Characterization of NF-κB Expression in Hodgkin's Disease: Inhibition of Constitutively Expressed NF-κB Results in Spontaneous Caspase-Independent Apoptosis in Hodgkin and Reed-Sternberg Cells

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Although the neoplastic cells of classical Hodgkin's disease (CHD) demonstrate high levels of constitutively active nuclear NF-κB, the precise physiologic and clinical significance of NF-κB expression is currently undefined. Expression of active NF-κB p65(Rel A) was evaluated in patient samples of CHD and nodular lymphocyte predominance Hodgkin's disease. The action of the chemical NF-κB inhibitors gliotoxin and MG132 and the effect of NF-κB inhibition utilizing an adenovirus vector carrying a dominant-negative IκBα mutant (Ad5IκB) were then demonstrated in CHD cell lines (L428, KMH2, and HS445). Hodgkin and Reed-Sternberg (HRS) cells from all patient and cell line specimens showed strong immunopositivity for active p65(Rel A). Expression was also seen in lymphocytic/histiocytic cells from all cases of nodular lymphocyte predominance Hodgkin's disease. After chemical NF-κB inhibition, p65(Rel A) was significantly reduced in nuclear extracts from cultured HRS cells as revealed by electrophoretic mobility shift assays. Furthermore, chemical NF-κB inhibition resulted in time- and concentration-dependent apoptosis in HRS cells. With the exception of MG132-induced apoptosis in HS445, apoptosis by chemical NF-κB inhibition was not significantly altered by preincubation with various caspase inhibitors (z-DQMD-FMK, z-DEVD-FMK, z-VAD-FMK, z-VEID-FMK, and z-IETD-FMK). Regardless of the chemical inhibitor used, no significant change in caspase-3 functional activity was found in CHD cell lines. HRS cells infected with Ad5IκB also showed a marked increase in spontaneous apoptosis compared with wild type adenovirus-infected and control cells. Overall, the inhibition of active NF-κB in HRS cells resulting in spontaneous caspase-independent apoptosis demonstrates a critical role for NF-κB in HRS cell survival and resistance to apoptosis.

KEY WORDS: Rel/NF-κB transcription factors, Ubiquitin-proteasome, MG132, Gliotoxin, p65(Rel A).

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Classical Hodgkin's disease (CHD) is a malignant disorder characterized morphologically by the presence of neoplastic Hodgkin and Reed-Sternberg (HRS) cells surrounded by a polymorphous stromal background comprised of lymphocytes, histiocytes, plasma cells, and eosinophils. Despite the apparent heterogeneity in studies addressing the origin and clonality of HRS cells, the histopathologic and clinical features of CHD consistently correlate well with the deregulated, high expression of various cytokines, growth factors, and cell surface receptors typical for HRS cells (1). HRS cells characteristically express CD30, a cell surface marker also found on activated B and T cells, CD40, and several cytokine receptors such as CD71, the interleukin-2 receptors p55 (CD25) and p75, and the interleukin-6 receptor (2–8). In addition to the expression of these cell surface receptors, HRS cells are able to produce several cytokines including tumor necrosis factor-α (TNF-α), lymphotoxin-α (LTα), and interleukin-1 (IL-1) (5, 9). Stimulation of TNF-α, LTα, IL-1, CD30,

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and CD40 receptors by their respective ligands typically results in activation of the transcription factor NF-κB (6, 10).

The NF-κB family of dimeric transcription factors is an important regulator of genes expressed during inflammatory responses, Ig class switching, cellular differentiation, and apoptosis. The activated NF-κB prototype typically consist of a heterodimer composed of p50 and p65 (Rel A). Other family members include Rel B, c-rel, v-rel, and p52. With the exception of mature B lymphocytes and plasmacytomas, inactive NF-κB is sequestered in the cytoplasm of most cell types by noncovalent interactions with a family of inhibitory proteins called IκB that mask the nuclear localization domain of Rel proteins (11–13). Of the seven IκB proteins known to date, the roles of only IκBα and IκBβ have been described in detail (14). After cellular stimulation by a number of agents including TNF-α and IL-1, NF-κB is activated by signal induced, site-specific phosphorylation and rapid degradation of IκB, mediated by the ubiquitin-proteasome pathway (15, 16). This process allows for release of the active NF-κB complex, which translocates to the nucleus where it plays an important role in the transcriptional activation of a large number of inducible target genes, including growth-promoting cytokines (17). As both the degradation of the NF-κB inhibitor IκBα and the processing of the p105 precursor of p50 are controlled by the ubiquitin-proteasome pathway, chemical inhibition of the proteasome results in NF-κB inhibition (16, 18). More specifically, both MG132 and gliotoxin have been shown to specifically prevent proteasome-mediated degradation of IκBα, resulting in NF-κB inhibition in other experimental models (16, 18, 19).

Several investigations have suggested a role for NF-κB and IκB gene products in cell proliferation, transformation, and tumor development (20, 21). Associations between members of the NF-κB and IκB gene families and various lymphoid neoplasms have been described including c-rel amplification in follicular and diffuse large cell lymphoma (22, 23), gene rearrangement and point mutations of Rel A in multiple myeloma (24, 25), and bcl-3 rearrangement in B-cell chronic lymphocytic leukemia (CLL), and non-Hodgkin's lymphoma (26). Importantly, recent investigations of CHD have shown that a common feature of HRS cells is the expression of high levels of constitutively active NF-κB p50-Rel A (9, 27, 28). Furthermore, the constitutive expression of NF-κB has been shown to play a vital role for proliferation and resistance to apoptosis of HRS cells (27, 28). Previous investigations have also shown an important role for NF-κB gene family members in regards to apoptosis resistance in other cell types. In fibroblasts, NF-κB demonstrated a protective role against TNF-α-induced apoptosis as has been shown with cells from Rel-A deficient mice (29). Furthermore, Jurkat and fibroblastic cells showed enhanced apoptosis after exposure to TNF-α, ionizing radiation, or daunorubicin when nuclear translocation of NF-κB was inhibited (30–32). Similarly, down-regulation of c-rel activity in the murine B-cell lymphoma line, WEHI 231 resulted in spontaneous apoptosis (33).

In this investigation, we demonstrate that chemical inhibition of the ubiquitin-proteasome pathway results in both significantly reduced levels of activated NF-κB and markedly increased apoptosis in CHD cell lines. We also show that both CHD and nodular lymphocyte predominance Hodgkin's disease (NLPHD) have active NF-κB p65(Rel A), as demonstrated by immunohistochemistry in HRS and lymphocytic/histiocytic (L&H) cells, respectively. These findings suggest an important role for NF-κB in the maintenance of cell growth and survival of HRS cells. Previously, we determined a critical role for caspase-3 in apoptosis induced by stimulation of CD95(fas), which is a very important mediator of apoptosis in CHD (34). Here we demonstrate the lack of inducible increased caspase-3 functional activity and the failure of the various caspase inhibitors to alter the apoptotic effect caused by chemical proteasome inhibition. These findings show that apoptosis resulting from decreased nuclear NF-κB expression via proteasome inhibition occurs independent of caspase-mediated mechanisms in CHD cell lines.

MATERIALS AND METHODS

Patient Samples

Formalin-fixed paraffin-embedded tissue sections from 16 cases of CHD (10 nodular sclerosis subtype; six mixed cellularity subtype) and six cases of NLPHD were selected from the surgical pathology files of Loyola University Medical Center, Maywood, IL and the University of Michigan, Ann Arbor, MI for immunohistochemical determination of active p65(Rel A).

Cell Culture

The CHD cell lines KMH2, L428, and HS445 were utilized in this study. KMH2 and L428 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). HS445 and Jurkat cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cell lines were cultured in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO),

1 mM sodium pyruvate (Biofluids, Inc., Rockville, MD), 5.5 $\times$ 10^{-5} M 2-mercaptoethanol (GIBCO-BRL), 2 mM L-glutamine (GIBCO-BRL), 25 mM Hepes (Sigma), and antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, Sigma). All cell lines were maintained at 37°C in a humidified incubator at 5% CO_2.

