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Spleen Tyrosine Kinase (Syk), a Novel Target of Curcumin, Is Required for B Lymphoma Growth

Murali Gururajan,*†† Trivikram Dasu,*† Seif Shahidain,*† C. Darrell Jennings,§ Darrell A. Robertson, ‡ Vivek M. Ranjanekar,*†¶ and Subbarao Bondada2*†‡¶

Curcumin (diferuloylmethane), a component of dietary spice turmeric (Curcuma longa), has been shown in recent studies to have therapeutic potential in the treatment of cancer, diabetes, arthritis, and osteoporosis. We investigated the ability of curcumin to modulate the growth of B lymphomas. Curcumin inhibited the growth of both murine and human B lymphoma in vitro and murine B lymphoma in vivo. We also demonstrate that curcumin-mediated growth inhibition of B lymphoma is through inhibition of the survival kinase Akt and its key target Bad. However, in vitro kinase assays show that Akt is not a direct target of curcumin. We identified a novel target for curcumin in B lymphoma viz spleen tyrosine kinase (Syk). Syk is constitutively activated in primary tumors and B lymphoma cell lines and curcumin down-modulates Syk activity accompanied by down-regulation of Akt activation. Moreover, we show that overexpression of Akt, a target of Syk, or Bcl-xL, a target of Akt can overcome curcumin-induced apoptosis of B lymphoma cells. These observations suggest a novel growth promoting role for Syk in lymphoma cells. The Journal of Immunology, 2007, 178: 111–121.

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2 Address correspondence and reprint requests to Dr. Subbarao Bondada, Center on Aging, University of Kentucky, Room 329A, Sanders Brown Building, Lexington, KY 40536-0230. E-mail address: bondada@uky.edu
3 Abbreviations used in this paper: NHL, non-Hodgkin’s lymphoma; DLBCL, diffuse large B cell lymphoma; mTOR, mammalian target of rapamycin; Syk, spleen tyrosine kinase; PI, propidium iodide; MyrAkt, myristoylated Akt; GSK, glycogen synthase kinase-3; PDK, phosphoinositide dependent kinase-1; siRNA, small interfering RNA; PARP, poly(ADP-ribose) polymerase.

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FIGURE 1. Curcumin inhibits growth of B lymphoma. B lymphoma cells (WEHI-231, murine B lymphoma in A and OCI Ly-10, human DLBCL in B) were cultured for 48 h with vehicle (DMSO) alone or with indicated concentrations of curcumin and proliferation measured as described in Materials and Methods. Ly-10 human B lymphoma cells (DLBCL) were cultured in 6 well plates at 1 × 10^6 cells/well in the presence of 0, 5, and 10 μM doses of curcumin. Two days later, cell cycle analysis was performed on these cultured cells by Propidium Iodide staining as described in Materials and Methods.
we show here for the first time that curcumin directly inhibits activation of Syk in vitro as well as in vivo, an enzyme essential for BCR signal transduction. Moreover, we find that Syk is constitutively activated in a variety of B lymphoma cell lines and primary B lymphoma samples but not in primary B cells. Thus, we demonstrate a novel growth promoting role for Syk, which has been considered a tumor suppressor in certain nonlymphoid cancers.

Materials and Methods

Reagents

Curcumin (diferuloylmethane) obtained from Sigma-Aldrich was dissolved in DMSO and was then diluted to 2 mM in culture medium as needed. Phosphospecific Abs against Syk (525/526), Lyn, Akt (Ser473), glycogen synthase kinase-3 (GSK3β) and Bad, and Ab against Akt were obtained from Cell Signaling Technologies. Ab to Bcl-x was obtained from BD Pharmingen. Monoclonal anti-β-actin Ab was obtained from Sigma-Aldrich. Caspase inhibitor ZVAD-fmk was obtained from Kamiya Biochemicals and primary B lymphoma samples but not in primary B cells. Thus, we demonstrate a novel growth promoting role for Syk, which has been considered a tumor suppressor in certain non-lymphoid cancers.

Materials and Methods

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Cells and mice

The panel of B cell lymphomas included cells of murine and human origin. Of murine origin were BKS-2, CH31, WEHI-231, and the WEHI-231 Bcl-xL; those of human origin included Ramos and the human DLBCL cell lines OCI-Ly7 and OCI-Ly10. The immature B lymphoma cell line BKS-2 was isolated and maintained in vivo as a splenic tumor in our laboratory (25). Female CBA/N (Xid) mice were obtained from The Jackson Laboratory. B lymphoma cells were incubated with indicated doses of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Abs to cleaved capase-9, cleaved PARP, and PARP, which were then stripped and probed for β-actin. Results are representative of three experiments. G. Ly-10 B lymphoma cells were incubated with indicated doses of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Abs to cleaved capase-9, cleaved PARP, and PARP, which were then stripped and probed for β-actin. Results are representative of two experiments.

