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Curcumin induces cell-arrest and apoptosis in association with the inhibition of constitutively active NF-κB and STAT3 pathways in Hodgkin's lymphoma cells

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Although treatment of Hodgkin's lymphoma (HL) with a multidrug approach has been very successful, its toxicity becomes evident after several years as secondary malignancies and cardiovascular disease. Therefore, the current goal in HL treatment is to find new therapies that specifically target the deregulated signaling cascades, such as NF-κB and STAT3, which cause Hodgkin and Reed-Sternberg (H-RS) cell proliferation and resistance of apoptosis. Based on the above information, we investigated the capacity of curcumin to inhibit NF-κB and STAT3 in H-RS cells, characterizing the functional consequences. Curcumin is incorporated into H-RS cells and acts inhibiting both NF-κB and STAT3 activation, leading to a decreased expression of proteins involved in cell proliferation and apoptosis, *e.g.* Bcl-2, Bcl-xL, cFLIP, XIAP, c-IAP1, survivin, c-myc and cyclin D1. Interestingly, curcumin caused cell cycle arrest in G2-M and a significant reduction (80–97%) in H-RS cell viability. Furthermore, curcumin triggered cell death by apoptosis, as evidenced by the activation of caspase-3 and caspase-9, changes in nuclear morphology and phosphatidylserine translocation. The above findings provide a mechanistic rationale for the potential use of curcumin as a therapeutic agent for patients with HL.

Key words: Hodgkin's lymphoma; H-RS cells; NF-κB; STAT3; curcumin; cell cycle arrest; apoptosis

Extensive research over recent decades has indicated that curcumin (diferuloylmethane) can both, prevent and treat cancer (reviewed in Ref. 1). Curcumin, a lipid-soluble yellow compound isolated from the rhizomes of the plant *Curcuma longa,* has been shown to suppress cancer initiation and promotion as well as metastasis.[1,2] Besides its anticancer effects, curcumin has known antiinflammatory and antioxidant properties.[3,4] Moreover, curcumin can inhibit several signaling pathways at multiple levels, including NF-κB and STAT pathways.[5]

Hodgkin's lymphoma (HL) is one of the most frequent lymphomas in the Western world[6] with an incidence of about 2–4 new cases per 100,000 people per year in the United States.[7] The hallmark of this disease is the presence of large mononuclear Hodgkin and multinuclear Reed-Sternberg cells (H-RS cells).[8] H-RS cells derive from B cells in nearly all cases of HL and only rarely from T cells. HL is characterized by an abnormal expression of cytokines mainly produced by the malignant H-RS cells, which attract the surrounding reactive cells.[9] Cytokine secretion may trigger signaling cascades, which support the proliferation of tumor cells and prevent cell death by apoptosis. Indeed, simultaneous deregulation of signaling pathways (NF-κB, STAT3, AP-1) that are normally transiently induced are characteristic for H-RS cells.[10–13]

The capacity of NF-κB to modulate the expression of genes involved in inflammation, cell proliferation and survival, and the finding of its constitutive activation in many different cancer types suggest that NF-κB plays a major role in carcinogenesis.[14,15] In addition to HL, the activation of NF-κB has been also described in solid tumors (breast, gastric, colonic cancers) (reviewed in Ref. 16). Transcription factor NF-κB is formed by homo- and heterodimers of the Rel/NF-κB family of proteins, and resides in the cytosol in an inactive form. Interaction with members of the inhibitory IκB proteins prevents its translocation to the nucleus.[17] In the classical pathway, 2 conserved serines in IκB are phosphorylated by specific IκB kinases (IKK), which target IκB for ubiquitination and degradation by the proteasome.[18] Degradation of IκB unmasks the nuclear localization signal allowing the nuclear translocation of NF-κB for the subsequent regulation of gene expression.

Besides NF-κB, another family of transcription factors is found to be constitutively active in HL cells: the signal transducer and activator of transcription (STAT) family.[13] Seven STATs have been identified in mammals, designated STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6.[19] STAT signaling activation involves the formation of a signaling-competent receptor complex that on binding leads to the activation of the receptor-associated Janus family of tyrosine kinases (JAKs).[20] JAKs phosphorylate STAT proteins that once phosphorylated, form homo or heterodimers. This allows its translocation to the nucleus, where it regulates gene transcription.[8] Constitutive STAT activation is associated with malignant tumor transformation[20] and tumor progression.[21] STAT3 regulates several pathways important in tumorigenesis including cell cycle progression, apoptosis, tumor angiogenesis, invasion and metastasis.[22] Elevated activities of STAT proteins are a frequent finding in blood malignancies (leukemias, lymphomas and multiple myeloma) as well as solid tissues (such as head and neck, breast and prostate cancer).[21] STAT3 is constitutively activated in H-RS cell lines, suggesting that deregulated STAT3 may play a causative role in HL.[13] In contrast to other malignancies, it is still unknown to what extent constitutively STAT3 activation is involved in the transformation of H-RS cells.

Although multidrug approaches have been very successful in HL treatment, its toxicity becomes evident after approximately 20 years in the form of secondary malignancies and cardiovascular disease (reviewed in Ref. 23). Therefore, the current goal in HL treatment is to find new therapies that specifically target the deregulated signaling cascades that cause H-RS cell proliferation and resistance of apoptosis. The hypothesis tested in this study is that, through the inhibition of NF-κB and STAT3, curcumin could be a potential therapeutic agent in the treatment of HL. For this purpose, the effects of curcumin on NF-κB and STAT3 constitu-

Abbreviations: DDT, dithiothreitol; EDTA, ethylenediamine tetraacetate; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HL, Hodgkin's lymphoma; H-RS cells, Hodgkin and Reed-Sternberg cells; IKK, IκB kinase; IL-6, interleukin-6; NF-κB, nuclear factor-κB.

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tive activations and the subsequent consequences on H-RS cell proliferation and apoptosis were tested in 4 well-characterized H-RS cell lines: KM-H2, L-428, L-540 and L-1236.

Material and methods

Materials

The H-RS cells (KM-H2, L-428, L-540, L-1236 and HDLM-2 cells) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) while Jurkat (T cell leukemia, human) cells were obtained from the American Type Culture Collection (Rockville, MA). Cell culture media and reagents were from Invitrogen Life Technologies (Carlsbad, CA). The CellTiter-Glo Luminiscent Cell Viability assay, the oligonucleotide containing the consensus sequence for NF-κB, and the reagents for the electrophoretic mobility shift assay (EMSA) were obtained from Promega (Madison, WI). The protease inhibitor cocktail was obtained from Roche Applied Science (Mannheim, Germany). The oligonucleotide containing the consensus sequence for STAT3 and the antibodies for $FLIP_{S/L}$, Bcl-xL, IκBα, RelA, p50, Bfl/A1, PARP (H-250), c-IAP2, and β-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2, XIAP, c-IAP1, survivin, cyclin D1, c-Myc, cleaved-caspase-9, phospho-JAK2, STAT3, phospho-STAT3 (tyr705) and phospho-IκB α antibodies were obtained from Cell Signaling Technology (Beverly, MA). PVDF membranes were obtained from BIO-RAD (Hercules, CA) and Chroma Spin-10 columns were obtained from Clontech (Palo Alto, CA). The ECL western blotting system was from GE Healthcare (formerly Amersham Pharmacia Biotech) (Piscataway, NJ). Curcumin, with purity greater than 98%, was kindly provided by Dr. B. B. Aggarwal (MD Anderson Cancer Center, University of Texas). The JAK2 inhibitor AG490 was obtained from Calbiochem, (San Diego, CA). Hoechst reagent and all other reagents were from the highest quality available and were purchased from Sigma (St. Louis, MO).

