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p21 (Waf1/Cip1) and FasL gene activation via Sp1 and NFκB is required for leukemia cell survival but not for cell death induced by diverse stimuli

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Abstract

The molecular mechanisms of the cellular response to different apoptotic effectors are only partially understood. Herein, the role of transcription factors, Sp1 and NFκB in differentiation-related and etoposide-induced apoptosis was examined in a number of human leukemia cell lines (HL-60, NB4, HEL, THP-1, K562). This was investigated with respect to the recruitment of one cell-cycle regulating gene, p21 and one cell death gene, FasL. Using electrophoretic mobility shift assay (EMSA), we consistently observed Sp1 and NFκB binding activity to the promoter of either gene during cell differentiation and the decrease associated with apoptosis upon long-term treatment with differentiation inducers in HL-60, NB4 and HEL cells. By contrast, Sp1 and NFκB binding capacities were lost in all myeloid cell lines undergoing etoposide-induced fast apoptosis. This effect was eliminated by the broad-spectrum caspase inhibitor, benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethylketone, thus restoring transcription factors' binding activity. However, sustained NFκB binding to the FasL promoter was noticed in apoptosis undergoing HEL cells treated by etoposide. Our results suggest that p21 and FasL gene activation is required for myeloid leukemia cell survival or maturation but not for cell death via Sp1 and NFκB as regulators of these genes. The findings also support the idea of a common mechanism for cellular responses to different apoptotic effectors in malignant hematopoietic cell lines.

Keywords: Apoptosis; Differentiation; Leukemia; Transcription factors; Etoposide

Abbreviations: DMSO, dimethylsulfoxide; ATP, adenosine-5′-triphosphate; FasL, Fas ligand; NFκB, nuclear factor kappa B; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonylfluoride; RA, all-trans retinoic acid; RAR, retinoic acid receptor; RNase, ribonuclease; TRAIL, TNF-related apoptosis-inducing ligand; z-VAD.fmk, benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethylketone

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1. Introduction

A balance between the signals supporting cell survival and inducing cell death regulates cell homeostasis, which is abrogated in malignant transformed cells. One of the approaches to cancer therapy is the prevention of uncontrolled growth and selective induction of malignant cell death. This may be achieved either with differentiation inducers or cytotoxic drugs. Retinoids are promising agents for leukemia therapy due to anticancer properties by modulating cell proliferation, differentiation and apoptosis (Altucci & Gronemeyer, 2001; Collins, 1987). The topoisomerase II inhibitor, etoposide is an important drug in the treatment of leukemias and efficient inducer of apoptosis (Froelich-Ammon & Osheroff, 1995). However, the molecular mechanisms of cellular response to different apoptotic effectors, such as retinoic acid (RA) or etoposide, may differ.

Malignant cells usually respond to drug-induced stress either by cell cycle arrest and/or by undergoing apoptotic cell death. The response depends on the activation of p53 or the induction of certain transcription factors (Harwood et al., 2000; Nelson & Kastan, 1994; Piret & Piette, 1996). The Fas receptor (CD95), a 48 kDa cell surface glycoprotein, is regarded as a major mediator of apoptosis. Ligation of Fas receptor with anti-Fas antibody or its specific ligand (FasL) induces a death signal in many cell types including those of haematopoietic origin (Dirks et al., 1997; Kim, Lee, Hong, & Park, 2000; Salih et al., 2002). Little is known about the regulation of Fas signaling in various types of myeloid leukemia's. Conflicting data have also been reported concerning the expression of Fas and sensitivity to Fas-induced apoptosis in acute promyelocytic leukemia (APL) cell lines (Kikuchi et al., 1996; Ohashi, Iwase, & Nagumo, 2000; Salih et al., 2002). The significance of Fas expression in chemotherapy-induced apoptosis has been questioned as well (Eischen et al., 1997; Gamen et al., 1997), and some reports suggest that cytotoxic drug-induced apoptosis is independent of Fas (Siitonen et al., 2000; Siitonen, Mantymaa, Sally, Savolainen, & Koistinen, 2000; Tolomeo et al., 1998; Villunger et al., 1997). The promoter region of the FasL gene has recently been cloned and found to contain binding sites for a number of transcription factors including NFκB, AP-1, Sp1 (Harwood et al., 2000; Kasibhata et al., 1998; Kavurma, Santiago, Bonfoco, & Khachigian, 2001). Topoisomerase II poisons can induce apoptosis via activation of NFκB or AP-1 and Fas signaling (Kasibhata et al., 1998; Piret & Piette, 1996). Sp1 involvement in apoptosis regulation via extracellular Fas/FasL engagement in smooth muscle cells has been recently demonstrated (Kavurma et al., 2001). However, the regulatory role of Sp1 in the process of apoptosis in many cell types is presently unknown.

The p21 (Waf1/Cip1) protein induces growth arrest by blocking cyclin-dependent kinases (CDK) or the activity of proliferating cell nuclear antigen (PCNA), which influences cell proliferation, differentiation and apoptosis (Gartel & Tyner, 1999; Harper, Adami, Wei, Keymarsi, & Elledge, 1993; Harper & Elledge, 1996). Exit from the cell cycle is a prerequisite for terminal differentiation, and treatment of myeloid cells with inducers of differentiation, such as PMA, okadaic acid or RA, enhances p21 gene transcription (Casini & Pelicci, 1999; El-Deiry et al., 1994; Harper et al., 1993; Jiang et al., 1994). In some systems, p21 either protects cells from apoptosis (Gorospe et al., 1997; Polyak et al., 1996) or promotes apoptosis (Gartel & Tyner, 1999). Agents that cause DNA damage, such as UV radiation or certain carcinogens, activate tumor suppressor p53-dependent p21 gene transcription (Dulic et al., 1994; El-Deiry et al., 1994). p53-independent expression of p21 occurs during terminal differentiation in a number cell types (Jiang et al., 1994; Macleod et al., 1995). The majority of these modulators affect p21 gene activity via Sp family proteins, which bind to regulatory elements in the proximal p21 gene promoter (Gartel & Tyner, 1999). The Sp family of structurally and functionally related transcription factors (Sp1, Sp2, Sp3 and Sp4) recognizes GC-rich or GT-rich DNA and regulates the promoters of several genes (Bouwman & Philipsen, 2002; Lania, Majello, & De Luca, 1997). The human p21 (Waf1/Cip1) gene contains six Sp1 binding sites (Gartel & Tyner, 1999). The ubiquitously expressed and closely related Sp1 and Sp3 regulate p21 gene by binding with different affinities as has been shown in rat liver (Koutsodontis, Moustakas, & Kardassis, 2002) and in Caco-2 cells (Gartel, Goufman, Najmabadi, & Tyner, 2000).