Ubiquitin Proteasome and Caspase Inhibitors

For evaluation of apoptosis due to NF-κB inhibition and its relationship to the caspase pathway, 1 $\times$ 10^6/mL cells from each cell line were cultured in 24-well tissue culture plates (Falcon, Lincoln Park, NJ) and incubated with either 10 μM z-Leu-Leu-Leu-aldehyde (MG132, Biomol Research Laboratories, Inc., Plymouth Meeting, PA), 10 μM gliotoxin (Calbiochem, La Jolla, CA) or 500 ng/mL of agonistic anti-CD95 monoclonal antibody (mAb) (clone CH11, mouse IgM, Upstate Biotechnology, Inc., Lake Placid, NY) for indicated time periods. Each reaction was performed with or without 1 hour preincubation with 100 μM concentration of one of the following peptide caspase inhibitors: z-Asp-Glu-Val-Asp-fluromethyl ketone (caspase-3 and-7 inhibitor z-DEVD-FMK, Enzyme Product Systems [EPS], Livermore, CA), z-D(O-Me)-M-Q-D(O-Me)-fluromethyl ketone (caspase-3 inhibitor z-DMQD-FMK, EPS), z-Val-Ala-Asp-fluromethyl ketone (caspase-1 inhibitor z-VAD-FMK, EPS), z-Val-Glu-Ile-Asp-fluromethyl ketone (Caspase-6 inhibitor z-VEID-FMK, EPS), z-Ile-Glu-(O-Me)-Thr-(O-Me)-Asp-fluromethyl ketone (caspase-8 inhibitor z-IETD-FMK, EPS). All reactions were performed at least in duplicate.

Immunohistochemical Determination of p65(Rel A)

Cytospins from CHD cell lines were fixed in a 1:1 mixture of acetone and methanol for 10 minutes. Staining was performed using a monoclonal mouse anti-human p65 (clone 12H11, 1:75 titer, Boehringer Mannheim Corporation, Indianapolis, IN), which recognizes only the unbound, active form of p65(Rel A) dissociated from IκBα (35) and a secondary polyclonal goat anti-mouse IgG/IgM antibody conjugated to fluorescein isothiocyanate (Biosource International, Camarillo, CA). Analysis of patient material for expression of active p65(Rel A) was performed using a Ventana NEXES automated stainer (Ventana Medical Systems, Tucson, AZ) and streptavidin/horseradish peroxidase detection kit (DAKO, Carpinteria, CA). The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride. Cases were considered positive if greater than 50% of HRS cells (or L&H cells in NLPHD cases) showed immunopositivity for p65(Rel A). All staining was performed at least twice with isotypic controls.

Detection of Apoptosis

For propidium iodide (PI) staining, treated and untreated cells (1 $\times$ 10^6 cells/mL) were washed in cold FACS buffer (500 mL PBS without Mg^{2+} or Ca^{2+}, 2% sodium azide/ deionized water and 5% [vol/vol] heat-inactivated fetal bovine serum [Sigma]) and resuspended in a 1:6 mixture of 100% heat-inactivated fetal bovine serum and absolute ethanol. After 30-minute incubation on ice, the cells were washed once again in FACS buffer. The cell pellet was resuspended and incubated at 37°C with 10 μg/mL RNase (Boehringer Mannheim) for 15 minutes. After brief cooling at room temperature, 100 μg/mL PI was added to each cell suspension followed by overnight incubation at 4°C in the dark. PI fluorescence of individual nuclei from each sample (10,000 events per sample) was analyzed on an Epics XL-MCL flow cytometer (Coulter, Miami Lakes, FL) and expressed on a logarithmic scale as a measure of DNA content using Coulter System II software. Apoptosis was quantitatively measured by PI/cell cycle analysis for the detection of a hypodiploid (sub-G_0) population within the cell suspensions.

The Annexin V/7-Amino Actinomycin D (7-AAD) staining method was performed per manufacturer's protocol using Annexin V-PE (PharMingen, San Diego, CA) and Via-Probe™ 7-AAD (PharMingen). Treated and untreated cells (0.5 $\times$ 10^6 cell/mL) were washed twice in cold PBS and resuspended in 1$\times$ binding buffer (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$). Resuspended cells were then incubated for 20 minutes at 20 to 25°C in the dark with 2 μl Annexin V-PE and 5 μl of 7-AAD. Samples (10,000 events per sample) were then quantitated on a Coulter Epics XL-MCL flow cytometer, recorded in LIST mode, and registered on logarithmic scales. 7-AAD emission was detected in the FL-3 channel (>650 nm). Analysis was performed using Coulter System II software.

Determination of Caspase-3 Activity in CHD Cell Lines Exposed to NF-κB Inhibitors

Caspase-3 activity was determined using the ApoAlert™ CPP32/Caspase-3 colorimetric assay kit (Clontech, Palo Alto, CA). After incubation with MG132 or gliotoxin, duplicate samples of untreated and treated cells (2 $\times$ 10^6 cells) were washed in cold PBS, resuspended in 50 μl Cell Lysis Buffer and incubated on ice for 10 minutes. Cell lysates were pelleted, followed by transfer of the supernatants to microcentrifuge tubes. 50 μl of 2$\times$ Reaction Buffer with 5 mM 1,4 dithiothreitol and 5 μl of 1 mM

DEVD-p-nitroanilide conjugated caspase-3 substrate were added to each tube, followed by 1 hour incubation in a 37°C water bath. For each run, the CD95-sensitive CHD cell lines (L428 and HS445) or Jurkat T cell line were incubated with anti-CD95 mAb, the results of which served as positive controls for caspase-3 functional activity. A control reaction of treated cells without DEVD-p-nitroanilide was included. Optical densities for each specimen were determined at 405 nm using the EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT). For quantification of protease activity, sample values were plotted on a calibration curve derived from the optical density values obtained from each of five standards (range: 0 to 20 nmole p-nitroanilide). For each assay, CPP32 activity were determined by the following formula

$$\text{Units of CPP32 activity} = (\Delta\text{OD}) \times (\text{calib. Curve slope})^{-1} \times (\text{nmole pNA/OD})$$

Where ΔOD = the change in optical density from the control reaction without conjugated substrate.

Determination of Active NF-κB by the Electrophoretic Mobility Shift Assay

Nuclear protein was extracted using the method of Gerder *et al.* (36). Protein content was measured by the Bradford method. A consensus double-stranded NF-κB probe, 5'- AGT TGA GGG GAC TTT CCC AGG C-3' (Integrated DNA Technologies, Inc., Coralville, IA) was end-labeled using γ-^{32}P-adenosine-5-triphosphate. 5 μg of nuclear extract from investigated cells were then incubated in binding buffer consisting of 10 mM HEPES (pH 7.9), 60 mM KCl, 4% ficoll, 1 mM dithiothreitol, 1 mM EDTA (pH 8.0), and 1 μg/mL poly dI:dC (Pharmacia Biotech Inc., Piscataway, NJ) in 20 μl volume. Afterward, the end-labeled probe was added (200,000 cpm). Samples were then incubated for 30 minutes on ice followed by loading on 4% nondenaturing polyacrylamide gel (0.25×Tris-borate-EDTA [TBE]). Electrophoresis was run for 3 hours in 0.25 TBE buffer at 100V. Protein complexes were identified by autoradiography. Hela cells before and after stimulation with TNF-α were used as negative and positive controls, respectively.

Recombinant Adenovirus Ad5IκB

The recombinant replication-deficient adenovirus Ad5IκB and wild type adenovirus (Ad wt) were generous gifts provided by Dr. David A. Brenner, University of North Carolina, Chapel Hill, North Carolina. Construction of the IκBα dominant-negative by contransfection of 293 embryonic human kidney cells with the pACCMV/IκB plasmid and the purified fragment of DNA from E1-deleted adenovirus type 5 (Ad5) was previously described by Graham *et al.* (37, 38).

Viral stocks were propagated in 293 cells, and viral titers were determined by optical densitometry (particles per milliliter). Ad5IκB or Ad wt stock solutions were added to medium containing L428 cells at a ratio of 10,000:1 viral particles to each HRS cell. To determine the *in vitro* apoptotic effect of NF-κB inhibition by Ad5IκB infection of L428, HRS cells were cultured for 96 hours, followed by quantitative measurement of apoptosis by Annexin V staining as described above.