Cell culture and incubations

H-RS and Jurkat cells were cultured in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (10 U/ml penicillin and 10 μg/ml streptomycin). Cells were incubated without or with curcumin (2.5–100 μM) for different periods of time (0–72 h). Since curcumin was resuspended in dimethylsulfoxide (DMSO), controls for cells incubated only with DMSO were run for each condition [maximum concentration of DMSO in the media was 0.25% (v/v)].

Determination of cell curcumin content

KM-H2 and L-1236 cells (4×10^6 cells) were incubated with 25 μM curcumin for 0, 1, 2, 4 or 24 h. After incubation, cells were collected by centrifugation at 800*g* for 10 min, and washed 4 times with PBS 1X. After the final wash and before freezing, an aliquot was taken to measure the number of cells by trypan blue exclusion. After 2 cycles of freezing/thawing, curcumin was extracted from the cells with 100 μl methanol. After filtering the methanol extract through a 0.22 μM nylon filter, curcumin was determined by HPLC with a diode array detector. Separation was carried out isocratically using a 250×4.6 mm^2, 4 μM Supelcosil LC-18 column. Mobile phase was 50% (v/v) acetonitrile, 45% (v/v) water containing 0.1% (v/v) trifluoroacetic acid, and 5% (v/v) methanol.

Electrophoretic mobility shift assay

After the corresponding treatments, nuclear fractions were isolated from cells (2×10^6 cells) as previously described.[24] For the EMSA, the oligonucleotides containing the consensus sequences for NF-κB and STAT3 were end labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1X binding buffer (10 mM Tris-HCl buffer, pH 7.5, containing 4% (v/v) glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl and 0.05 mg/ml poly(dI-dC). The products were separated by electrophoresis in a 6% (w/v) nondenaturing polyacrylamide gel using 0.5 × TBE (45 mM Tris/borate, 1 mM EDTA, pH 8.3) as the running buffer. The gels were dried and the radioactivity was quantified in a Phosphoimager 840 (Amersham Pharmacia Biotech, Piscataway, NJ).

Western blot analysis

Nuclear fractions were obtained as described above, and total cell fractions as previously described.[24] Aliquots of nuclear or total fractions containing 25–40 μg protein were separated by reducing 10–12.5% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. To evaluate the bcl-2, bcl-xL, c-IAP1, c-IAP2, survivin, XIAP, cFLIP, caspase-9, phospho-IκBα, IκBα, phospho-JAK2, JAK2, phospho-STAT3 and STAT3 proteins, membranes were immunoblotted with the corresponding primary antibody (1:500 dilution) in 5% (w/v) bovine serum albumin in TBS overnight at 4°C and the following day for 90 min at room temperature in the presence of the corresponding secondary antibody (HRP-conjugated). For PARP and β-tubulin, membranes were blocked overnight in 5% (w/v) nonfat milk in TBS and subsequently incubated in the presence of the corresponding antibodies (1:1,000 dilution) for 90 min at 37°C. After incubation, for 90 min at room temperature, in the presence of the secondary antibody (HRP-conjugated;1:5,000 dilution), the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840.

Cell viability assay

The effect of curcumin on cell viability for the different H-RS cell lines was determined by CellTiter-Glo Luminiscent Cell Viability Assay. Briefly, cells (50,000 cells/100 μl medium) were incubated in the absence or in the presence of 2.5–50 μM curcumin or 2.5–50 μM AG490 for different periods of time (0–72 hr).

Evaluation of cell cycle progression

To synchronize the cells, KM-H2 and L-428 cell lines were washed with PBS and then serum-starved for 48 hr in RPMI medium containing 0.1% (v/v) FBS. Re-entry into G1 phase of cell cycle was initiated by replacement of the starvation medium with complete medium containing 10 μM curcumin. After 0, 12 and 24 hr, cells were collected and washed with PBS. The analysis of nuclear DNA was performed using the Cycletest^{TM} Plus DNA Reagent Kit. Propidium iodide-stained nuclei were analyzed by flow cytometry and the percentages of cells in the G_0-G_1, S, and G_2-M phases of the cell cycle were determined by using the Mod Fit LT cell cycle analysis software (Verity Software, Topsham, ME).

Hoechst staining

H-RS cells (1.0×10^5 cells) were cultured in 200 μl of RPMI media in 24-well plate and treated without or with curcumin (25 μM) for 24 hr. The Hoechst reagent (5 μl/ml) was added to the media and incubated in the dark for 15 min at 37°C. The cells were transferred to previously l-polylysinated coverslips and centrifuged at 800*g* for 10 min. The media was discarded and the cells were washed with PBS. Cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, and after washing with PBS, the cells were dried at room temperature. Finally, cells were mounted with Fluorsave and visualized by fluorescence microscopy (Olympus IMT-2).

Annexin V staining

Cells were seeded at 1.0×10^5 cells per well and incubated in the presence of 10 μM curcumin or equivalent volumes of DMSO for 0, 16 and 48 hr. Cells were harvested, washed with cold PBS, resuspended in cold calcium binding medium (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$), and stained with Alexa 488-labeled annexin V and propidium iodide. Following incuba-

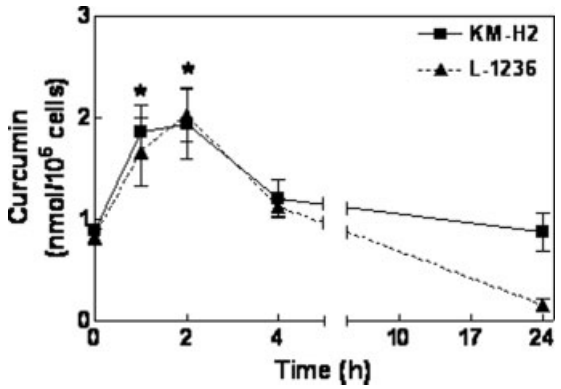

FIGURE 1 – Time dependent incorporation of curcumin in H-RS cells. KM-H2 and L-1236 cells (4×10^6 cells) were incubated with 25 μM curcumin for different times. Curcumin cell content values are shown as means ± SEM of at least 3 independent experiments. *Significantly different compared to both zero time and 24 h time ($p < 0.05$, one-way ANOVA test).

tion at room temperature for 15 min in the dark, cells were counted in a Becton Dickinson flow cytometer using the Win MDI version 2.8 software.