The NFκB family of transcription factors is often proposed as a key regulator of differentiation and survival. In mammals, this protein family includes p50/p105, p52/p100, p65 (RelA), c-Rel and RelB (Glosh, May, & Kopp, 1998). NFκB is activated by

multiple physiological or environmental stresses (Pahl, 1999). In the nucleus, NFκB dimers bind to a set of related 10 bp DNA sites, collectively called κB sites, thereby regulating the expression of many genes. However, interestingly, NFκB can exert both pro- and anti-apoptotic effects in different cells types (Barkett & Gilmore, 1999).

In this study, we have explored the regulatory role of Sp1 and NFκB during differentiation-associated and etoposide-induced apoptotic processes in leukemia cell lines with different p53 and Fas receptor status.

2. Materials and methods

2.1. Materials

RA, PMA, NBT, RNase A, proteinase K, ethidium bromide, hemin chloride and etoposide were purchased from Sigma (St. Louis, MO), and z-VAD.fmk from Promega Corp. (Madison, WI). The stock solutions of RA (500 μM) in ethanol and hemin (25 mg/ml) in 1.4 M ammonium hydroxide were stored at −20 °C.

2.2. Cell culture and cell lines

The human leukemia cell lines (promyelocytic HL-60, acute promyelocytic NB4, chronic myeloid K562, erythroleukemia HEL and acute monocytic THP-1) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere and used for assays during exponential phase of growth.

2.3. Assessment of cell differentiation

Granulocytic differentiation of HL-60 and NB4 cells was determined by nitroblue tetrazolium (NBT) reduction (Collins, 1987). Hemoglobin production during erythroid differentiation of HEL cells was established by benzidine staining (Jeannesson, Ginot, Manfait, & Jardillier, 1984).

2.4. Morphologic assessment of apoptotic cells

After any incubation, cells were pelleted at 500 × *g* for 5 min and resuspended (5×10^6 cells/ml) in 100 μl PBS. Cells were stained with 0.01% acridine orange/0.01% ethidium bromide (AO/EtBr) mixture (1:1, v/v), 6 μl per 100 μl cell suspension (Mercille & Massie, 1994). Apoptotic bodies containing cells were counted using a fluorescence microscope by scoring at least 300 cells.

2.5. Isolation and electrophoresis of fragmented DNA

Cells were harvested, washed with PBS and pelleted by centrifugation. Cell pellets were then treated for 10 s with lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5), 10 μl per 10^6 cells. After centrifugation for 5 min at 1600 × *g*, supernatants were collected and the extraction was repeated with the same amount of lysis buffer. Fragmented DNA fraction was prepared according Herman et al., 1994. The DNA fragments were analysed by 1.5% agarose gel electrophoresis and visualised by UV illumination. DNA marker of 123 bp was used as a molecular weight standard (Sigma).

2.6. Preparation of nuclear extracts

Cells (5×10^6 to 5×10^7) were harvested and pelleted at 500 × *g* for 6 min, then washed twice in ice cold PBS and lysed in Nuclei EZ lysis buffer (Sigma) for 5 min on ice. After centrifugation at 500 × *g* for 5 min at 4 °C, nuclei were washed in the same cold buffer, vortexed briefly and set on ice for 5 min. Nuclei were pelleted at 500 × *g* for 5 min, then completely suspended in Nuclei EZ storage buffer (Sigma) and frozen at −70 °C. Nuclear protein extracts for electrophoretic mobility shift assay (EMSA) were prepared by lysis of nuclei in buffer (20 mM Tris–HCl, pH 8.0, 200 μM EDTA, 2 mM EGTA, 20% glycerol, 400 mM NaCl) containing 3 mM DTT, 1 mM PMSF and 1× protease inhibitor cocktail P2714 (Sigma). After incubation for 1 h on ice, the extracts were centrifuged at 18,000 × *g* for 20 min, and used immediately. Protein concentrations were determined by RCDC protein assay as recommended by the manufacturer (Bio-Rad).

2.7. Oligonucleotides

Te probes used were synthetic oligonucleotides (MWG-Biotech AG, Sweden) representing bind-

ing sites: (5′-AGTTGAGGGGACTTTCCCAGGC-3′) consensus NFκB; (5′-AAGCCTGG GCAACATAGA-AAGTCCCCATCTGTACAAAAA-3′) NFκB from the FasL promoter; (5′-GCCTGGGCCCCGGGAGG-GCGGTCCCGGGCGGCGC-3′) Sp1 (elements 1 and 2) from the p21 promoter; (5′-ATCAGAAAATTG-TGGGCGGAAACTTCCAGG-3′) Sp1 from the FasL promoter; (5′-TTTGGGGCTCGAGGTCCCG-3′) mutated Sp1 (element 1) from the p21 promoter.