Table I. Cell cycle analysis of WEHI-231 B lymphoma cells treated with curcumin for 48 h

<table>
<thead>
<tr>
<th>WEHI-231</th>
<th>Sub-G₁</th>
<th>G₁</th>
<th>S</th>
<th>G₂-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>3 ± 1</td>
<td>37 ± 2</td>
<td>32 ± 4</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>Curcumin (5 μM)</td>
<td>10 ± 2</td>
<td>48 ± 1</td>
<td>22 ± 3</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Curcumin (10 μM)</td>
<td>60 ± 1</td>
<td>32 ± 2</td>
<td>5 ± 1</td>
<td>3 ± 3</td>
</tr>
</tbody>
</table>

Values presented are percentage of cells in indicated phases of cell cycle. Phase of cell cycle was determined by flow cytometric analysis of PI-stained cells. Experiments were performed three times with similar outcomes.

Table II. Apoptosis analysis of WEHI-231 B lymphoma cells (murine) treated with curcumin for 24 h

<table>
<thead>
<tr>
<th>Control</th>
<th>MyrAkt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V-Positive Cells (%)</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Curcumin (5 μM)</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Curcumin (10 μM)</td>
<td>74 ± 4</td>
</tr>
</tbody>
</table>

Values presented are percentage of cells that bind annexin V as determined by flow cytometric analysis. Experiments were performed two to three times with a similar outcome.

of triplicate cultures ± SE and statistical significance of different treatments was evaluated by the Student t test.

Western blotting and flow cytometry

B lymphoma cells were treated with curcumin at concentrations from 2.5 to 20 μM in 1 ml cultures of 1 × 10⁶ cells in 6-well plates (Corning/Costar). Cell lysates were prepared in 1× SDS sample buffer or 1% Triton X-100 as described earlier and were subjected to SDS-PAGE and Western blot analysis (26). The Western blots were analyzed by probing the membrane using various primary Abs followed by HRP-conjugated secondary Abs (Santa Cruz Biotechnology). The blots were developed with Pico Chemiluminescence substrate (Pierce Biotechnology) and exposed to Kodak X-O mat film which was scanned with a flat-bed scanner (UMAX Technologies). Alternatively, the blots were scanned by a Kodak Image Station 2000RT (Eastman Kodak). For reprobing, membranes were stripped using a solution containing 62.5 mM Tris-HCl, 2% SDS, and 100 mM 2-ME at 65°C for 20 min. For flow cytometry, 1 × 10⁶ cells were stained with B220-PE and Annexin VFITC (BD Pharmingen).

In vitro kinase assays for Akt and Syk

For immunoprecipitation, cell lysates were precleared for 1 h at 4°C by protein G-Sepharose (Sigma-Aldrich) and then incubated with Abs to Akt, mTOR, Syk, or Igo. For Akt kinase assays, immunoprecipitated Akt or mTOR was incubated with 10 μM ATP, GSK3β (direct substrate for Akt), or RAK (for mTOR-mediated Akt phosphorylation) and then the reaction was stopped after 30 min by adding 1× SDS sample buffer. For Syk kinase assay, immunoprecipitated Syk was incubated with ATP and different doses of curcumin or vehicle for 30 min at room temperature and then the reaction was stopped by adding 1× SDS sample buffer. Western blotting of the lysates was performed as described earlier in this section.

Apoptosis and cell cycle analysis

The cell cycle status and apoptosis were analyzed using propidium iodide (PI) and annexin V staining. Cultured B lymphoma cells (1 × 10⁶/2 ml) were treated with varying doses of curcumin and then fixed in ethanol for at least 1 h at 4°C, after which cells were incubated in a mixture of 1 μg/ml PI (Sigma-Aldrich) and 25 μg/ml RNase A (Sigma-Aldrich) at 37°C for at least 30 min. The level of PI fluorescence was measured with a MoFlo flow cytometer (DakoCytomation). Cells in the sub-G₁ region were considered to be apoptotic.