RESULTS

HRS Cells Demonstrate Strong Immunopositivity for Active p65(Rel A)

Immunofluorescence staining of HRS cells from all three cell lines showed strong immunopositivity for nuclear and cytoplasmic expression of p65(Rel A) (Fig. 1, A-B). Similarly, HRS cells from all patient samples also showed strong nuclear and cytoplasmic immunopositivity (12 cases) or predominantly cytoplasmic expression (four cases) of active p65(Rel A) indicating abundant levels of activated NF-κB in HRS cells regardless of subtype (nodular sclerosis or mixed cellularity) (Fig. 1, C-F). Six cases of NLPHD were also analyzed for active p65(Rel A) expression. In all six cases, L&H cells showed predominantly nuclear expression of p65(Rel A) (Fig. 1, G-H).

Ubiquitin Proteasome Inhibition and Ad5IκB Infection Result in Substantial Increases in Apoptosis in CHD Cell Lines

To investigate the potential apoptotic effect of MG132 and gliotoxin on CHD cell lines, determinations of apoptotic activity were measured at 24-hour intervals for three consecutive days. In each cell line, the apoptotic effect of both gliotoxin and MG132 showed a time-dependent apoptotic effect (Fig. 2). After 72-hour incubation with gliotoxin, the maximum apoptotic effect was seen with HS445, L428, and KMH2 demonstrating mean increases of 79.7%, 76.8%, and 59.0%, respectively, as determined by PI/cell cycle analysis. Although less than gliotoxin, increased apoptosis was also seen in each cell line after 72-hour incubation with MG132. Once again significant apoptotic activity was seen characterized by mean increases of 62.8%, 56.2%, and 57.3% for HS445, L428, and KMH2, respectively. All 72-hour apoptosis data were confirmed by Annexin V/7-AAD staining performed in two separate experiments (data not shown). For each cell line, the effect of concentration on levels of induced apoptosis by MG132 and gliotoxin was ex-

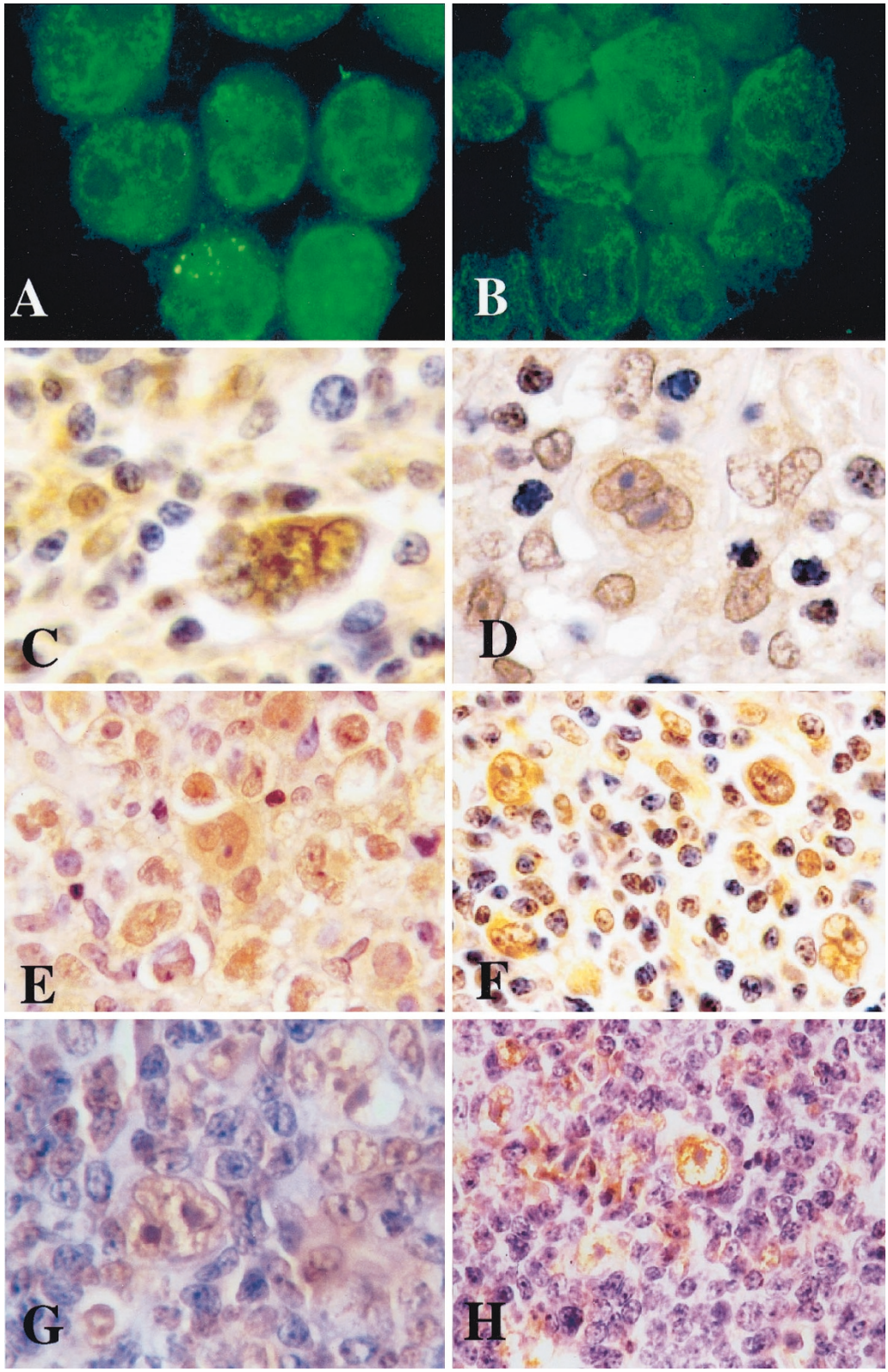

FIGURE 1. Hodgkin and Reed-Sternberg (HRS) cells from all patient samples and cell lines showed strong immunopositivity for active p65(Rel A). HRS cells from classical Hodgkin's disease (CHD) cell lines L428 (**A**, ×1000, p65(Rel A)/polyclonal anti-mouse-FITC) and HS445 (**B**, ×1000, p65(Rel A)/polyclonal anti-mouse-FITC) showed prominent nuclear and cytoplasmic expression for p65(Rel A). Similar patterns of positive staining were obtained in patient samples with no differences seen between nodular sclerosis (**C**, **D**, ×1000, p65(Rel A)/diaminobenzidine tetrahydrochloride) and mixed cellularity (**E**, **F**, ×500, p65(Rel A)/diaminobenzidine tetrahydrochloride) subtypes. Positive staining for p65(Rel A) also was found in all cases of nodular lymphocyte predominance Hodgkin's disease analyzed. Typically, lymphocytic/histiocytic cells showed a predominate nuclear staining pattern (**G**, **H**, ×1000,p65(Rel A)/diaminobenzidine tetrahydrochloride.