2.8. Electrophoretic mobility shift assay

Complementary oligonucleotides were annealed and labeled at their 5′ ends using [γ-^{32}P-ATP] (Amersham, UK) and T4 polynucleotide kinase (MBI Fermentas Inc., Vilnius, Lithuania). Standard DNA reactions were performed with 12–15 μg nuclear extracts in a 20 μl of reaction buffer (10 mM HEPES, pH 7.9, 3 mM $MgCl_2$, 0.1 mM EDTA, 40 mM NaCl, 10% glycerol) containing 2 μg BSA, 1 μg poly(dI-dC), 1 pM labeled oligonucleotide for 30 min at room temperature. When desired, unlabeled competitor oligonucleotide was added to protein extracts at 50- or 100-fold molar excess for 15 min preincubation. DNA-protein complexes were resolved on 6% polyacrylamide gel containing 1× Tris–borate buffer. After electrophoresis, gels were dried and then exposed to X-ray films.

3. Results

3.1. Sp1 and NFκB binding sites in the p21 and the FasL promoters of leukemia cells

To assess whether the putative binding sites in the p21 promoter might bind to Sp1 transcription factors, we chose to analyze the p21 promoter region between nucleotides −135/−101 using EMSA. It includes elements 1 and 2 (Sp1-1 and 2) containing GC-rich motifs (Koutsodontis et al., 2002). As shown in Fig. 1A, incubation of RA-stimulated NB4 cell nuclear extracts with the Sp1-1 and 2 probe revealed the formation of three specific protein-DNA complexes. Two complexes closely resemble the previously observed Sp1 and Sp3 using specific antibodies for these proteins (Koutsodontis et al., 2002). Binding of Sp1 to the p21 promoter was efficiently competed by a 50-fold molar excess of unlabeled oligonucleotide (cold). The band

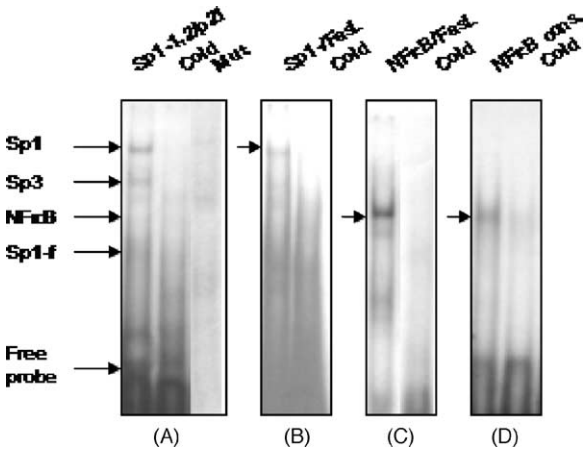

Fig. 1. Sp1 and NFκB binding to the p21 and the FasL promoters of myeloid origin. EMSA was performed using nuclear extracts from 2h RA-activated NB4 cells and a labeled oligonucleotide representing: Sp1 binding sites (A) from the p21 promoter and (B) from the FasL promoter; NFκB binding site (C) from the FasL promoter and (D) consensus NFκB motif. Sp1 and NFκB binding was eliminated competitively by addition of a 50–100-fold molar excess of self, unlabeled competitor (cold) or using a probe containing mutated Sp1 binding site (mut) from the p21 promoter. Arrows indicate DNA complexes with the full length Sp1 and Sp3 proteins, or with faster migrating form of Sp1 protein (Sp1-f), and with NFκB proteins.

specificity was confirmed using oligonucleotide containing mutated Sp1 binding site. Sp1-f, the faster migrating band, is proposed to be Sp1-related protein, being eliminated in the competition assay. Similar results were found using nuclear extracts from HEL, K562 and THP-1 cell lines (Figs. 3, 7 and 8).

To determine if Sp1 binds specifically to the FasL promoter, we used a nucleotide recognition element for Sp1, located in the FasL promoter between −296/−265 bp relative to the transcription start site. The nuclear extracts from myeloid cell lines consistently produced three DNA-protein complexes (Fig. 1B). Two slowly migrating bands correspond to the position of full length Sp1 and Sp3 by its electrophoretic mobility and molecular weight, as determined by Rao, Zhang, Donnelly, Spector, & Studzinski, 1998. The faster migrating band (Sp1-f) can likely be attributed to truncated form or splicing isoform of Sp1 (Rao et al., 1998). The upper complexes appear to be eliminated competitively by addition of unlabeled Sp1/FasL probe, whereas only weak competition was observed in binding of the third (Sp1-f) complex.

To test the role of NFκB, we used a nucleotide corresponding to a putative atypical NFκB binding sequence from the FasL promoter (Israel et al., 1989). As shown in Fig. 1C, nuclear extracts from leukemic cells formed one DNA–protein complex with this probe, as for the probe possessing a consensus NFκB motif (Fig. 1D). The nuclear proteins may represent a complex of p50 and p65 subunits of NFκB according to its electrophoretic mobility. The binding was abolished by corresponding unlabeled oligonucleotides.

3.2. Sp1 and NFκB binding activity to the p21 and NFκB promoters during leukemia cell differentiation and leading apoptosis

