PMA-induced cell differentiation despite the induction of NF- κ B nuclear translocation (Fig. 7), supports the role of p21^{WAF1/Cip1} as an NF- κ B-dependent survival gene required for macrophage differentiation.

Inhibition of NF- κ B reduces p21^{WAF1/Cip1} expression in primary macrophages. To investigate whether the NF- κ B-dependent upregulation of p21^{WAF1/Cip1} in monocytic cells undergoing macrophage differentiation is also present in primary human cells, we optimized an adenovirus transduction model through which NF- κ B could be inhibited by genetic approaches during the process of differentiation from monocytes to macrophages. Highly purified primary CD14⁺ peripheral human blood monocytes were isolated by negative selection and separated into two aliquots, one of which was lysed. The remaining cells were allowed to differentiate in tissue culture to macrophages for 5 days. At this time point adherent macrophages were transduced with adenovirus expressing either alkaline phosphatase (control) or HA-I κ B α 32/36A. After 48 h the cells were harvested, lysed, and analyzed by SDS-PAGE in parallel with lysates obtained upon initial monocyte isolation for p21^{WAF1/Cip1} levels. As shown in Fig. 8, a small amount of p21^{WAF1/Cip1} is present in freshly isolated monocytes compared to that observed following their differentiation to macrophages. More importantly, transduction and expression of HA-I κ B α 32/36A but not of alkaline phosphatase reduces the level of p21^{WAF1/Cip1} in differentiated macrophages, once more supporting the regulatory role of NF- κ B on p21^{WAF1/Cip1} during macrophage differentiation.

DISCUSSION

The present study provides a number of novel observations pertaining to the regulation and functional role of NF- κ B in cell differentiation. First, we have documented that the persistent activation of NF- κ B that ensues following the initiation of monocyte differentiation is dependent on the continuous activation of the IKK complex and hence the phosphorylation and/or degradation of I κ B molecules. Second, we have demonstrated the necessary role of NF- κ B in allowing the cell to survive the differentiation process. Finally, we have shown that p21^{WAF1/Cip1} is indeed regulated by IKK-dependent NF- κ B

FIG. 6. Activation of NF- κ B is necessary for expression of p21^{WAF1/Cip1}. (A) U937 cells induced to differentiate were lysed at the indicated times after PMA treatment, and cytosolic and nuclear extracts were collected. Cytosols were probed for p21^{WAF1/Cip1} expression by Western blot (small arrow). β -Actin was blotted as a loading control. Similar results were seen with whole-cell extracts (data not shown). Nuclear NF- κ B activity was detected by EMSA, and the identity of the NF- κ B components was confirmed by supershift assay as for Fig. 1 (data not shown). RelA-p50 and p50-p50 complexes are indicated by filled circles and arrows, respectively. (B) Vector control (SFFV) or cells stably expressing an SRD mutant I κ B α (32/36) were treated with PMA and harvested at various times posttreatment. The cell cycle distribution is shown. The apoptotic cells were scored by FACS analysis as the sub-G₁ DNA fraction following fixation and PI staining as for Fig. 4. (C) U937 cells stably transfected with either empty vector (SFFV) or the I κ B α SRD mutant (32/36) were treated with PMA (2 ng/ml) or TNF- α (10 ng/ml) for 6 h, and the total RNA was harvested. p21^{WAF1/Cip1} mRNA levels were determined by Northern analysis, as were the GAPDH levels as a loading control. p21^{WAF1/Cip1} mRNA levels were enhanced only following PMA treatment despite the activation of NF- κ B with both TNF- α and PMA treatments (data not shown).

FIG. 7. Ablation of p21^{WAF1/Cip1} expression results in failure to survive the differentiation program, even in the presence of NF-κB activation. U937 cells stably transfected with either empty vector (pREP4) or an antisense p21^{WAF1/Cip1} construct (F4) were treated with 2 ng of PMA per ml to initiate the differentiation program. pREP4 or F4 cells were collected at the indicated time points post-PMA treatment, and nuclear and cytosolic fractions were collected as for Fig. 6. F4 cells demonstrate a marked reduction in p21^{WAF1/Cip1} expression. β-Actin detection was included as a control for loading. EMSA analysis reveals roughly equivalent levels of nuclear NF-κB dimers. RelA-p50 and p50-p50 complexes are indicated by filled circles and arrows, respectively. Viable cells were detected by PI staining as for Fig. 5 and are expressed as the percent survival over time. A representative experiment is shown. Similar results were obtained with a second p21(AS) clone.

activation of p21^{WAF1/Cip1} in differentiating monocytes and may be the effector protein required to protect the cell from differentiation-induced cell death.