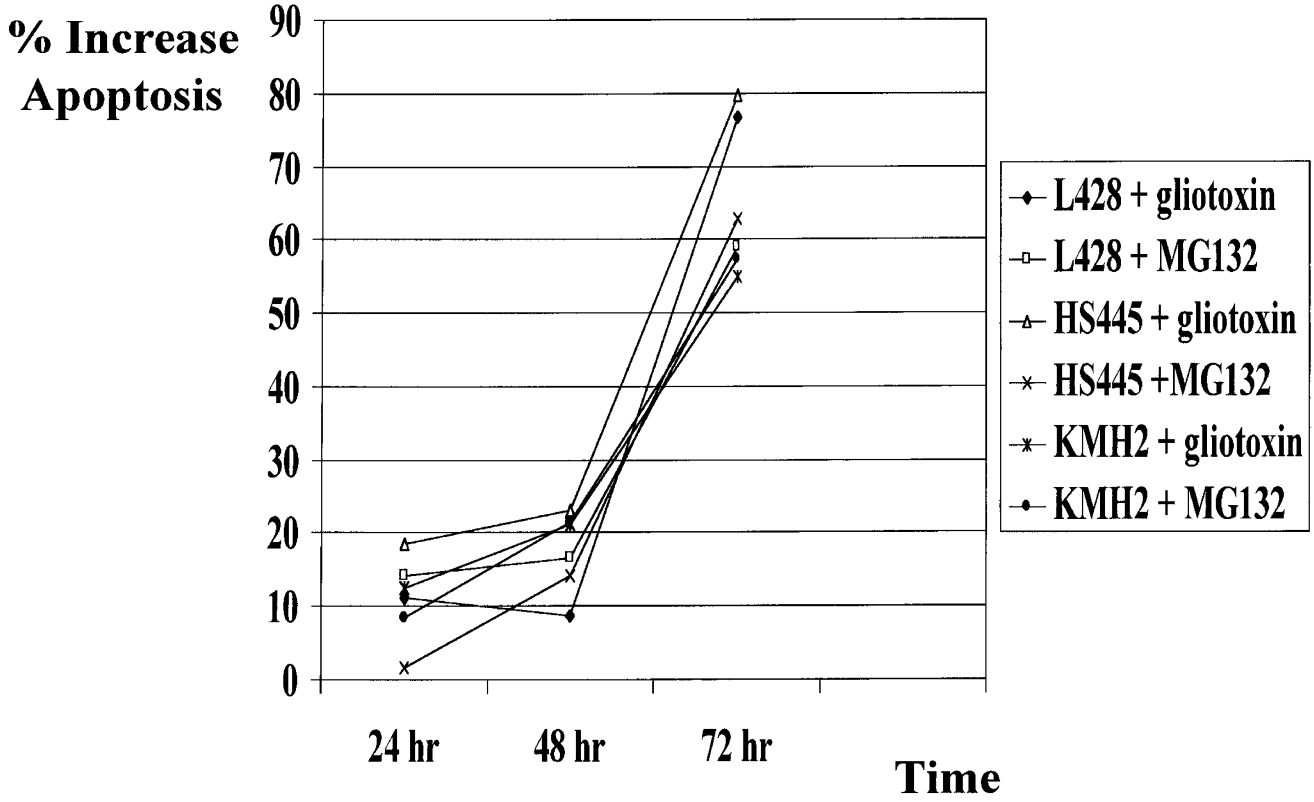

FIGURE 2. Time-dependent apoptosis produced by gliotoxin and MG132 in classical Hodgkin's disease cell lines. For each cell line, the mean apoptotic effect showed a progressive increase over a 72-hour time course. The data are representative of three separate experiments.

amined over a range of 0.01 to 20 μM. For both compounds, the apoptotic effects demonstrated dose-dependence in each cell line with the maximum effects achieved at 10 μM and 20 μM (Fig. 3 and data not shown).

To directly investigate the biologic consequences of NF-κB inhibition in HRS cells, L428 cells were infected with Ad5IκB, an adenovirus strain containing a dominant-negative IκBα mutant. After 96-hour incubation under normal cell culture conditions, L428 cells infected with Ad5IκB demonstrated an approximate 3-fold increase in spontaneous apoptosis compared with both untreated controls and Ad wt infected cells (Fig. 4).

Apoptosis Induced by Proteasome Inhibition Occurs Independently of Caspase-Mediated Pathways

Each cell line was preincubated with 100 μM concentrations of the caspase inhibitors z-DMQD-FMK, z-DEVD-FMK, z-VAD-FMK, z-VEID-FMK, or z-IETD-FMK. For each run, the ability of the caspase inhibitors to decrease CD95-induced apoptosis in sensitive CHD cell lines (HS445 and L428) and a Jurkat cell line served as functional controls; however, the findings of these control experiments are alone significant. In HS445, L428, and the Jurkat control reproducible inhibition of CD95-induced apoptosis by the caspase inhibitors z-DMQD-FMK, z-VEID-FMK, and z-IETD-FMK not only support the results of our previous investigation confirming the critical role of caspase-3 in CD95-induced apoptosis in CHD, but also suggests that caspase-6 and caspase-8 are important as well (Fig. 5). As expected, the caspase-3 deficient CHD cell line KMH2 showed no increase in apoptosis after incubation with agonistic anti-CD95 mAb.

Regardless of the caspase inhibitor used, there was no significant decrease in apoptosis after 72-hour incubation with 10 μM gliotoxin for each cell line tested (Fig. 6, A-B). Similarly, the caspase inhibitors also failed to decrease the apoptotic effect induced by 72-hour incubation with 10 μM MG132 for L428 and KMH2 (Fig. 7, A-B). Interestingly, preincubation with the majority of the caspase inhibitors resulted in significant concentration-dependent decreases (>20%) in 72-hour MG132-induced apoptosis in cell line HS445, suggesting that the caspase pathway was involved, at least in part, in MG132-induced apoptosis (Fig. 7B).

To further investigate the relationship between MG132- and gliotoxin-induced apoptosis and caspases, caspase-3 functional activity was measured at 24 and 48 hours postincubation with each compound. Both 24- and 48-hour incubation with either MG132 or gliotoxin showed no significant

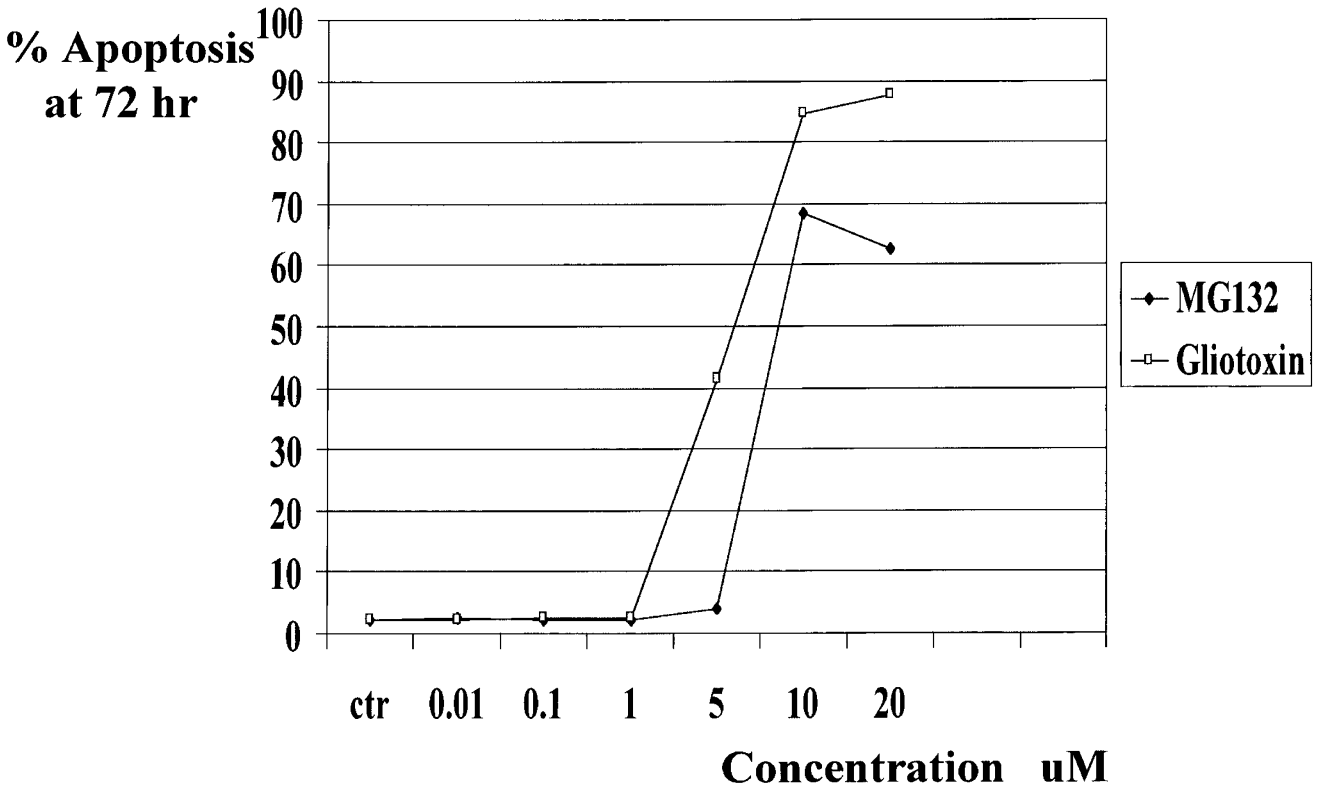

FIGURE 3. Concentration-dependent apoptosis produced by MG132 and gliotoxin in classical Hodgkin's disease cell line HS445. At concentrations below 1.0 μM, apoptosis in HS445 showed no significant difference from untreated control cells. Maximum apoptosis in both compounds was obtained at 10 μM and 20 μM concentrations. The data are representative of three separate experiments. Similar concentration-dependent apoptosis was found in L428 and KMH2.