To begin investigating a possible role of Sp1 and NFκB in programmed cell death of terminally differentiated cells, we compared differentiation and subsequent apoptosis in different leukemia cell lines: HL-60, NB4 and HEL. The promyelocytic leukemia cells NB4 and HL-60 differ in some characteristics: NB4 cell line has a *t*(15;17) translocation associated with the APL, whereas HL-60 cells lack this chromosomal translocation and p53 (Collins, 1987). Both cell lines showed granulocytic differentiation after treatment with 1 μM RA. HL-60 cells were stained positively with NBT on day 5 (about 60%), while NB4 cells reached this level on day 3 (Fig. 2C). The staining of the cells with EtBr/AO revealed induction of apoptosis, increasing from 3–5% (spontaneous) to more than 40% in HL-60 and around 30% in NB4 cell population on day 5 (Fig. 2C). EMSA revealed that RA treatment caused a time-dependent decrease in Sp1 binding to the p21 and the FasL promoters in NB4 cells (Fig. 2A). Two bands, which competed with unlabeled probe, were visible in untreated NB4 cells (Fig. 2A) representing full length Sp1 and Sp3. The formation of the third, faster migrating complex (Sp1-f) was noticed in untreated and RA-treated NB4 cells. However, Sp1-s complex was more prominent after day 1 of RA-treatment and gradually decreased during NB4 cell differentiation. Sp1 binding to the FasL promoter slightly decreased only in the cells undergoing apoptosis (Fig. 2A). NB4 cell differentiation process was associated with a gradual increase in NFκB binding activity to consensus motif and with sustained affinity for the FasL promoter. The binding reduction to these sites was accompanied by the increase in apoptosis on day 5 (Fig. 2B). Using nuclear extracts from proliferating and differentiating HL-60 cells, we did not find any interaction of full length Sp1 with the p21 or the FasL promoters (Fig. 2A). By contrast, Sp1-f binding to the FasL promoter was detected in control and RA-induced HL-60 cells at the commitment stage only. In RA-treated both cell lines, similar effects were observed for the association of NFκB to consensus motif and to the FasL promoter (Fig. 2B). Thus, the down-regulation of Sp1 and NFκB binding activities was associated with apoptosis following long-term RA-treatment, most likely without the involvement of the Fas/FasL pathway.

To examine whether this is a general phenomenon for leukemic cell differentiation, we performed EMSA using extracts from human erythroleukemia HEL cells induced to differentiate to erythrocytes by 60 μM hemin. A typical HEL cell population contained, on day 6, about 80% of differentiated cells and 20% of cells undergoing apoptosis (Fig. 3C). Furthermore, EMSA demonstrated a gradual decrease in Sp1 and NFκB binding to the p21 and FasL promoters during cell differentiation (Fig. 3A and B). The mobility of complexes was the same as in other cell lines but bands corresponding to these complexes were prominent on day 6. This could reflect a lower proportion of cells undergoing apoptosis at the same time-point, as compared to more apoptosis sensitive HL-60 and NB4 cells.

Thus, our observations demonstrate a positive role of Sp1 and NFκB in the differentiation process and a negative one in apoptosis of terminally differentiated cells of myeloid or erythroid origin.

3.3. The loss of Sp1 and NFκB binding activity to the p21 and the FasL promoters during etoposide-induced leukemia cell apoptosis

To further characterize the influence of DNA damage on NFκB and Sp1 activity, we treated promyelocytic leukemia HL-60 cells having a null-p53 phenotype, or p53-carrying NB4 cells with etoposide (68 μM) for different times. In NB4 cells, the drug caused Sp1 and NFκB binding to the p21 and the FasL promoters during 3 h of treatment, as well as drastic decrease at 6–18 h (Fig. 4A and B). To inhibit caspases in intact cells, we employed the irreversible, cell-permeable, the broad-spectrum caspase inhibitor z-VAD.fmk (25 μM). The time of the inhibitor addition was chosen to yield maximum changes in transcription

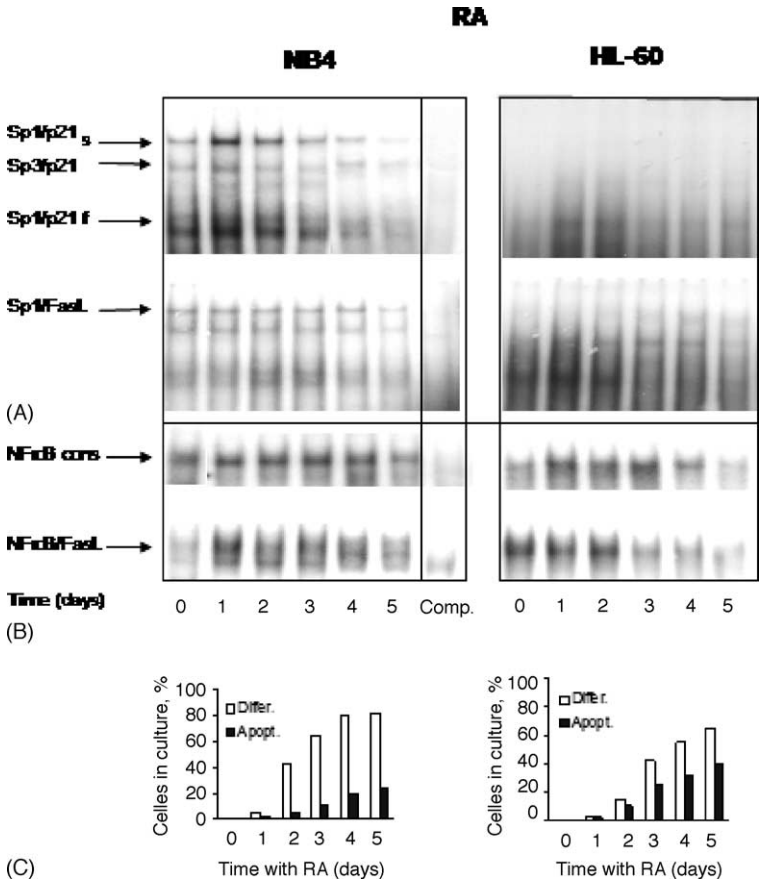

Fig. 2. Sp1 and NFκB binding activity during granulocytic differentiation and apoptosis. Nuclear extracts were prepared from control or 1 μM RA-treated (A) NB4 and (B) HL-60 cells. EMSA was performed using a total 12–15 μg protein from each nuclear extract and Sp1 probe, containing binding site (Sp1-1 and 2) from the p21 or the FasL promoter, and NFκB probe harboring a consensus NFκB motif, or the FasL binding site. Arrows indicate specific bands, the decrease of which is shown by competition (comp.) analysis. (C) Time course of HL-60 and NB4 cell differentiation and apoptosis after treatment with RA. Results are from one representative experiment of three.

factors activity between 6 and 18 h of etoposide treatment. As expected, z-VAD.fmk produced a marked inhibitory effect and restored transcription factors binding activity seen at 6–18 h (Fig. 4A and B) confirming caspase-dependent events. Identical results were obtained with extracts from etoposide-treated HL-60 cells (Fig. 5A and B). There was a strong relationship between the changes in transcription factors binding activity and the number of apoptotic cells (Fig. 5D), the appearance of internucleosomal fragmentation at 6–18 h and the absence of the ladders using z-VAD.fmk (Fig. 5C).