Transfection of plasmids and small interfering RNA (siRNA) into B lymphoma cells

For myristoylated Akt (MyrAkt) overexpression studies, WEHI-231 B lymphoma cells were transfected with GFP alone (pEGFP-N1) or cotransfected with GFP and MyrAkt expression vector (29) by electroporation at

D. Burkitt’s B lymphoma cells from human patients were cultured for 48 h with vehicle (DMSO) alone or with indicated concentrations of curcumin and proliferation measured as described in Materials and Methods. Results are representative of three experiments. E. Ly-10 human B lymphoma cells (DLBCL) were cultured as in C in the presence or absence of 10 μM curcumin and the caspase inhibitor, ZVAD-fmk. Two days later, cell cycle analysis was performed on these cultured cells by PI staining. F. Ly-10 human B lymphoma cells (DLBCL) were cultured in 96-well plates at 3 × 10⁴ cells/well in the presence or absence of 5 μM dose of curcumin and 10 μM caspase inhibitor. Two days later, proliferation was measured as described in Materials and Methods. *, p < 0.05 when comparing response with curcumin to solvent only treatment. Results are representative of three experiments. G. Ly-10 B lymphoma cells were incubated with indicated doses of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to cleaved capase-9, cleaved PARP, and PARP, which were then stripped and probed for β-actin. Results are representative of two experiments.
250 mV, 960 μF, and 200 Ohm with a Gene Pulser electroporator (Bio-Rad). Two days later, cells were treated with 5 and 10 μM curcumin. Forty-eight hours after treatment, cells were analyzed for apoptosis by Annexin V staining. For RNA interference studies, lymphoma cells were cotransfected with a GFP plasmid and control or Syk specific siRNA (Dharmacon). Forty-eight hours after transfection, cells were treated with curcumin and were analyzed for cell cycle 48 h after treatment by Hoechst staining.

**In vivo studies**

Female CBA/N mice were administered i.v. with 10 × 10⁶ BKS-2 B lymphoma cells on day 0. From day 1, mice were injected i.p. either with 40 mg/kg body weight curcumin or the vehicle (DMSO) alternate days for 10 days. On day 11, mice were sacrificed and the number of nucleated cells was determined.

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### Table III. Apoptosis analysis of OCI-Ly10 B lymphoma cells treated with curcumin for 24 h

<table>
<thead>
<tr>
<th>OCI-Ly10</th>
<th>Annexin V-Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Curcumin (5 μM)</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Curcumin (10 μM)</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

* Values presented are the percentage of cells that bind annexin V as determined by flow cytometric analysis. Experiments were performed three times with similar outcome.

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**Results**

**Curcumin causes growth arrest and apoptosis of B lymphoma cells**

Previously, we reported that curcumin inhibited the growth of murine B lymphoma BKS-2. Here, we tested the effect of curcumin on multiple cell lines including those of human origin to study the importance of curcumin as a potential antilymphoma agent and to identify its intracellular target. We demonstrate here that curcumin causes a dose-dependent decrease in proliferation of WEHI-231 (mouse B lymphoma) and OCI-Ly-10 (human DLBCL) cells as measured by \(^{3}H\)thymidine incorporation. DMSO is used as vehicle control and the concentration of DMSO used here has no toxicity for these cells (Fig. 1, A and B). Similarly, growth of DLBCL cells OCI-Ly-7 and SUDHL-4 was also inhibited by 10 μM curcumin by 90% (data not shown). We investigated whether the decrease in proliferation was due to growth arrest, apoptosis, or both. Cell cycle analysis performed by staining DNA with PI demonstrated an increase in sub-G₁ population, an indicator of apoptotic cells and a decrease in cells in the S phase of cell cycle (Fig. 1C for OCI-Ly10 and Table I for WEHI-231). The apoptotic population increased from 11 ± 1% to 23 ± 2% in Ly-10 B lymphoma cells (DLBCL) when treated with 10 μM curcumin for 48 h whereas the cells in S phase decreased from 54 ± 2% to 26 ± 3%.

**FIGURE 2.** Bcl-xL is down-regulated in curcumin-treated B lymphoma cells and its overexpression rescues WEHI-231 B lymphoma cells from curcumin-induced apoptosis. Ly-10 DLBCL B lymphoma cells (A) or WEHI-231 mouse lymphoma cells (B) were incubated with indicated doses of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to Bcl-xL which were then stripped and probed for β-actin. C, PI analysis of WEHI-231 or WEHI-231 stably transfected with Bcl-xL (WEHI-Bcl-xL) in the presence or absence of indicated concentrations of curcumin. Similar results were obtained in two other experiments. Numbers indicate the ratio of Bcl-xL to β-actin in A and B.
Because the sub-G1 population identified by PI staining could represent apoptotic or necrotic cells, we performed annexin V staining, which distinguishes apoptotic and necrotic cells. Apoptotic population increased from 16/10062% to 74/10064% in WEHI-231 B lymphoma cells and from 4/10061% to 16/10061% in OCI-Ly10 B lymphoma cells treated with 10/1006M curcumin (Tables II and III).