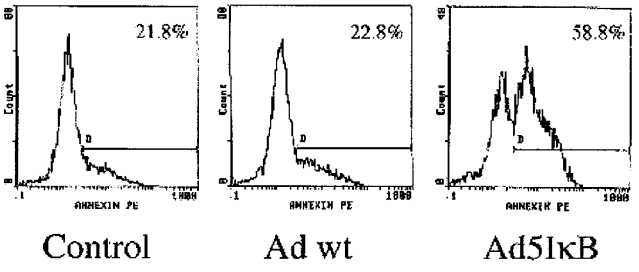

FIGURE 4. Infection of L428 cells with recombinant adenovirus Ad5IκB. Annexin V staining and flow cytometric analysis of Ad5IκB-infected cells showed a marked increase in spontaneous apoptosis compared with both Ad wt-infected cells and untreated control cells. The percentage of apoptotic cells 96 hours after treatment is shown in the upper right corner of each box.

increase in caspase-3 functional activity for all three cell lines compared with untreated controls (Fig. 8). These findings suggest that caspase-3 does not play a significant role in MG132- or gliotoxin-induced apoptosis in HRS cells.

Incubation with MG132 or Gliotoxin Results in a Significant Decrease in Active NF-κB

To investigate whether MG132- or gliotoxin-induced apoptosis correlated with inhibition of NF-κB activity in CHD cell lines, NF-κB activity was tested from nuclear extracts prepared from each cell line after 48-hour incubation with either MG132 or gliotoxin. In all three cell lines, both MG132 and gliotoxin significantly decreased NF-κB DNA binding activity compared with untreated controls (Fig. 9).

DISCUSSION

Constitutive activation of NF-κB p50-p65(Rel A) has been observed in several CHD cell lines and in HRS cells from primary patient samples (27, 28). In support of these previous investigations, we found strong nuclear and cytoplasmic expression of p65(Rel A) by immunohistochemistry in HRS cells from patient samples and cell lines and high nuclear DNA-binding activity of activated NF-κB by electrophoretic mobility shift assay in unstimulated HRS cells from each of our cell lines. Unlike HRS cells, Rel A does not contribute to the constitutive NF-κB activity observed in mature B cells, which consists mainly of c-rel or Rel B coupled with p50 or p52 (11, 12, 39). Because of these and other past observations, it was once believed that constitutive activation of NF-κB p50-Rel A was unique to HRS cells (27). Recent investigations, however, have also shown the involvement of the p50-Rel A heterodimer in promoting survival of various hematopoietic and non-hematopoietic neoplasms includ-

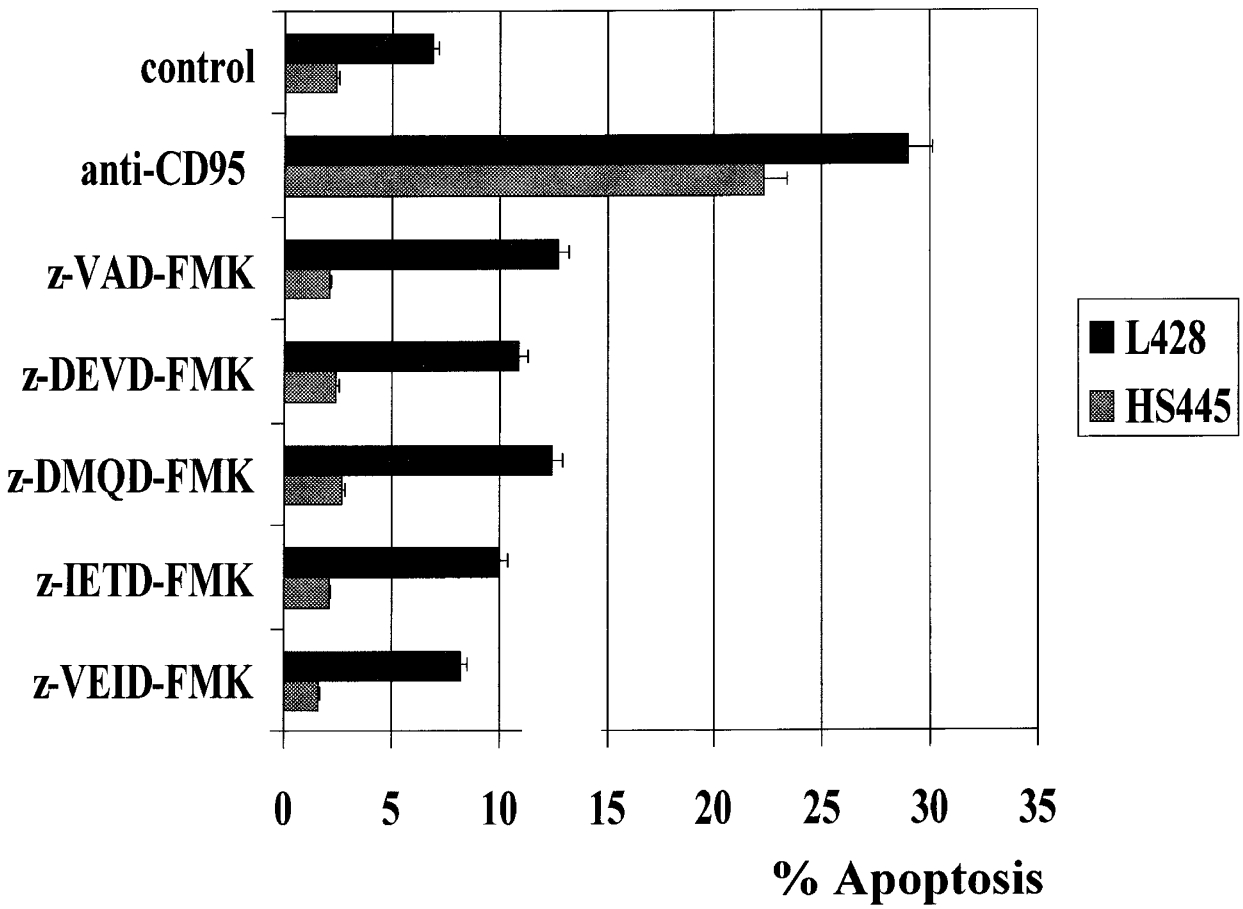

FIGURE 5. The effect of various caspase inhibitors (100 μM) on CD95-induced apoptosis in sensitive classical Hodgkin's disease cell lines. For both HS445 and L428, each caspase inhibitor produced a significant, and often marked, decrease in CD95-induced apoptosis, demonstrating the importance of caspase proteases in mediating CD95-induced apoptosis. These data show the mean increase in apoptosis ± SE from three separate experiments.

ing estrogen-independent human breast cancer cell lines (40, 41), CLL (42), multiple myeloma (43), adult T-cell lymphoma/leukemia cells (44), and thyroid carcinomas (45). Additionally, for the first time, we demonstrated expression of active p65(Rel A) in L&H cells from cases of NLPHD. The presence of active p65(Rel A) in CHD is consistent with investigations by Bargou *et al.* and others who showed constitutive activation of NF-κB p50-Rel A in HRS cells and revealed its functional role in proliferation and survival of HRS cells (27, 28, 46, 47). In our investigations, increased apoptosis in CHD cell lines correlated with markedly decreased expression of activated NF-κB by electrophoretic mobility shift assay, supporting the critical role of activated NF-κB for HRS cell survival.