Similar results with a decrease in Sp1 binding to the p21 promoter or NFκB to consensus motif were obtained using etoposide-treated HEL cell extracts (Fig. 6). However, in these cells NFκB binding capacity to the FasL promoter was detected after 18 h exposure to the drug (Fig. 6B), suggesting that, in HEL cells, NFκB might participate in DNA damage-induced FasL-mediated apoptosis.

K562 cell line is normally resistant to the induction of apoptosis by a number of agents (McGahon et al., 1994). We thus investigated whether etoposide was able to trigger cell death in K562 cells via NFκB or Sp1. Fig. 7 demonstrates an inducible formation of NFκB binding complexes with consensus motif and the FasL promoter within 18 h. There was a decrease at 56 h, likely to be due to an augmented number of

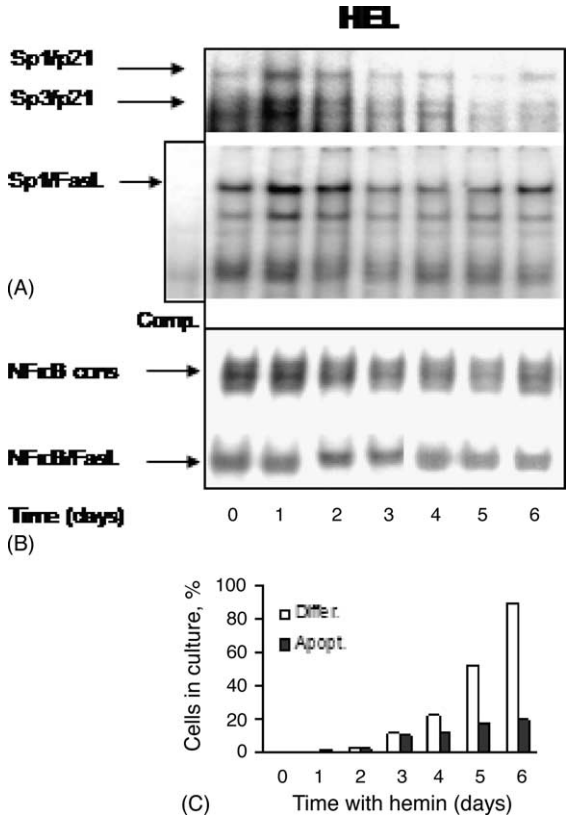

Fig. 3. Sp1 and NFκB activity during hemin-induced HEL cell differentiation and apoptosis. Nuclear extracts were prepared from control or 60 μM hemin-treated cells. EMSA was performed by using a total 12–15 μg of protein from each nuclear extract and (A) Sp1 probe, containing binding site (Sp1-1 and 2) from the p21 or the FasL promoter and (B) NFκB probe, harboring a consensus NFκB motif or the FasL binding site. Arrows indicate specific bands. (C) Time course of HEL cell differentiation and apoptosis after treatment with hemin. Results are from a representative experiment of three.

apoptotic cells. Similar results were obtained for the acute monocytic leukemia THP-1 cells (Fig. 8). In these cells, the delay of etoposide-induced apoptosis and a corresponding decrease in Sp1 or NFκB binding activities occurred at 36 h of treatment (Fig. 8A and B). This is earlier than in K562, but later than in NB4 cells.

In summary, the results of our study explain the requirement of activation of p21 (Waf1/Cip1) and FasL genes for cell survival but not for DNA damage-induced cell death. It involves Sp1 and NFκB as regulators of these genes, thereby suggesting a common mechanism of cellular response to diverse apoptotic effectors (RA or etoposide) in malignant hematopoietic cell lines.

4. Discussion

In this study, we have investigated whether transcription factors, Sp1 and NFκB are connected to apoptosis via the engagement of p21 and FasL genes in leukemia cell lines with different status of p53 and Fas receptor. It is widely expected that the Fas/FasL pathway is involved in apoptotic cell death in many cell types. Many hematopoietic cell lines express cell surface protein Fas (Kim et al., 2000). Cell lines not expressing Fas (K562) or expressing it at moderate (HL-60) or low (THP-1) levels have been found to be resistant to Fas/FasL-mediated apoptosis (Dirks et al., 1997; Kim et al., 2000). Current existing data concerning Fas signal regulation during differentiation leading to apoptosis is controversial (Kikuchi et al., 1996; Ohashi et al., 2000). In our study, we found that terminal differentiation leads to the induction of apoptosis in cells of myeloid (HL-60, NB4) and erythroid (HEL) origin. This correlated positively with a significant decrease in the binding capacity of Sp1 and NFκB to the FasL promoter. Our results are in agreement with those of others, demonstrating a reduction in both Fas and FasL in RA-treated promyelocytic HL-60, monocytic U-937 cells or APL cells from patients, and indicating that differentiation leading apoptosis through Fas is unlikely to play a major role in these cells (Kikuchi et al., 1996; Salih et al., 2002). If granulocytic differentiation is mainly regulated through retinoic acid receptors of the RAR type, the apoptotic process in HL-60 cells is modulated by activation of retinoid receptor RXR and pro-caspases (Nagy et al., 1995; Watson et al., 1997). It is known that NB4 cells undergo paracrine programmed cell death through the functional death ligand TRAIL and up-regulation of pro-caspase genes upon treatment with RA (Altucci et al., 2001).