Statistical analysis

One way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe was performed using Statview 512+ (Brainpower, Calabazas CA). A p value < 0.05 was considered statistically significant. Values are shown as means ± SEM.

Results

The goal of the present study was to investigate the effects of curcumin on NF-κB- and STAT3-pathways, and the subsequent consequences on H-RS cell proliferation and apoptosis.

Time-dependent incorporation of curcumin in H-RS cells

The steady state level of curcumin, which result from curcumin uptake, metabolization and export, was determined in KM-H2 and L-1236 cells exposed to 25 μM curcumin. Cell curcumin content was time-dependent. After 2 hr of incubation, curcumin content was 1.94 ± 0.35 and 2.03 ± 0.26 nmoles/10^6 cells in KM-H2 and L-1236 cells, respectively (Fig. 1). These values represent a 100% increase over the values obtained at zero time ($p < 0.01$). After 24 hr of incubation curcumin content was significantly reduced in both cell types.

Curcumin inhibits NF-κB- and STAT3-DNA binding in a dose- and a time-dependent manner in H-RS cells

All the studied H-RS cell lines presented high constitutive levels of NF-κB- and STAT3-activation. This was evidenced by a higher NF-κB- and STAT3-DNA nuclear binding (Fig. 2*a*) and higher levels of STAT3 phosphorylation (Fig. 2*b*) compared to Jurkat T cells. As previously reported,[25,26] KM-H2 and L-428 cells showed the absence of functional IκBα (Fig. 2*b*). The capacity of curcumin to down-regulate the constitutive NF-κB and STAT3 activation in H-RS cells was next investigated. For this purpose, H-RS cells were incubated with different concentrations of curcumin (5–25 μM) for 24 hr. Curcumin inhibited nuclear NF-κB-DNA binding in all the H-RS cell lines in a dose-dependent manner (Fig. 2*c*). In KM-H2 cells, a significant inhibition of NF-κB-DNA binding was observed at 5 μM curcumin concentration (Fig. 2*d*). A significant inhibition was observed at 10 μM curcumin in L-428 and L-1236 cells, and at 25 μM curcumin in L-540 cells (Fig. 2*d*).

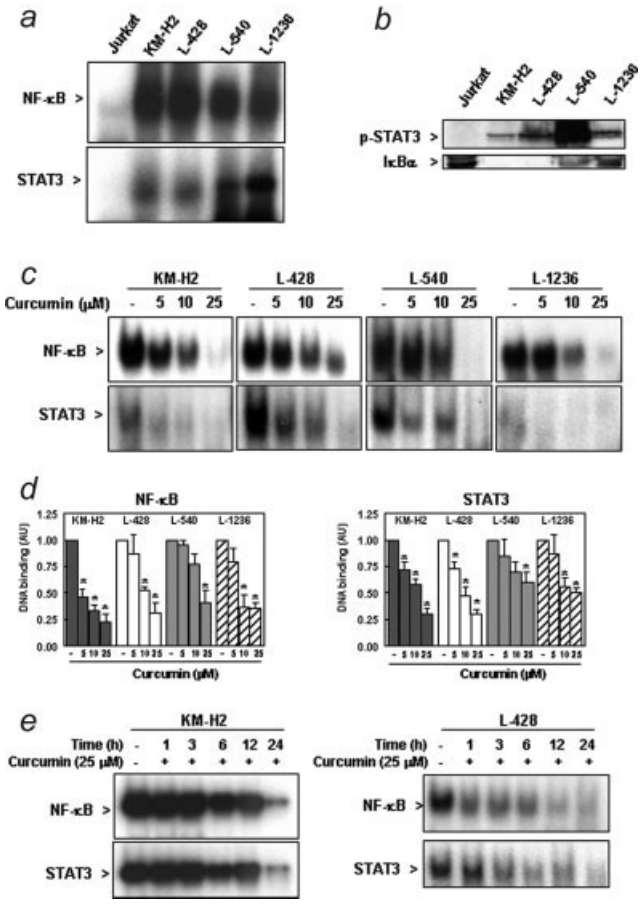

FIGURE 2 – Curcumin inhibits NF-κB- and STAT3-DNA binding in H-RS cells in a dose- and time-dependent manner. (*a*) Constitutive NF-κB and STAT3 activation in H-RS cells was evaluated by EMSA in nuclear fractions isolated from KM-H2, L-428, L-540 and L-1236 cells. Jurkat T cells were used as a control of cells with low constitutive activation of either NF-κB or STAT3. (*b*) Western blot analysis of p-STAT3 (tyr705) and IκBα, in total fractions isolated from KM-H2, L-428, L-540 and L-1236 cells. (*c*) NF-κB- and STAT3-DNA binding were measured by EMSA in nuclear fractions isolated from KM-H2, L-428, L-540 and L-1236 cells that were incubated for 24 h without (−) or with 5, 10, or 25 μM curcumin. (*d*) After the EMSA, bands were quantitated and results are shown as means ± SEM of 4 independent experiments. *Significantly different compared to untreated cells ($p < 0.05$, one-way ANOVA test). (e) Time-dependent inhibition of NF-κB- and STAT3-DNA binding by curcumin in KM-H2 and L-428 cells. Cells were incubated with 25 μM curcumin for the indicated times, and the DNA binding was evaluated in nuclear fractions by EMSA. Representative images out of two independent experiments are shown.

Figure 2*c* shows that curcumin inhibited STAT3-DNA binding in a dose-dependent manner (5–25 μM). The inhibitory action of curcumin was observed at concentrations as low as 5 μM in KM-H2 and L-428 cells, but higher concentrations (10 and 25 μM, respectively) were needed to inhibit STAT3-DNA binding in L-1236 and L-540 cells (Fig. 2*d*).