Several lines of evidence support the hypothesis that persistent NF-κB activation in a defined cell in the absence of punctual stimuli is in part due to a continued degradation of IκB molecules (35, 55). B cells at different stages of differentiation exhibit a structured sequence of NF-κB activation as they progress through the various steps of B-cell differentiation, ultimately leading to mature B cells and plasma cells which contain significant levels of NF-κB in the nucleus in a constitutive manner (3, 22). Our results suggest a key role for the IKK complex in mediating this continuous level of NF-κB translocation. Therefore, the IKK complex can be in a continuous state of activation, separate from the well-characterized punctual inducibility and rapid regression of activity seen with activators such as TNF or IL-1. In addition to the process of

FIG. 8. NF-κB activation is necessary for p21^{WAF1/Cip1} expression in differentiated human monocytes. CD14⁺ cells were isolated from the buffy coat by negative depletion. FACS analysis indicated 84% enrichment. Cells were either lysed immediately after isolation or differentiated by plastic adherence. At day 5 postadherence, cells were transduced with adenovirus (Adv) expressing either HA-IκBα 32/36 (32/36) or alkaline phosphatase (AP). Cells were lysed 48 h posttransduction and analyzed for p21^{WAF1/Cip1} by immunoblotting. IκBα levels are included to show the relative expression of endogenous versus transduced protein. β-Actin was included as a loading control.

cell differentiation, pathological conditions such as cell transformation are also associated with a continuous and persistent level of NF-κB in the nucleus (12, 19, 36, 41). Whether the IKK complex is persistently activated in cells undergoing transformation such as shown in this study is unknown and deserves to be addressed in future studies since the persistent levels of NF-κB in the nuclei of cancer cells impact the response to chemotherapeutic and ionizing agents.

It should be noted that it is unlikely in this model that PMA itself acts directly upon signal transduction pathways converging on the IKK complex. First, in contrast to the case of TNF, IKK activity is not upregulated immediately following the treatment of monocytic cells with relatively low doses (2 ng/ml) of PMA. Second, IKK activation, as well as differentiation, is seen within 24 h and peaks at 3 to 5 days following a single treatment dose of PMA, time points in which it is unlikely that significant levels of PMA remain in the culture media. Lastly, cells extensively washed after the initial PMA treatment and recultured in PMA free media proceed with the differentiation program (data not shown). The identity of the PMA-dependent stimulus or stimuli which drives chronic IKK activation allowing macrophage differentiation is unknown. However, it is possible that different cytokines produced as a result of PMA treatment may act in an autocrine fashion on the cells to reinforce and perpetuate the differentiation program. This possibility is supported by the observation that monocyte differentiation induces TNF production which, in turn, drives apoptosis in the absence of NF-κB. However, it should be noted that a single dose of TNF in the absence of PMA min-

imally upregulates p21^{WAF1/Cip1} expression (Fig. 6). Therefore, it is likely that additional differentiation-inducing signals are initiated by PMA independent of TNF production. Although not exclusive of autocrine factors, it is also possible that internal perturbations initiated by PMA treatment may increase IKK activity. For example, treatment of U937 cells with okadaic acid, an inhibitor of phosphatases 1 and 2A, induces differentiation, cell cycle arrest, and eventual cell death (28, 42, 50). It has previously been suggested that phosphatase 2A may play a role in inhibiting basal IKK activity (13).

The necessary role of NF- κ B in macrophage differentiation is relevant and justifies at a functional level the long-term observations that NF- κ B is activated during the differentiation of macrophages and that it is present at very high levels in the nuclei of fully differentiated macrophages (24). While we cannot exclude that NF- κ B is also influencing specific steps of the cell differentiation process, such as promoting exit from the cell cycle, its role in protecting the cell from undergoing death is clearly highlighted. Overexpression of certain transcription factors alone can be sufficient to drive cell differentiation in a number of cell models (21, 26, 32, 33). During the differentiation of promonocytic cells, NF- κ B is potently and persistently activated. Whether this activation is driving differentiation or merely a consequence thereof was unknown. Our data suggest that NF- κ B may not be driving differentiation but rather is necessary for surviving the differentiation program. Abundant information supports the role of NF- κ B as an antiapoptotic molecule following cell stimulation with apoptosis-inducing ligands such as TNF or chemotherapeutic and ionizing agents (6, 10, 54, 58, 59). However, to our knowledge this study provides the first evidence that the cell differentiation process triggered by chemical agents such as phorbol esters is a significant cell injury that leads to cell death unless NF- κ B is concomitantly activated. It remains to be seen whether constitutive, forced activation of NF- κ B in promonocytes, in the absence of PMA, alone is sufficient to either force differentiation or confer resistance to subsequent apoptotic stimuli. These experiments are currently under way.