A number of studies have suggested that anticancer drugs up-regulate FasL expression and initiate Fas/FasL-mediated signal transduction, activation of caspases and apoptotic cell death (Villunger et al., 1997). Presently, little is known about factors that link the ability of etoposide to induce DNA damage and FasL activation. By contrast, some reports have proposed that the induction of apoptosis by this drug is not

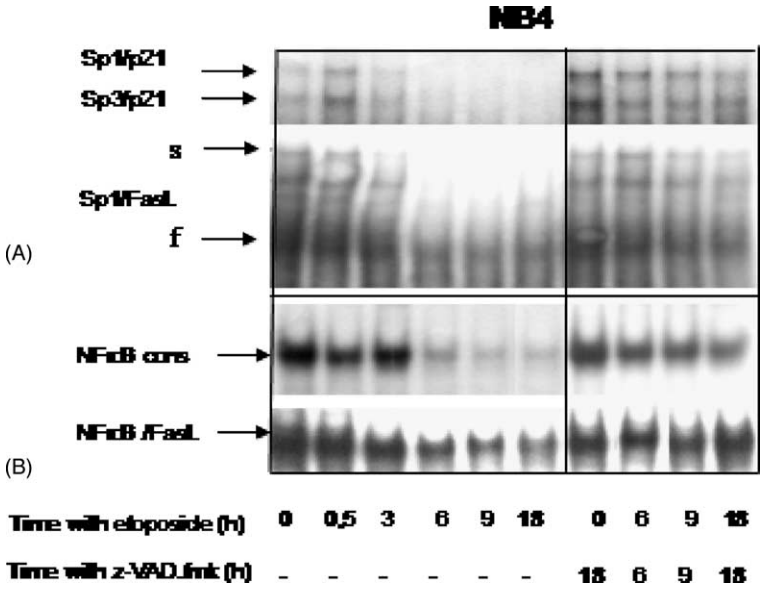

Fig. 4. The loss of Sp1 (A) and NFκB (B) binding activity during etoposide-induced apoptosis and the recovery by caspase inhibitor z-VAD.fmk in NB4 cells. Nuclear extracts were prepared after exposure to 68 μM etoposide in the absence or the presence of 25 μM z-VAD.fmk for time period as indicated and analyzed by EMSA. Arrows indicate specific bands. Results are from a representative experiment of two.

mediated via the Fas pathway in lymphoid and myeloid cells (McGahon, Costa Pereira, Daly, & Cotter, 1998; Siitonen et al., 2000). Our data shows that etoposide-mediated apoptosis is likely to be independent of the cell Fas status. At first, we have observed that untreated cell lines, non-expressing (K562) and low-expressing (THP-1) Fas have relatively high Sp1 and NFκB binding activities to the FasL promoter. Later, decreases in these activities are associated with delayed apoptosis in those cell lines. Thus, our observations are consistent with the idea that the recruitment of Fas/FasL system is not necessary for etoposide-induced apoptosis. The death process of etoposide-sensitive cells, such as HL-60 and U937, has been shown to up-regulate Casp-2 and Casp-3 genes associated with enhanced synthesis of related procaspases (Droin et al., 1998). The permanent peptide z-VAD.fmk, which is homologues to sequences targeted by ICE-like caspases, efficiently suppresses Casp gene expression (Droin et al., 1998), thereby preventing apoptotic DNA fragmentation.

K562 cell resistance to the induction of apoptosis is attributed to the activity of $p210^{bcr\text{-}abl}$ tyrosine kinase encoded by the Bcr-Abl fusion gene and is associated with a delayed activation of procaspase-3 and target protein cleavage in response to DNA damage (Dubrez et al., 1998). However, multiple Fas-resistant cell lines (such as K562) remain sensitive to chemotherapy-induced apoptosis (Eischen et al., 1997). Interestingly, Fas resistance could be overcome by cycloheximide in some Fas-resistant or etoposide-resistant cell lines (Kim et al., 2000; Siitonen et al., 2000), suggesting the presence of short-lived apoptosis-inhibitory proteins (FLIP, RIP, XIAP, cIAP2) (Fulda, Meyer, & Debatin, 2000; Willems et al., 2000) that can account for the deficient functioning of the Fas system in those cells. Moreover, etoposide-induced, Fas-independent and Fas-mediated apoptosis lead to the activation of caspase-3 in many cell lines and the late stages of apoptosis after such treatments proceed through a common caspase pathway (Eischen et al., 1997; Zhuang & Cohen, 1998).

Topoisomerase poisons, including etoposide, activate NFκB and induce apoptosis (Bessho et al., 1996; Piret & Piette, 1996). In our study, NFκB binding activity to consensus motif and to the FasL promoter occurred during early hours of etoposide treatment. The decrease was associated with cell death in myeloid cell lines. No decrease in NFκB binding to the FasL promoter in HEL cells was observed during 18 h of etoposide treatment, suggesting a long lasting NFκB