To investigate the time dependence of the inhibition of NF-κB- and STAT3-DNA binding by curcumin, KM-H2 and L-428 cells were incubated with 25 μM curcumin for 1–24 hr. Curcumin inhibited NF-κB-DNA binding in a time-dependent manner in L-428 and KM-H2 cells (Fig. 2*e*). In L-428 cells, a significant inhibition of NF-κB-DNA binding was observed after only 1 hr, whereas in KM-H2 cells the shortest time to obtain a significant inhibition was 6 hr. Similarly, STAT3-DNA binding was also inhibited by 25 μM curcumin in L-428 and KM-H2 cells in a

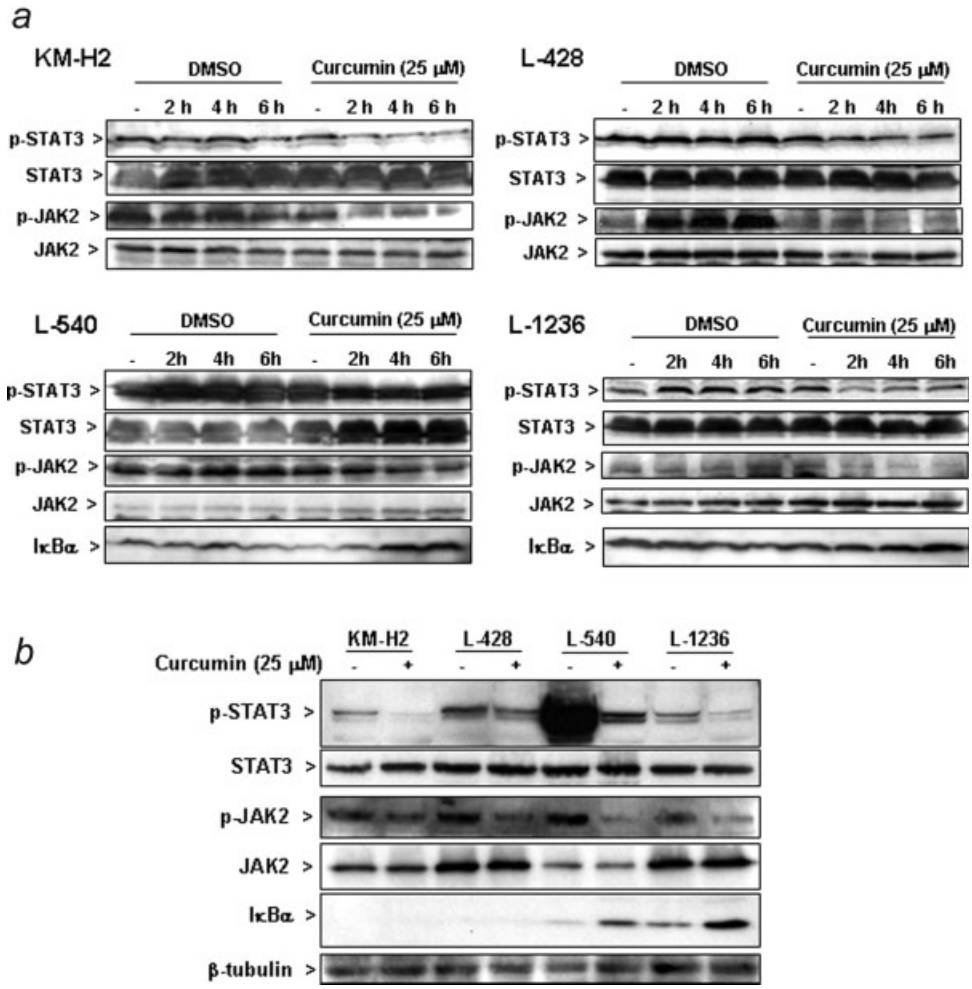

FIGURE 3 – Curcumin inhibits upstream events in the STAT3 and NF-κB pathways in H-RS cells. (*a*) Levels of phosphorylated STAT3 (tyr705) (p-STAT3), phosphorylated JAK2 (p-JAK2) and IκBα were measured by Western blot in total cell extracts isolated from KM-H2, L-428, L-540 and L-1236 cells incubated for the indicated periods of time, with or without DMSO or 25 μM curcumin. STAT3, JAK2 and β-tubulin levels are shown as loading controls. (*b*) p-STAT3, p-JAK2 and IκBα levels measured by Western blot in total cell extracts isolated from KM-H2, L-428, L-540 and L-1236 cells incubated with 25 μM curcumin for 18 h. STAT3, JAK2 and β-tubulin levels are shown as loading controls. Representative images are shown.

time-dependent manner (Fig. 2*e*). In L-428 cells, a significant inhibitory effect on STAT3-DNA binding was observed after 3 hr of incubation, whereas in KM-H2 cells, a significant inhibition was observed after 24 hr.

Curcumin inhibits upstream events of NF-κB and STAT3 pathways in H-RS cells

The effects of curcumin on upstream events of NF-κB and STAT3 activation signaling pathways were next investigated. As shown in Figure 3, curcumin (25 μM) inhibited STAT3 phosphorylation (tyr 705) as early as after 2 hr of incubation, and the inhibition was maintained throughout the period of time studied. Members of the JAK family of protein tyrosine kinases, phosphorylate and activate cytoplasmic STAT proteins. JAK2 is a recognized activator of STAT3.[27] In accordance with the results obtained for p-STAT3, curcumin (25 μM) also inhibited JAK2 phosphorylation in a time-dependent manner in all H-RS cells studied (Fig. 3*a*). Regarding the NF-κB pathway, IκBα degradation is needed for the activation of NF-κB. After 18 hr of incubation, we observed that curcumin prevented IκBα degradation in L-540 and in L-1236 cells (Fig. 3*b*).

Curcumin inhibits NF-κB- and STAT3-regulated gene expression

The effect of curcumin on NF-κB- and STAT3-dependent gene expression was next investigated. As depicted in Figure 4, the studied H-RS cells were characterized by a remarkably different pattern of expression of antiapoptotic proteins. Nevertheless, Curcumin down-regulated the expression of Bcl-xL, Bcl-2, Bfl 1/A1, c-IAP1, XIAP, survivin and c-FLIP in all H-RS cell lines (Fig. 4). c-IAP2 expression was affected by curcumin in L-428 cells, but not in KM-H2, L-540 and L-1236 cells (Fig. 4).

Curcumin decreases cell viability in a dose- and time-dependent manner

To investigate whether curcumin affects H-RS cell viability, cells were incubated in the absence or the presence of different concentrations of curcumin (2.5–50 μM) for 24, 48 and 72 hr. Results show that curcumin causes a dose- and time-dependent decrease in cell viability in the H-RS cell studied (Fig. 5). After 24 hr of incubation with 25 μM curcumin a significant decrease in cell viability is observed for KM-H2 and L-540 cells. A concentration of 50 μM curcumin significantly decreased viability in L-428 and L-1236 cells (Fig. 5*a*). After 48 and 72 hr of incubation, 2.5 μM curcumin significantly decreased KM-H2 cell viability

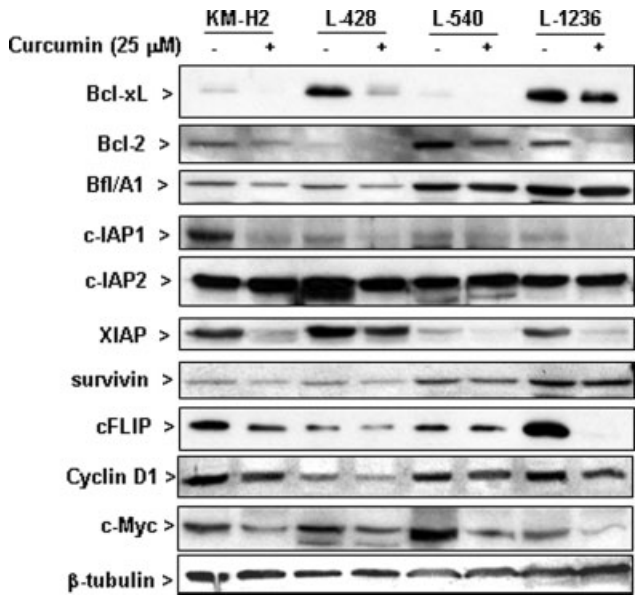

FIGURE 4 – Curcumin inhibits the expression of NF-κB- and STAT3-regulated proteins involved in the regulation of H-RS cells cell proliferation and survival. KM-H2, L-428, L-540 and L-1236 cells were incubated for 24 h in the absence or presence of 25 μM curcumin. The content of different antiapoptotic and pro-proliferative proteins was evaluated by Western blot in total cell fractions. Representative images out of 3 independent experiments are shown.

while 5 μM curcumin significantly decreased cell viability in the other 3 cell lines studied (Fig. 5*a*).