Our data show that the ubiquitin-proteasome pathway plays an essential role in the maintenance of cell survival in HRS cells, such that the inhibitors used in this study (MG132 and gliotoxin) were capable of inducing markedly increased apoptosis in HRS cells from all cell lines analyzed. Previous investigations involving the proteasome inhibitors used in this study have revealed that the effects of these compounds are tightly linked to suppression of activated NF-κB (16, 18, 19). The proteasome has an essential role for two proteolytic processes required for activation of NF-κB: the processing of p105, which generates the active p50-Rel A heterodimer of NF-κB, and the complete degradation of IκBα, which is necessary for the translocation of NF-κB into the nucleus, where it stimulates gene expression (16, 48, 49). MG132 has been shown to block the function of both the 20s and 26s proteasome complexes in HeLa cells, which results in potent inhibition of both processes required for NF-κB activation (16). Chandra *et al.* recently demonstrated the induction of apoptosis by MG132 in glucocorticoid-resistant CLL cells (19). Similar to our findings in HRS cells, these investigators demonstrated a relationship between suppression of active NF-κB and MG132-induced apoptosis in CLL cells. Furthermore, they showed the apoptotic effect to be independent of other proteasome-regulated factors implicated in the control of cell survival including p53, Myc, Fos, Jun, and p27. Although the exact mechanism has not been fully defined, the immunosuppressive fungal metabolite,

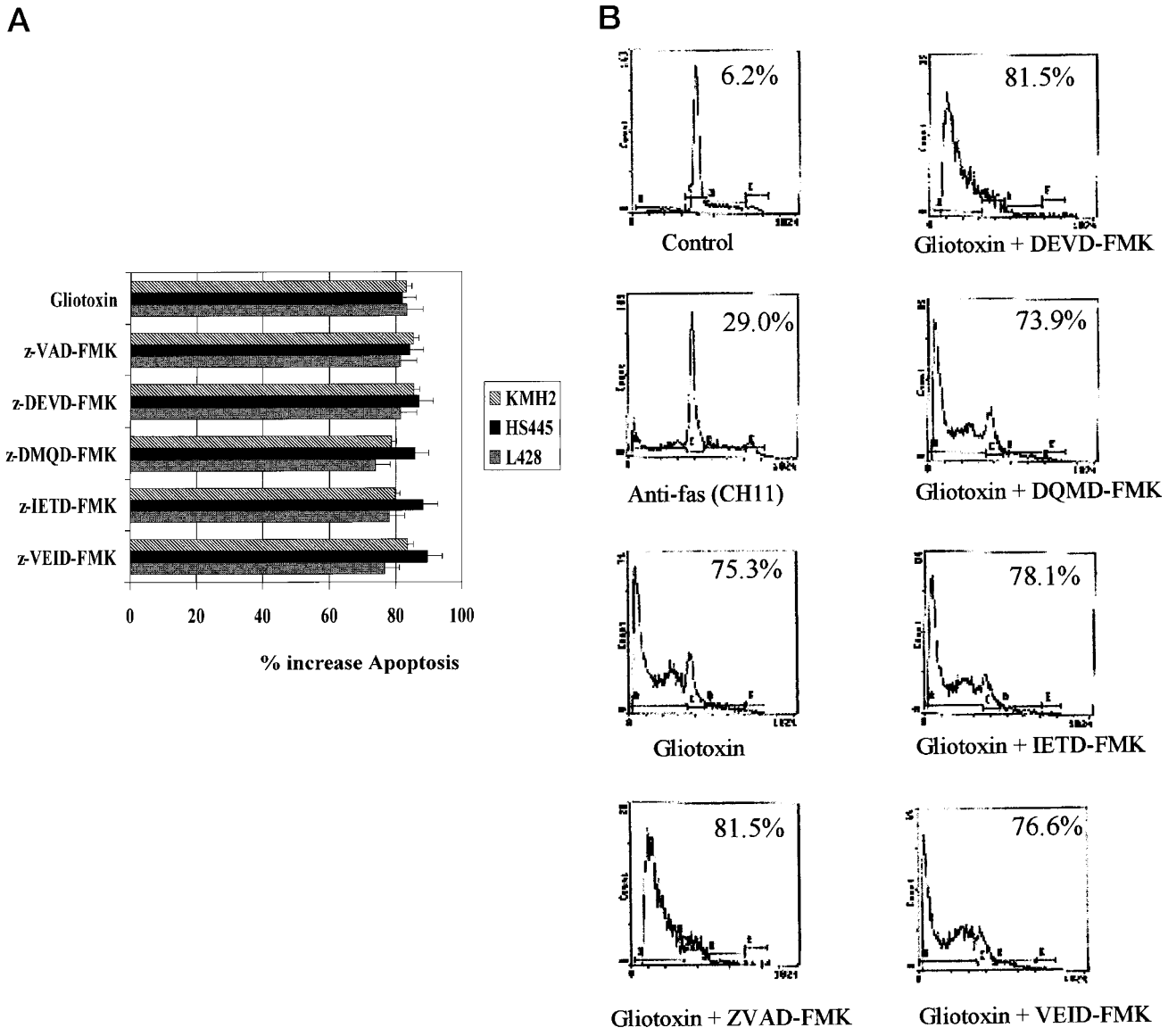

FIGURE 6 **A**, The effect of preincubation with various caspase inhibitors (100 μM) on 72-hour gliotoxin-induced apoptosis in classical Hodgkin's disease cell lines. For each cell line, preincubation with the above caspase inhibitors resulted in no significant decrease in gliotoxin-induced apoptosis. These data show the mean increase in apoptosis ± SE from three separate experiments. **B**, DNA fragmentation analysis by propidium iodide staining and flow cytometry. L428 was treated with 10 μM gliotoxin in the presence or absence of one of several caspase inhibitors. The DNA content in the cells was determined 72 hours after treatment. Marker B identifies apoptotic cells with hypodiploid DNA content, and C, D, and E represent stage G_1,S, and G_2/M of the cell cycle, respectively. The percentage of apoptotic cells 72 hours after treatment is shown in the upper right corner of each box.

gliotoxin has been shown to act through specific suppression of active NF-κB by preventing the proteolytic degradation of IκBα in several different cell types (18). As a whole these studies consistently demonstrate specific inhibition of active NF-κB by MG132 and gliotoxin. The marked apoptotic effect rendered in HRS cells by MG132 and gliotoxin and infection with adenovirus Ad5IκB, and the marked decrease in active NF-κB in HRS after proteasome inhibition strongly suggests that NF-κB is a key factor for preventing apoptosis in HRS cells.

Both MG132 and gliotoxin prevent the degradation of IκBα by the proteasome, which was suggested as a possible mechanism responsible for the decreased expression of active NF-κB seen in HRS cells. Considering this possibility, Bargou *et al.* successfully demonstrated specific and efficient suppression of constitutive NF-κB activity by stable transfection of HRS cells with a dominant-negative IκBα mutant (28). Additionally, these investigators revealed low-steady state wild-type (wt) IκBα protein expression in HRS cells, indicating high turnover of IκBα (27). It is possible that the continuous high level degradation of IκBα may be responsible, at least in part, for constitutive NF-κB activity in HRS cells. As previously mentioned, HRS cells express several cytokines and cell surface activation receptors and are able to produce high levels of