It is unclear whether NF- κ B knockouts have an impairment in macrophage differentiation since RelA animals are nonviable. Further, double knockouts of the p50 and p52 NF- κ B components, which theoretically would render RelA inactive, have other deficiencies which could impose upon a deficiency in macrophage development (27). Our data, however, suggest that NF- κ B activity is important for macrophage development and raise the question as to whether NF- κ B activity is involved in differentiation of other specific cell lineages. Knockout experiments, although a valuable tool, may be unable to completely address this issue (26, 33). In vivo, it is possible that other members of the Rel family may compensate for the differentiation of specific cell lineages. Overlying mortalities and morbidities such as seen with the nonviable RelA knockout could make observations difficult and confusing. Finally, NF- κ B activity may be important for discrete steps in cell-lineage-specific development. For example, PU.1 knockout mice lack both mature macrophages and granulocytes since PU.1 expression is necessary for myeloid lineage commitment of pluripotent progenitors (47). However, overexpression of PU.1 in cells cannot drive further differentiation of myeloblasts to monocytes (43, 48), suggesting that PU.1 has a distinct role

at a specific stage of the differentiation program of pluripotent progenitors. Knockout experiments may therefore mask the specific cell type and cell stage requirements of a gene because of underlying pathologies.

A most intriguing observation is the fact that p21^{WAF1/Cip1}, generally considered a protein restricted to the nuclear compartment in which it exerts its inhibition of CDK and thus regulates cell cycle progression, is in the unique case of macrophages, predominantly located in the cytosol, where it exerts antiapoptotic functions (1). Based on our results demonstrating that NF- κ B is required for cell survival, the link confirmed in this study between NF- κ B and p21^{WAF1/Cip1} during the process of macrophage differentiation is noteworthy for several reasons. First and foremost, our results support that, in cells of monocytic lineage, including primary macrophages, p21^{WAF1/Cip1} is an NF- κ B-dependent gene and thus a potential effector of the antiapoptotic effects of NF- κ B. The mechanism whereby NF- κ B ultimately leads to p21^{WAF1/Cip1} upregulation is unknown. NF- κ B may directly drive the transcription of p21^{WAF1/Cip1}, as suggested by Fig. 6, or have other effects on p21^{WAF1/Cip1} expression, such as on protein or mRNA stability. Experiments are currently under way to explore the link of NF- κ B to p21^{WAF1/Cip1}. Likewise, it will be of interest to investigate whether cells with constitutive levels of NF- κ B in the nuclei, such as mature B lymphocytes or transformed cancer cells, also demonstrate upregulation of p21^{WAF1/Cip1}. If this were the case, it could be postulated that under certain conditions, beyond those of macrophage differentiation, p21^{WAF1/Cip1} may exert important apoptotic functions relevant to, e.g., oncogenic processes.

The observation that endogenously produced TNF triggers the apoptosis seen during monocyte differentiation (Fig. 5), coupled with the need for p21^{WAF1/Cip1} expression for survival, suggests that NF- κ B-dependent p21 expression directly opposes TNF-induced apoptosis. This observation agrees with a number of previous reports demonstrating that p21^{WAF1/Cip1} opposes TNF-induced apoptosis (15, 29, 30). How this mechanism occurs is unknown and warrants further investigation. It should be noted that PMA fails to induce apoptosis in fully differentiated primary human macrophages even when NF- κ B is inhibited (data not shown). This implies that the sensitivity to TNF-induced apoptosis is unique to cells actively undergoing differentiation and is not seen in cells that have completed the maturational process. Alternatively, fully differentiated macrophages may have additional antiapoptotic mechanisms such as the upregulation of FLIP (46). Previous studies have suggested that TNF plays an active role in the differentiation of a number of hematopoietic cells. Whether this is true also in monocytes remains unclear. One possibility is that it is the careful balance of TNF-induced apoptosis versus direct differentiation signals which mediate the final maturational outcome.

In summary, we have shown that constitutive activation of NF- κ B is required for surviving the monocyte differentiation program and is dependent on the chronic activation of IKK targeting the I κ B α SRD. Based on our data with cells stably expressing transdominant I κ B α , we propose that the maturation of monocytes involves two distinct signals. The first signal drives the differentiation of the cell and may be independent of NF- κ B activity. The second signal, mediated by TNF- α , ini-

tiates a death signal in the differentiation monocyte. This death signal is directly opposed by NF- κ B activity. Finally, persistent NF- κ B activation induces the expression of p21^{WAF1/Cip1}, which may be the critical protein which protects the monocyte from differentiation-induced cell death.

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