The effects of the Jak2 inhibitor AG490 on H-RS cell viability were next investigated and compared to those of curcumin. As depicted in Figure 5*b*, 25 μM curcumin was more effective than 50 μM AG490 in decreasing viability in KM-H2 and L-540 cells. For each of the time periods studied, curcumin was remarkably more effective than AG490 in decreasing KM-H2 and L-540 cell viability. This could be explained by the fact that while curcumin inhibited both NF-κB and STAT3 transcription factors, AG490 inhibited only STAT3 (Fig 5*c*).

Since NF-κB and STAT3 promote cell cycle progression by regulating the expression of several genes involved in the cell cycle machinery, such as cyclin D1 and c-Myc, we next evaluated the effects of curcumin on the expression level of cyclin D1 and c-Myc by Western blot analysis. The treatment of the cells with 25 μM curcumin for 24 hr down-regulated the expression of both cyclin D1 and c-Myc in all the H-RS cell lines studied (Fig. 4).

Curcumin inhibits cell cycle progression

To investigate the mechanism involved in the decrease of cell viability induced by curcumin, we analyzed the cell-cycle distribution of KM-H2 and L-428 cells using flow cytometry. The treatment with 10 μM curcumin induced G2/M arrest in both, KM-H2 and L-428 cells. Figure 6 shows that the percentage of cells in G2/M phase was significantly higher in the cells treated with curcumin than in cells treated with DMSO (23% *vs*. 11%, and 39% *vs*. 17% for KM-H2 and L-428 cells, respectively).

Curcumin induces H-RS cell death by apoptosis

One hallmark of apoptosis is the activation of caspases. Figure 7*a* shows that curcumin treatment induced the activation of caspase-9, as evidenced by a higher content of cleaved caspase-9 in the H-RS cell lines studied. The incubation of cells with curcumin also led to caspase-3 activation, as evidenced by the cleavage of the 116 kDa PARP protein into an 85 kDa fragments. An increase in the 85 kDa peptide was observed in all the studied cell lines except for L-428 (Fig. 7*a*). However, a decrease of the 116 kDa peptide was observed in L-428 cells. Control cells (incubated with DMSO) do not show any detectable PARP cleavage.

The induction of H-RS cell apoptosis by curcumin was also investigated evaluating the apoptotic morphology of cell nuclei by Hoechst staining using fluorescence imaging. As an evidence of apoptosis, the nuclei of KM-H2, L-428 and L-540 cells incubated with 25 μM curcumin were smaller, and with a more compacted chromatin than the nuclei of cells treated with DMSO (Fig. 7*b*).

The ability of curcumin to induce apoptotic cell death in H-RS cells was further confirmed by Annexin V (an indicator of phosphatidylserine externalization) and propidium iodide staining. Figure 7*c* shows a time-dependent increase in the percentage of apoptotic cells (annexin V-positive cells) in KM-H2, L-428 and L-540 cells. After 48 hr incubation with curcumin, the percentage of apoptotic cells was 46%, 38% and 79% for the KM-H2, L-428 and L-540 cell lines, respectively (Fig. 7*c*). The obtained results on the activation of caspases, nuclear morphology changes, and phosphatidylserine externalization, show that curcumin induces apoptotic cell death in all the H-RS cells studied.

Discussion

Because of the central role in cell survival and proliferation, both NF-κB and STAT3 transcription factors are proposed to be important molecular markers in the malignant transformation process of cells.[28,29] Therefore in the current study, these transcription factors were characterized as targets for the treatment of HL. The novel finding that curcumin is rapidly incorporated into the cells inhibiting constitutively NF-κB and STAT3 activation, H-RS cell proliferation, and inducing cell death by apoptosis, provides rationale for the potential use of curcumin as a therapeutic agent for patients with HL. NF-κB and STAT3 transcription factors play a major role in the pathogenesis of HL. The oncogenic significance of activated NF-κB and STAT3 molecules is due to their effects on numerous parameters of the development and progression of malignancy, such as cell proliferation and apoptosis. In neoplastic and preneoplastic cells, inhibition of proliferation and induction of apoptosis can be regarded as a therapeutic function intended to eliminate malignant cells.[30]

Curcumin was incorporated into H-RS cells reaching a maximum steady state level within 1 and 2 hr of incubation. The values at time zero probably reflect a rapid incorporation into membranes, due to curcumin hydrophobic nature, or to its adsorption to membranes. After 24 hr, cell curcumin content was similar to time zero values (KM-H2) or almost undetectable (L-1236). The finding that the observed effects of curcumin on cell signaling, proliferation and apoptosis extend beyond the time of its maximal presence in the cells, can be explained by the fact that: (*i*) not only curcumin but curcumin metabolites could be the actual effectors; (*ii*) many of the observed effects require long term events, *e.g.,* protein synthesis, that are not expected to show the same kinetics than that of curcumin accumulation; and/or (*iii*) curcumin triggers irreversible events that drive long term effects on cell signaling as well as on cell fate.

Curcumin down-regulated the constitutive NF-κB activity in mantle cell lymphoma[31] and in human multiple myeloma cells.[32] Similarly, in the current study, a dose- and time-dependent suppression of NF-κB constitutive activity was observed in all H-RS cell lines. Curcumin inhibited NF-κB-DNA binding in KM-H2 and L-428 cells despite their lack of functional IκBα.[25,26] In mantle cell lymphoma cells, curcumin was shown to inhibit IKK activation, IkBα phosphorylation and RelA nuclear phosphorylation.[31] Thus, in KM-H2 and L-428 cells, curcumin could be inhibiting NF-κB-DNA binding by preventing RelA nuclear phosphorylation an important step before NF-κB-DNA binding. Regarding the STAT3 pathway, curcumin inhibits the constitutive activation of STAT3 in head and neck squamous cell carcinoma cells and