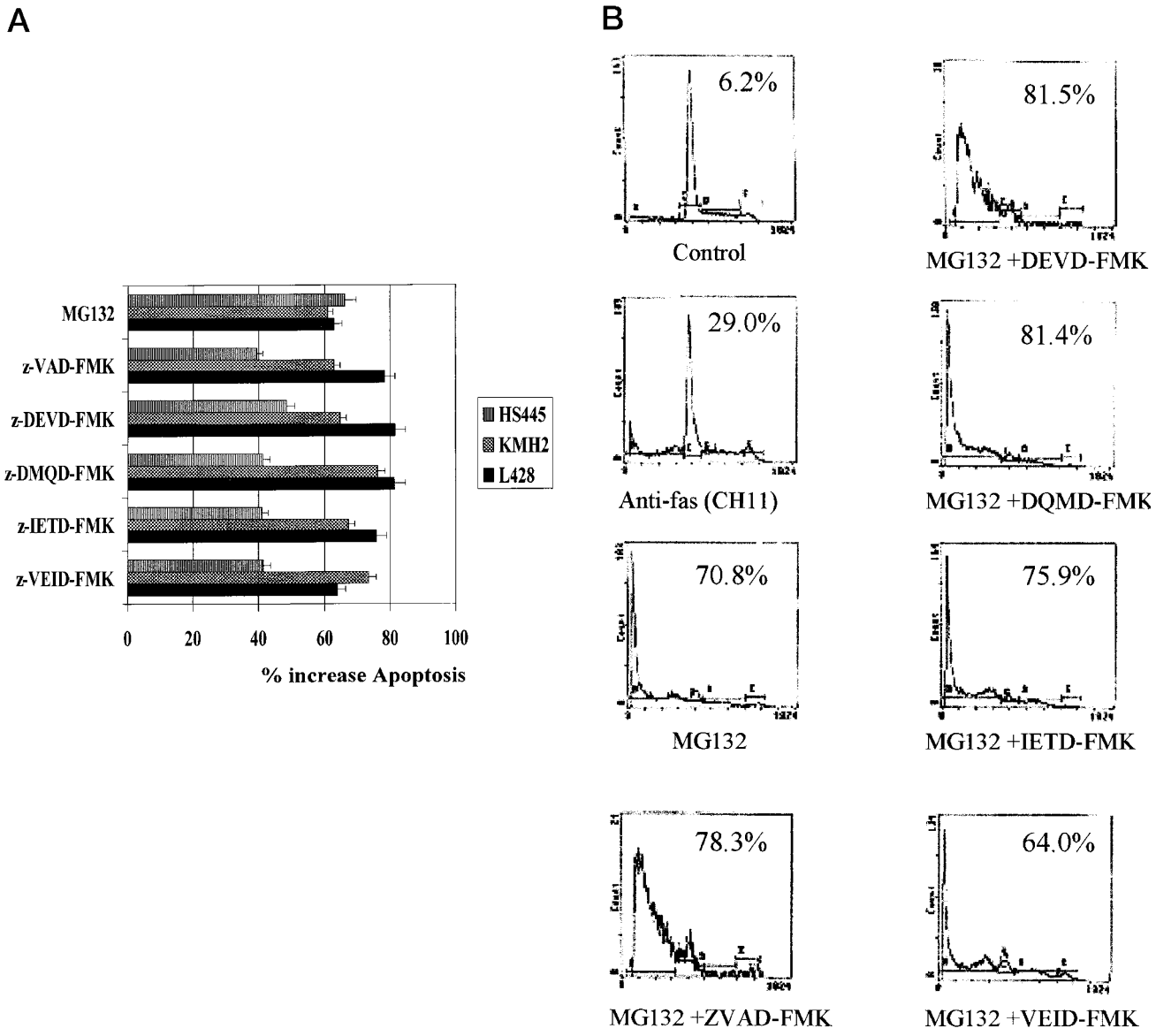

FIGURE 7 **A**, The effect of preincubation with various caspase inhibitors (100 μM) on 72-hour MG132-induced apoptosis in classical Hodgkin's disease cell lines. Similarly to gliotoxin, the caspase inhibitors failed to produce a significant decrease in MG132-induced apoptosis in L428 and KMH2; however, preincubation with the majority of the caspase inhibitors resulted in significant decreases in MG132-induced apoptosis. These data show the mean increase in apoptosis ± SE from three separate experiments. **B**, DNA fragmentation analysis by propidium iodide staining and flow cytometry. L428 was treated with 10 μM MG132 in the presence or absence of one of several caspase inhibitors. The DNA content in the cells was determined 72 hours after treatment. Marker B identifies apoptotic cells with hypodiploid DNA content, and C, D, and E represent stage G_1,S, and G_2/M of the cell cycle, respectively. The percentage of apoptotic cells 72 hours after treatment is shown in the upper right corner of each box.

TNF-α, LTα, and IL-1, all of which could stimulate NF-κB activity by an autocrine mechanism. In support of this possibility, Krappman *et al.* showed transient activation of NF-κB in Reh cells exposed to growth medium of HRS cells (47). Additionally, Gruss and colleagues demonstrated a relationship between activation of NF-κB and CD30 in HRS cells (6). Present in approximately one-third of CHD cases in North America and up to 90% of cases in South America, HRS cells are infected with Epstein-Barr virus. The HRS cells infected with Epstein-Barr virus express LMP-1 protein, which can activate NF-κB through tumor necrosis factor-associated factor, providing an additional explanation for constitutive activation of NF-κB in at least some cases of CHD (9, 50). Alternatively, HRS cells may acquire defects in regulatory components responsible for NF-κB/IκBα interaction, resulting in constitutive NF-κB activity. Interestingly, several recent investigations have shown that defective or complete absence of IκBα protein is responsible for constitutively active NF-κB in some CHD cell lines (46, 47, 51). Mutations in the IκBα gene have also been recently found in HRS cells from patient samples (51, 52). Unlike Bargou and colleagues, these investigators failed to observe wt IκBα protein in CHD

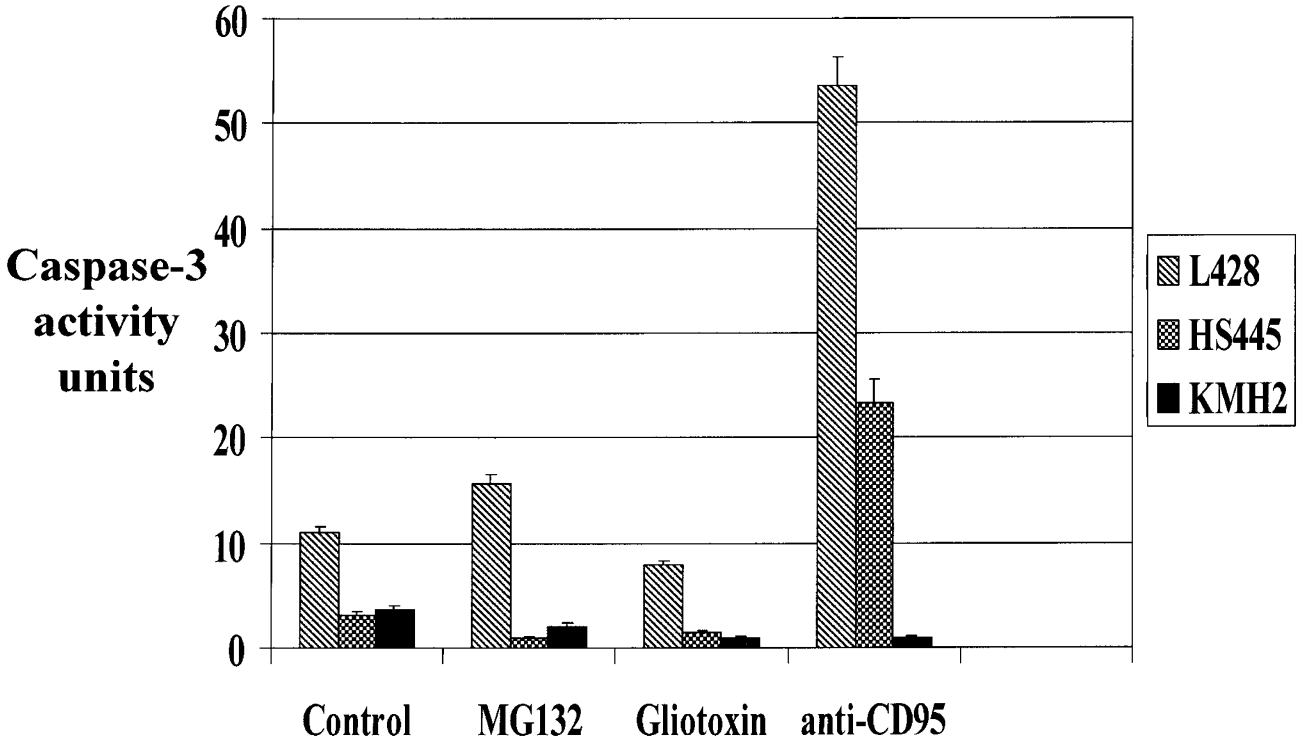

FIGURE 8. Caspase-3 activity in classical Hodgkin's disease cell lines treated with MG132, gliotoxin, or anti-CD95 mAb after 48-hour incubation. No significant increases in caspase-3 function activity were detected in all three cell lines after 48 hours of MG132 or gliotoxin treatment. As expected, significant increases in caspase-3 functional activity were obtained in the CD95 sensitive cell lines (HS445 and L428) after 48-hour treatment with agonistic CD95 mAb. Values are the means ± SE from three separate experiments.