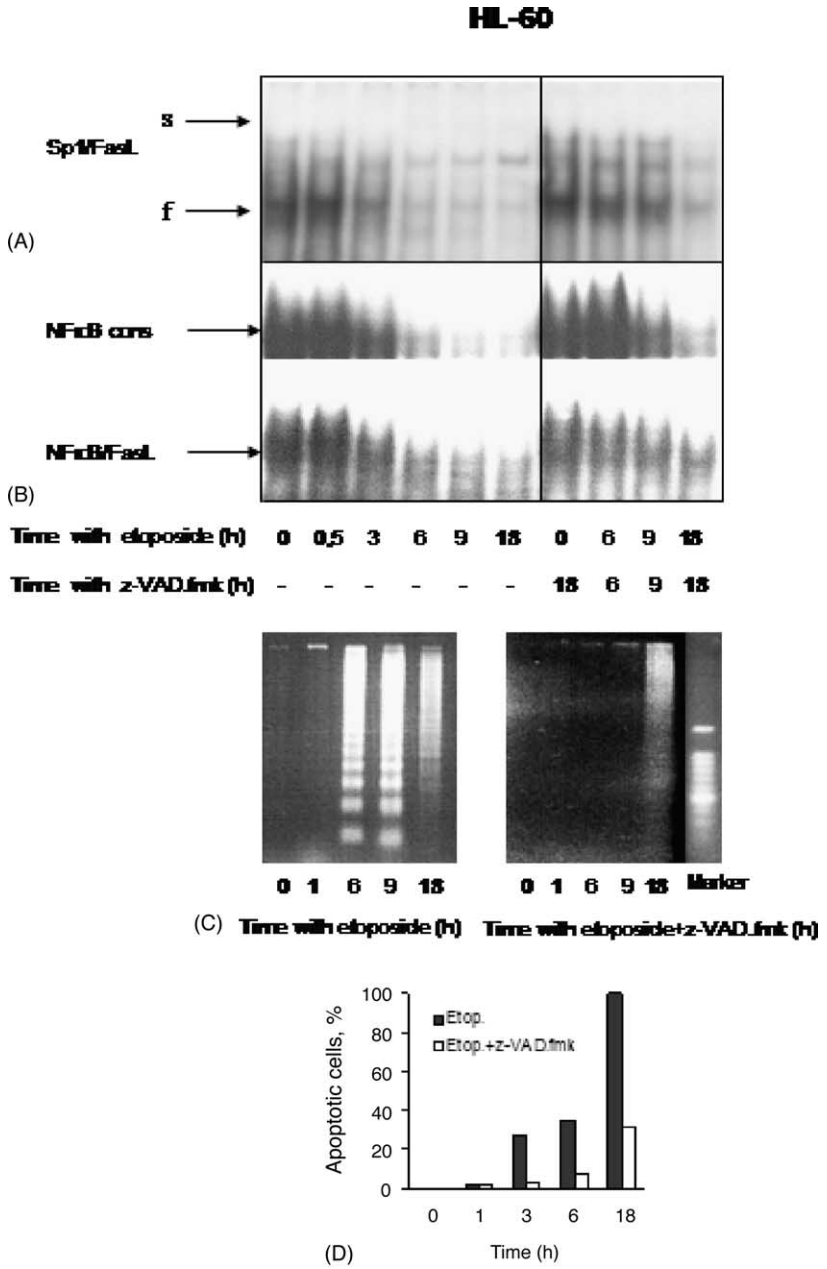

Fig. 5. The loss of Sp1 (A) and NFκB (B) binding activity during etoposide-induced apoptosis and the recovery by caspase inhibitor z-VAD.fmk in HL-60 cells. Nuclear extracts were prepared after exposure to 68 μM etoposide in the absence or the presence of 25 μM z-VAD.fmk for time period as indicated and analyzed by EMSA. Arrows indicate specific bands.(C) Effect of caspase inhibitor z-VAD.fmk on etoposide-induced DNA fragmentation of HL-60 cells. Etoposide-induced cells were treated without or with z-VAD.fmk for 6 h. Cells were lysed and DNA extracted for electrophoresis in 1.5% agarose gel. (D) Time course of etoposide-induced apoptoside in HL-60 cells. Results are from one representative experiment of two.

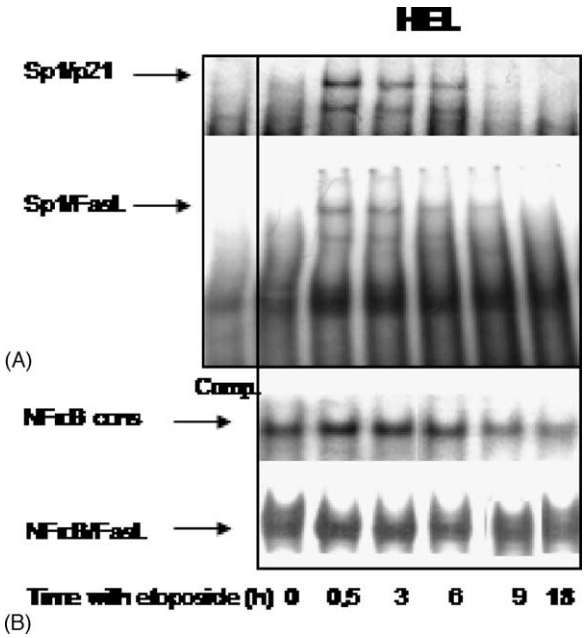

Fig. 6. Kinetics of Sp1 (A) and NFκB (B) binding activity to the p21 or the FasL promoters in HEL cells. Nuclear extracts were prepared after exposure to 68 μM etoposide for time period as indicated and analyzed by EMSA. Arrows indicate specific bands. Results are from a representative experiment of three.

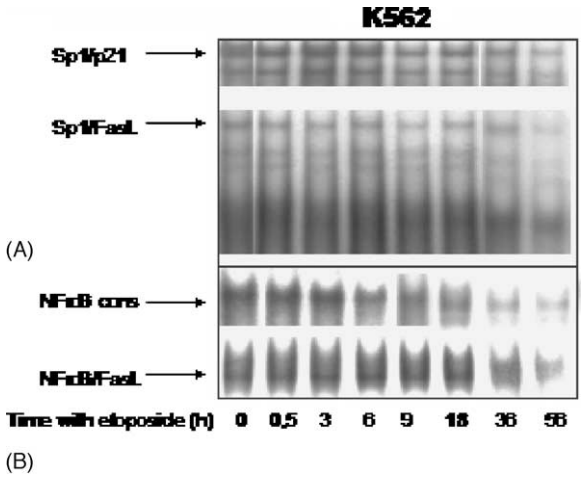

Fig. 7. Kinetics of etoposide-mediated Sp1 (A) and NFκB (B) binding activity to the p21 or the FasL promoters in K562 cells. Nuclear extracts were prepared after exposure to 68 μM etoposide for time period as indicated and analyzed by EMSA. Arrows indicate specific bands. Results are from one representative experiment of two.