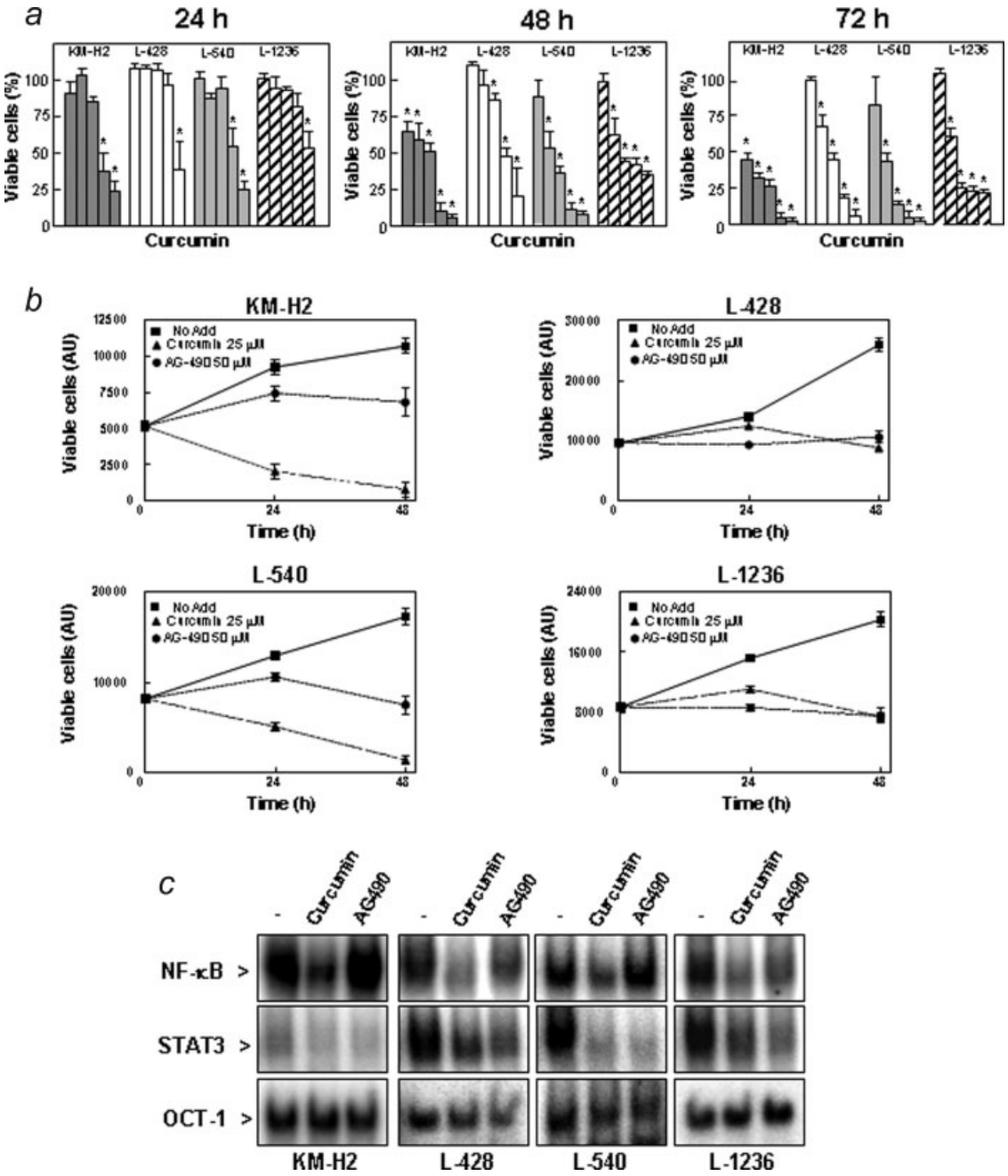

FIGURE 5 – Curcumin inhibits H-RS cell viability in a dose- and time-dependent manner. (*a*) Cell viability was determined in KM-H2, L-428, L-540 and L-1236 cells after 24, 48 or 72 h of incubation without or with DMSO or 2.5, 5, 10, 25 or 50 μM curcumin (bars from left to right for each type of cell). DMSO incubation did not significantly affect cell viability. Data for curcumin-treated cells are expressed as percentage of the DMSO control for each incubation time. Values are shown as means ± SEM of 5 independent experiments. *Significantly different from cells incubated in the absence of curcumin ($p < 0.05$, one way ANOVA test). (*b*) Comparative effects of AG490 and curcumin on KM-H2, L-428, L-540 and L-1236 cell viability determined after 0, 24 or 48 h of incubation without or with 25 μM curcumin, or 50 μM AG490. Results are shown as means ± SEM of 3 independent experiments. (*c*) NF-κB-, STAT3- and OCT-1-DNA binding were measured by EMSA in nuclear fractions isolated from KM-H2, L-428, L-540 and L-1236 cells that were incubated for 24 h without (−) or with 25 μM curcumin or 50 μM AG490. Representative images out of two independent experiments are shown.

human multiple myeloma cells, respectively.[33,34] In head and neck squamous cell carcinoma cells, curcumin inhibited the STAT3 constitutive activation as early as 15 min after incubation, and with 25 μM curcumin inhibition was complete. In L-428 cells, we observed a significant inhibitory effect on STAT3-DNA binding after 3 hr incubation. After 24 hr incubation with curcumin, the inhibition of STAT3-DNA binding was almost complete in KM-H2 and L428 cells. Furthermore, 5 μM curcumin was sufficient to significantly inhibit STAT3 constitutive activation in L-428 cells.

Among the kinases known to phosphorylate STAT3, those inhibited by curcumin are not fully elucidated. Treatment of T cells with curcumin resulted in the complete inhibition of the JAK2 phosphorylation.[35] In agreement, we observed that the incubation with curcumin led to the inhibition of the JAK2 phospho-