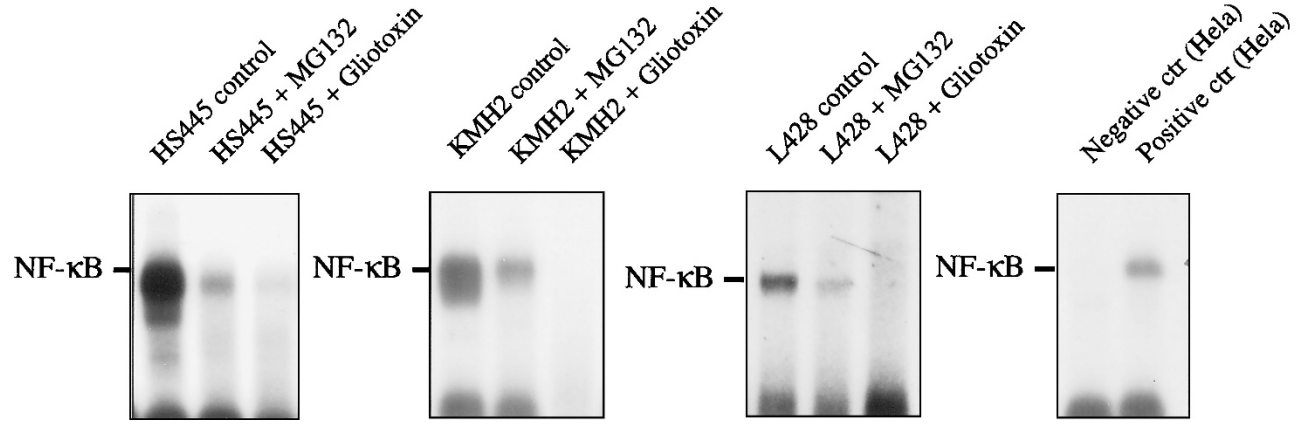

FIGURE 9. Nuclear expression of NF-κB in classical Hodgkin's disease cell lines after 48-hour incubation with MG132 or gliotoxin. As shown by electrophoretic mobility shift assay, marked decreases in active NF-κB were obtained in each cell line after 48-hour treatment with either compound. The Hela cells used as control cells do not constitutively express NF-κB (negative control); however, on stimulation with tumor necrosis factor-α, active nuclear expression of NF-κB is identified (positive control).

cell lines, KMH2 and HDLM-2. Such contradictory results, although unclear, may be due to sensitivity and specificity differences of the antibodies to IκBα employed by the two groups. Nevertheless, the addition of recombinant IκBα to nuclear extracts from each cell line and the transfection of HRS cells with a dominant-negative IκBα mutant again resulted in a significant decrease in NF-κB activity, as seen in the experimental models of Bargou *et al.*

The investigations of Bargou *et al.* (28) and Wood *et al.* (46) both successfully showed decreased NF-κB activity as a result of dominant-negative IκBα mutant transfections; however, neither group was able to demonstrate a significant increase in apoptosis without the addition of apoptosis-inducing stimuli or serum starvation. For the first time, we were able to demonstrate a marked spontaneous apoptotic effect in HRS cells after infection and incubation with Ad5IκB, without the addition of apoptosis-inducing stimuli or serum starvation. These results suggest that inhibition of NF-κB alone can significantly alter the survival of HRS cells. In

regards to proteasome inhibition, several investigators showed no decrease in NF-κB activity in CHD cell lines L428 and KMH2 after brief incubation with MG132 or gliotoxin (46, 51) or 8-hour incubation with ALLN (47). However, we clearly identified consistent and reproducible suppression of NF-κB activity in L428, KMH2, and HS445 after 48-hour incubation with MG132 or gliotoxin, suggesting that longer exposure to these compounds may be necessary to identify their physiologic effects on NF-κB activity. Overall, the results of this study and those of previous investigators suggest that multiple mechanisms contribute to constitutive NF-κB expression in HRS cells.

Having established that MG132 and gliotoxin decrease NF-κB activity and induce significant levels of apoptosis in HRS cells, we attempted to determine whether these effects were caspase mediated. Previous investigations by our group revealed a critical link between caspase-3 activity and CD95-induced apoptosis in HRS cells (34). Preincubation with z-VEID-FMK and z-IETD-FMK demonstrated significant decreases in CD95-induced apoptosis in sensitive CHD cell lines (L428 and HS445), suggesting that in addition to caspase-3, caspase-6, and caspase-8 are also important mediators of CD95-induced apoptosis in HRS cells. Two recent studies have shown a possible link between NF-κB activity and caspases. Ravi *et al.* demonstrated that inhibition of NF-κB activity in Jurkat cells undergoing CD95-induced apoptosis is a consequence of the proteolytic cleavage of both p50 and Rel A by caspase-3-related proteases (53). Furthermore, the apoptotic effect of proteasome inhibition demonstrated in CLL cells by Chandra *et al.* was found to be related to caspase activation (19). Specifically, the broad caspase inhibitor z-VAD-FMK completely blocked MG132-induced DNA fragmentation and surface exposure of phosphatidylserine (annexin staining), revealing that caspase activity was required for proteasome inhibitor-induced apoptosis in CLL cells (19). Although we preincubated the HRS cell from all CHD cell lines with a variety of caspase inhibitors, including z-VAD-FMK, no significant decreases in either MG132- or gliotoxin-induced apoptosis was observed with the exception of the concentration-dependent inhibition of MG132-induced apoptosis in HS445. This finding suggests that the caspase pathway was involved, at least in part, in MG132-induced apoptosis in HS445 cells. Previously, we have shown that KMH2 has markedly diminished caspase-3 compared with other CHD cell lines (34). Given this finding, a similar degree of proteasome inhibition-induced apoptosis was demonstrated in KMH2 compared with the other CHD cell lines, providing additional evidence that caspase-3 is not involved in this apoptotic process. Furthermore, HRS cells from all three cell lines failed to demonstrate a significant increase in caspase-3 functional activity after 24- and 48-hour incubation with MG132 or gliotoxin. As a whole, these findings suggest that caspase-3 does not play a significant role in MG132- or gliotoxin-induced apoptosis in HRS cells.

In summary, we demonstrate that NF-κB is constitutively expressed in both CHD and NLPHD and chemical inhibition of the ubiquitin-proteasome pathway by MG132 and gliotoxin results in both significantly decreased levels of NF-κB activity and markedly increased apoptosis of HRS cells. Furthermore, we also show that the effects of these proteasome inhibitors generally occur independent of caspase-mediated apoptosis in HRS cells. These findings support previous studies that demonstrate that constitutive expression of NF-κB activity is required for resistance to apoptosis in HRS cells. Additional studies of the effects of proteasome inhibitors on HRS cells may provide valuable insights into the pathogenesis of Hodgkin's disease and potentially create novel therapeutic opportunities for the treatment of these and other neoplasms.

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Book Review

Clement PB, Young RH: Atlas of Gynecologic Surgical Pathology, 360 pp, Philadelphia, W.B. Saunders, 2000 ($199.00).

Clement and Young, two well-known leaders in the field of gynecologic pathology have authored an outstanding book. This atlas is a valuable and timely addition to the popular series of pathology atlases published by Saunders.

The book is comprehensive and covers all major entities of gynecologic pathology, except placenta. It is composed of 20 chapters organized by site within the female genital tract and peritoneum. Gynecologic neoplasia receives more space than non-neoplastic diseases, but these are included as well. In a clear, well-organized, and authoritative manner, the book provides most of what the pathologist needs to know about gynecologic diseases. The main emphasis of this atlas is on the histopathologic diagnostic aspect of the neoplastic and pseudoneoplastic diseases. The editors gave readers good exposure to the different tumors in the particular organ, the histologic classification, criteria for diagnosis, and a large number of diagnostic, prognostic, and genetic biomarkers specific for that tumor. The text, even though brief, is quite informative and instructive. The differential diagnosis is discussed masterfully, providing valuable guidance to solving complex diagnostic problems. As expected in an atlas, the illustrations, most of which are in color, are of high quality and adequately labeled. The book is easy to navigate through and is logical in its layout with a complete list of up-to-date references. A personal delight is the final sections of the book, devoted to pattern-based approach to ovarian tumors and to the one for tumors with functioning stroma.

We have used the book in daily surgical pathology practice, and we found it very informative and entertaining. It is especially convenient that the text is in outline format with "bullets," allowing one to quickly grasp the most important points, essential for the diagnosis.

Although there are currently many textbooks of gynecologic pathology, this atlas provides a unique point of view and deserves to be included in the library of each pathologist. I found this book to be a must-have for pathology residents, fellows, and practicing pathologists. It also should be on the short list of assigned reading for gynecologists and gynecology residents preparing for their specialty Board examinations or during their rotations in surgical pathology.

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