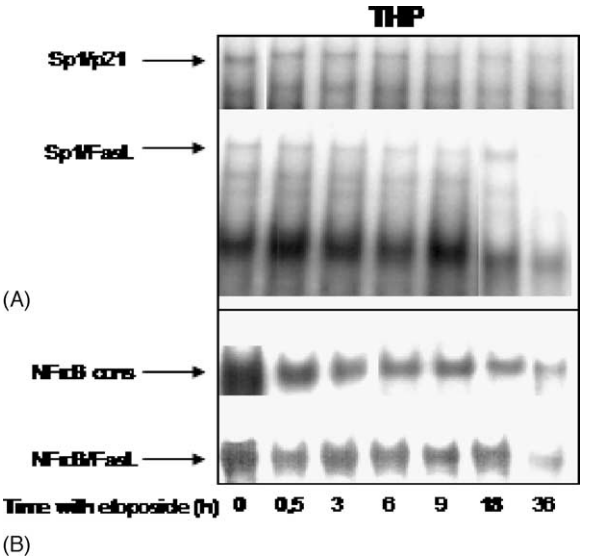

Fig. 8. Kinetics of etoposide-mediated Sp1 (A) and NFκB (B) binding activity to the p21 or the FasL promoters in THP-1 cells. Nuclear extracts were prepared after exposure to 68 μM etoposide for time period as indicated and analyzed by EMSA. Arrows indicate specific bands. Results are from one representative experiment of two.

activation of FasL in HEL cells undergoing apoptosis. Our observation that NFκB activity is significant for RA-induced NB4 and HL-60 cell differentiation is consistent with our earlier report (Navakauskiene, Kulyte, Treigyte, Gineitis, & Magnusson, 2003), indicating elevated nuclear levels of NFκB p65 and p50 proteins during granulocytic differentiation.

The present study demonstrates the absence of a slowly migrating band (Sp1-s) in both untreated and RA-treated HL-60 cell extracts. This may be explained by the fact that in untreated HL-60 cells, the full length 105 kDa Sp1 protein cannot be detected (Rao et al., 1998). Sp1 protein exists in a truncated, approximately 30 kDa fragment, which is able to bind to the GC-rich elements in DNA (Rao et al., 1998). This coincides with the presence of a serine protease, myeloblastin, in undifferentiated HL-60 cells and with a down-regulation of its expression by differentiating agents (Bories, Raynai, Solomon, Darzynkiewitz, & Coyre, 1989). By contrast, in undifferentiated NB4 cell extracts we found Sp1-s band, the intensity of which decreases during RA-induced apoptosis. In fact, a lower level of Sp1 protein and the appearance of the truncated Sp1 form were observed during retinoid-induced

T-cell apoptotic process (Piedrafita & Pfahl, 1997). Interestingly, apoptosis did not require de novo mRNA and protein synthesis, suggesting that cell death is a transcription-independent and caspase-dependent event. It is likely that Sp1 is cleaved during apoptosis with a loss of DNA binding activity and further inability to activate transcription. This has been demonstrated in IgM-induced B-cell apoptosis (Rickers et al., 1999) as well.

p21 (Waf1/Cip1) has been shown to inhibit cell proliferation, induce cell cycle arrest in G1, and may also mediate G2 arrest that is characteristic for apoptotic process (Dulic, Stein, Far, & Reed, 1998; Harper & Elledge, 1996). For example, the G1 to S transition at the commitment period and G1 block requirement for the differentiated phenotype formation has been shown in RA-induced NB4 cells (Casini & Pelicci, 1999). These events were accompanied by an induction of two peaks of p21 synthesis, one early (2–6 h) and one late (3–4 days). We have observed Sp1 binding activation to the p21 promoter during 2–3 days of NB4 and HEL cell differentiation, suggesting the role of Sp1 in p21 induction. This may occur by increased Sp1 protein expression and interaction with the promoter or changes in their post-translational modifications (Biggs, Kudlow, & Kraft, 1996; Gartel et al., 2000; Lania et al., 1997).

In some systems, p21 has been found to either protect cells from apoptosis or to promote apoptosis (Duttaroy, Qian, Smith, & Wang, 1997; Gartel & Tyner, 1999; Gorospe et al., 1997). In cell lines containing wild-type p53, DNA-topoisomerase drugs rapidly trigger p53 protein elevations (Nelson & Kastan, 1994), suggesting p21 involvement in apoptosis via p53-dependent mechanisms. Down-regulation of p21 expression by subsequent cleavage could, in part, contribute to the induction of apoptosis too. Since HL-60 cells have a null-p53 phenotype, p21 induction occurs during initiation of differentiation to granulocytes in a p53-independent manner (Jiang et al., 1994). This type of p21 transcription activation may occur by the involvement of different transcription factors, including for instance Sp1 and Sp3 (El-Deiry et al., 1994). Herein, we demonstrated Sp1 binding activity to the p21 promoter in differentiating cells and significant decrease in those triggered to apoptotic death by diverse stimuli irrespective of p53 gene status.

In summary, the results of the present study indicate the positive involvement of Sp1 and NFκB transcription factors in differentiation process and the negative one in apoptosis, acting on p21 and FasL genes via a common way in malignant hematopoietic cell lines.

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