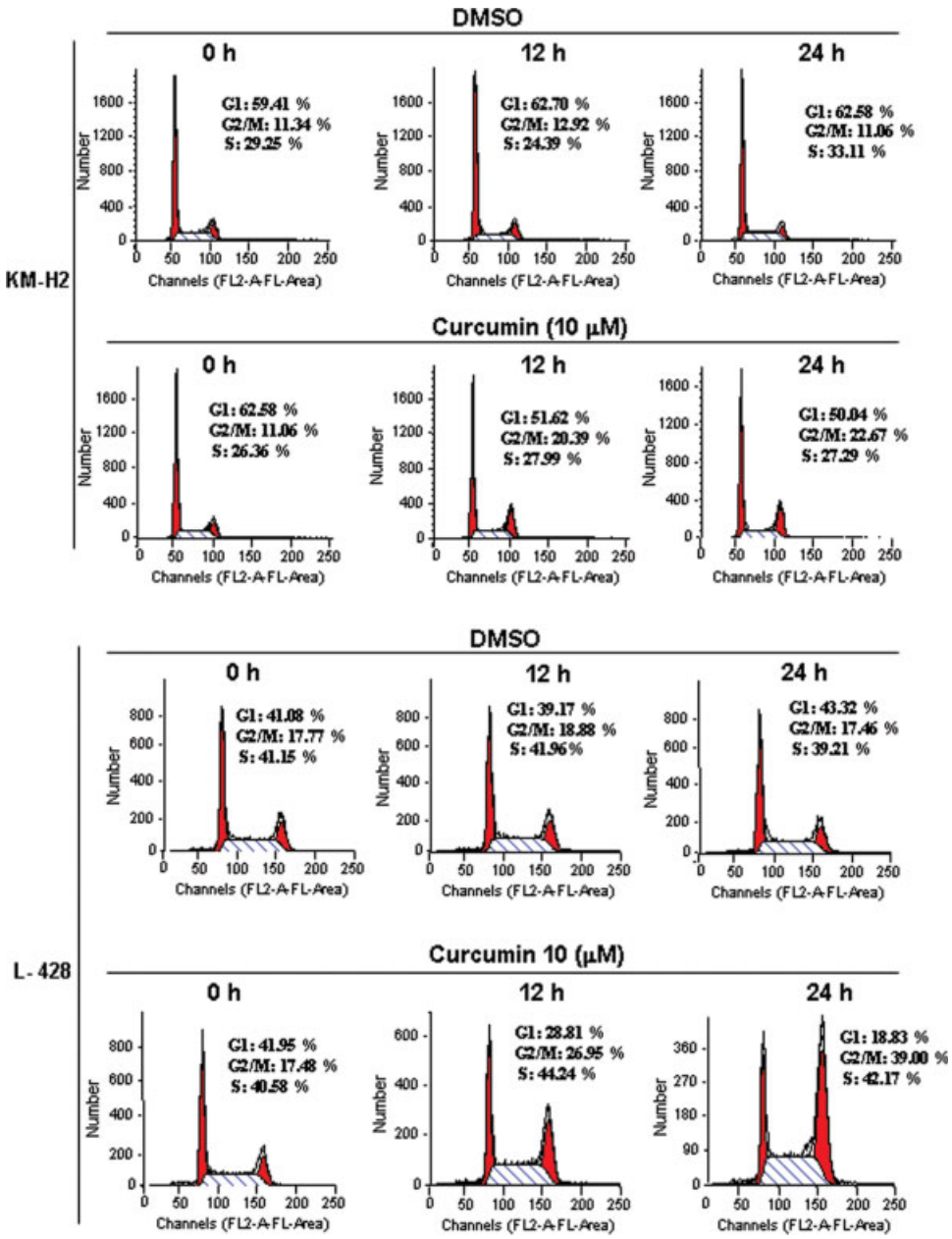

FIGURE 6 – Curcumin inhibits cell cycle progression in H-RS cells. Cell cycle progression was measured in KM-H2 and L-428 cells after 0, 12 or 24 h of incubation without or with 10 μM curcumin. DNA content was determinate from IP fluorescence. Representative profiles of the distribution of cells in G1, G2/M and S phases, out of 4 independent experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rylation in all the H-RS cells studied. JAK2 inhibition could explain the observed inhibitory effects of curcumin on STAT3 phosphorylation in all the H-RS cells studied. In accordance to our results, curcumin inhibited STAT3 phosphorylation in both MT-2 and SLB-1 T cell leukemia lines.[36] In contrast, in K562 chronic leukemia cells, although curcumin induced a decrease of nuclear STAT3, it had no effect inhibiting STAT3 phopsphorylation.[37] The capacity of curcumin to inhibit nuclear NF-κB- and STAT3-DNA binding was associated with the inhibition of NF-κB- and STAT3-regulated gene expression. In this regard, curcumin caused a reduction in the expression of genes involved in cell proliferation (cyclin D1 and c-Myc) and anti-apoptosis (IAPs, cFLIP, survivin, Bcl-xL and Bcl-2).

The treatment of H-RS cells with curcumin caused a dose- and time-dependent decrease in cell viability. Since curcumin can effectively inhibit the activation of both NF-κB and STAT3 activity in H-RS cell lines, curcumin should be expected to inhibit cell proliferation and induce apoptosis more efficiently than specific inhibitors of either transcription factor alone. In fact, we observed that AG490 was less effective in decreasing H-RS cell viability than curcumin. These results are in agreement with those observed in mantle cell lymphoma cells[31] and in multiple myeloma cells.[33,34] As AG490 acts in general decreasing cell viability in all the H-RS cells tested, it can be speculated that the STAT pathway is important in H-RS cell proliferation. AG490 is a potent inhibitor of the STAT3 pathway that is capable of reversing apoptosis resistance in HL by inducing cell cycle arrest and reducing cell proliferation.[8] Holtick *et al*. also raised the possibility that anti-apoptotic genes are regulated in H-RS cells in a redundant or cooperative manner by STAT and NF-κB transcription factors.[8]

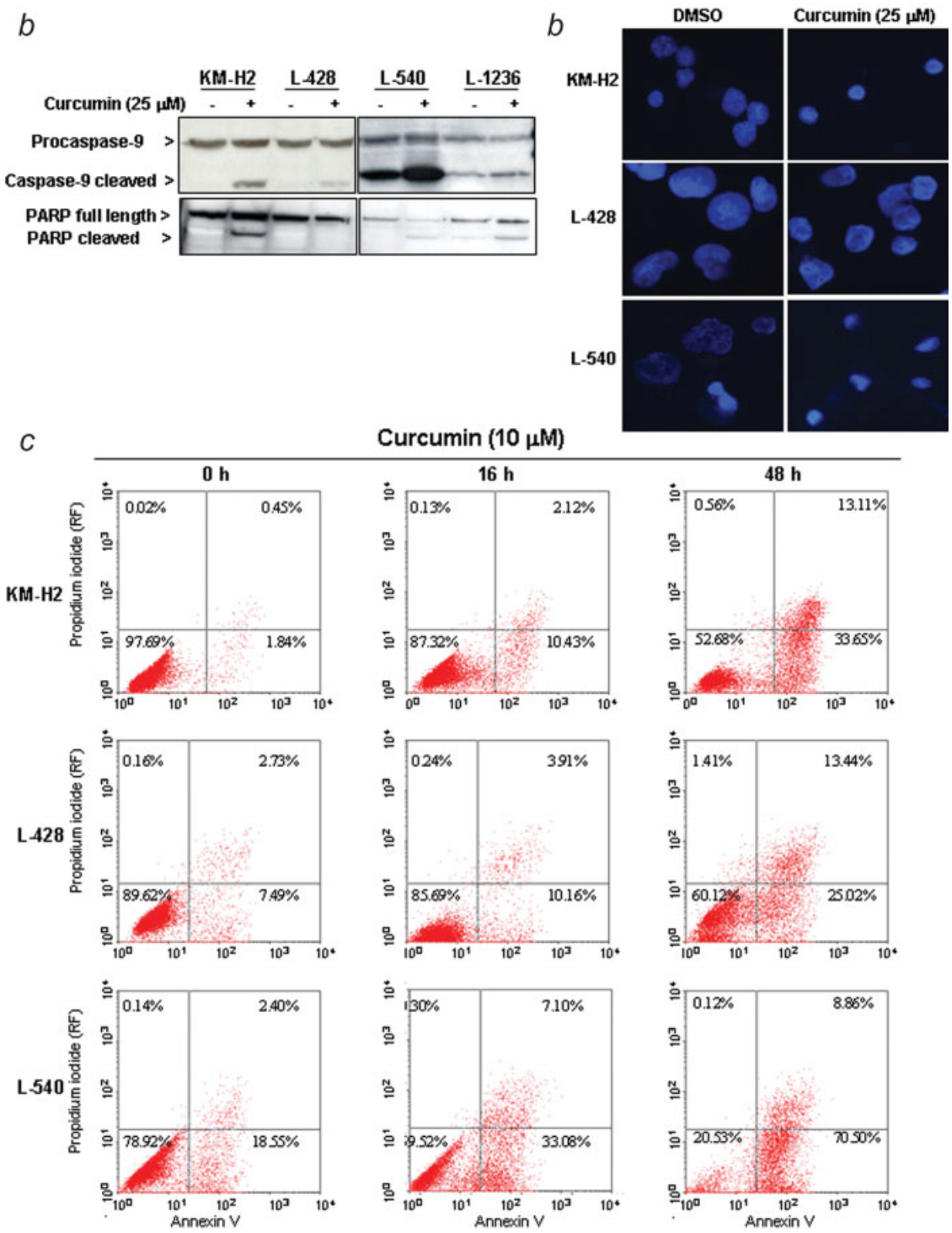

Figure 7 – Curcumin induces apoptosis in H-RS cells. (*a*) Caspase 9 and PARP protein levels were measured by Western blot in total cell extracts isolated from KM-H2, L-428, L-540 and L-1236 cells incubated for 24 h without (−) or with (+) 25 μM curcumin. Representative images are shown. (*b*) Nuclear morphology determined by Hoechst staining in KM-H2, L-428 and L-540 cells incubated for 24 h with DMSO or 25 μM curcumin. (*c*) Phosphatidylserine externalization as a marker of apoptosis, determined using annexin V; and cell necrosis determined by propidium iodide fluorescence were evaluated in KM-H2, L-428 and L-540 cells incubated with 10 μM curcumin for 0, 16 or 48 h. The percentages of apoptotic cells were determined using the dual staining with annexin V and propidium iodide and are indicated in each quadrant: left bottom quadrant, viable cells (annexin V-negative/PI-negative); right bottom quadrant, early apoptotic cells (annexin V-positive/PI-negative); right upper quadrant, late apoptotic cells (annexin V-positive/PI-positive); left upper quadrant, necrotic cells (annexin V-negative, PI-positive). Representative images out of 4 independent experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

While NF-κB activation was not affected by AG490, we observed that curcumin, which can inhibit both NF-κB and STAT3, is more effective in inhibiting cell proliferation than AG490 alone. These findings stress the relevance of inhibiting both NF-κB and STAT3 in the development of targeted therapies for HL.

The decrease in cell viability could be associated with a decreased cell proliferation and/or an increase in apoptotic cell death. We observed that curcumin caused a reduction in the expression of cyclin D1. Cyclin D1 plays a key role in cell proliferation through activation of cyclin-dependent kinases.[31] Furthermore, cyclin D1 is required for the progress of cells from the G1phase to the S phase of the cell cycle.[2] Mukhopadhyay *et al*. have shown that curcumin blocks the proliferation of various prostate, breast and squamous cell carcinoma cell lines by down-regulating the expression of cyclin D1 protein.[38] This could explain the results from earlier reports in several cancer cells showing that curcumin induces G1/S cell cycle arrest and thus inhibits cell proliferation.[2] However, we observed that in KM-H2 and L-428 cells, curcumin induced cell cycle arrest in G2/S phase, instead of G1/S phase. In concordance with our results, in human melanoma cells, curcumin arrested cell growth at the G2/M phase and induced apoptosis by inhibiting NF-κB activation.[4] On the other hand, in deregulated cyclin D1-expressed mammary epithelial carcinoma cells, curcumin failed to arrest these cells at G0 phase of cell cycle.[30] Another NF-κB and STAT3-regulated protein involved in cell proliferation is c-Myc. The proto-oncogene c-Myc regulates multiple aspects of cell proliferation, and its aberrant expression is frequently seen in multiple human cancers.[39] We also observed that curcumin down-regulated the expression of c-Myc in all the H-RS cells studied. The reduced expression of cyclin D1 and c-Myc as well as the associated cell cycle arrest can in part explain the decrease in the number of viable cells induced by curcumin in the H-RS cell lines.

A possible mechanism underlying the induction of apoptosis by curcumin could be its capacity to inhibit NF-κB- and STAT3-regulated anti-apoptotic proteins. Bcl-2 and Bcl-xL prevent the process of mitochondrial release of pro-apoptotic factors, such as cytochrome c.[40] Treatment of H-RS cells with curcumin caused a reduction in Bcl-2 and Bcl-xL expression. Interestingly, in H-RS cells both proteins have been implicated in cell survival; and the deregulation of Bcl-2 and Bcl-xL results in increased susceptibility to cell death.[33,41] In the current study, curcumin also down-regulated the expression of the other NF-κB-dependent anti-apoptotic proteins: A1/Bfl 1, c-IAP, XIAP, survivin and c-FLIP.[31] The overexpression of A1/Bfl 1, an hematopoietic-specific Bcl-2 homologue, prevents mitochondrial depolarization, the release of cytochrome c and caspase-9 activation.[42] c-IAPs are known for inhibiting both extrinsic and intrinsic pathways of apoptosis. c-IAPs, XIAP and survivin directly bind and inhibit effector caspases, acting downstream of initiator caspases. The constitutive expression of c-FLIP could be a critical mechanism for the survival of H-RS cells by providing resistance to CD95-mediated apoptosis.[43] Significantly, we observed that c-FLIP was down-regulated by curcumin in H-RS cells.

Similar to that observed in animal models, in human studies curcumin supplementation has mostly failed to allow a therapeutically relevant presence/accumulation of curcumin and curcumin metabolites in blood or organs.[44] In this regard, several methods to improve curcumin bioavailability are currently being investigated.[44] Targeted and triggered drug delivery systems accompanied by nanoparticle technology are emerging prominent solutions to the bioavailability of potential therapeutic agents, such as curcumin.

In summary, the current study demonstrates that curcumin has the ability to inhibit both NF-κB and STAT3 constitutive activation in H-RS cells. The ability of curcumin to decrease H-RS cell viability is due to its capacity to decrease cell proliferation by causing cell cycle arrest, and by inducing apoptotic cell death. The observed effects combined with the well-established pharmacological safety of curcumin, provides rationale for the potential use of curcumin as a new therapeutic agent for patients with HL.

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