Thus, curcumin inhibits the growth and survival of both murine and human B lymphoma cells. The effects of curcumin are not limited to cell lines, since curcumin induced a dose-dependent decrease in proliferation of primary human malignant Burkitt’s cells (Fig. 1D).

Curcumin-induced apoptosis is caspase dependent

Caspases are cysteine proteases which upon activation can induce apoptosis (30). Using thymidine incorporation and cell cycle analysis, we show here that curcumin-induced apoptosis is caspase dependent. At 10 μM concentration of a pan-caspase inhibitor, Z-VAD-fmk, the proliferation of OCI-Ly10 DLBCL lymphoma cells was not inhibited by curcumin. PI analysis revealed that curcumin-induced apoptosis was inhibited by the caspase inhibitor (Fig. 1E). Moreover, curcumin-induced cell cycle arrest was also reversed by ZVAD-fmk (Fig. 1, E and F) suggesting that cell cycle arrest is a secondary consequence of curcumin-induced apoptosis. Moreover, we demonstrate that curcumin-induced apoptosis is caspase dependent because poly(ADP-ribose) polymerase (PARP), a substrate of caspase, is cleaved in the presence of curcumin in addition to caspase-9 cleavage (Fig. 1G).

**Overexpression of Bcl-xL overcomes curcumin-induced apoptosis**

Expression of Bcl-xL, an antiapoptotic factor overexpressed in many B lymphomas, is regulated by NF-κB. Curcumin has been shown to inhibit the NF-κB pathway in many cancer cells including multiple myeloma (11, 12). Accordingly, we found that Bcl-xL is down-regulated in OCI-Ly10 and WEHI-231 cell lines upon treatment with curcumin suggesting that curcumin targets the NF-κB pathway in B lymphoma cells (Fig. 2, A and B). Then, we determined whether overexpression of Bcl-xL, an NF-κB target gene, overcomes curcumin-induced apoptosis in B lymphoma cells. Using a WEHI-231 cell line, which is stably transfected with Bcl-xL (31), we observed no difference in apoptosis between untreated and curcumin-treated cells. But WEHI-231 cells which

**FIGURE 3.** Curcumin-induced growth inhibition is Akt dependent. A, Ly-10 B lymphoma cells (DLBCL) were incubated with medium, 10 μM and 20 μM of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to Phospho-Akt. The blots were then stripped and probed with Akt or anti-β-actin Ab to correct for changes in protein loading in different lanes. Early time points are shown in the top panel and later points are shown in the bottom panel. B, Ly-10 B lymphoma cells (DLBCL) were incubated with different doses of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to phospho-Bad and then stripped and probed for β-actin. C, Ly-10 B lymphoma cells (DLBCL) were incubated with medium or 10 μM or 20 μM of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to phospho-GSK3β and then stripped and probed for β-actin. Numbers indicate the ratio of pGSK-3 to actin in C. Results from one of three similar experiments are shown.
Activation of key enzymes like GSK-3β phosphorylation at serine 473 and threonine 308 by upstream Akt is a serine/threonine kinase which critically regulates cell survival and cell growth in many cell types (17, 32). Akt is activated by phosphorylation at serine 473 and threonine 308 by upstream kinases. Akt is a constitute active form of Akt (MyrAkt) overcome curcumin-induced apoptosis. WEHI-231 B lymphoma cells (murine) were transfected with the GFP plasmid alone or cotransfected with the GFP and the MyrAkt plasmids. Two days later, cells were treated with indicated concentrations of curcumin. Twenty-four hours after curcumin treatment, GFP-positive cells were analyzed for Annexin V staining as described in Materials and Methods. B. Western analysis of lysates from control or MyrAkt transfected WEHI-231 (murine) by an Ab to phosphorylated Akt. Phosphorylated MyrAkt has been found to have lower mobility in several previous studies. The blots were then stripped and reprobed for Akt as a loading control. Results are representative of three experiments.

Curcumin inhibits phosphorylation of survival kinase Akt

Akt is a serine/threonine kinase which critically regulates cell survival and cell growth in many cell types (17, 32). Akt is activated by phosphorylation at serine 473 and threonine 308 by upstream kinases. Activated Akt has been shown to phosphorylate at least 20 different substrates within the cell including activation of key survival proteins, inactivation of proapoptotic proteins like Bad, and activation of key enzymes like GSK-3β (15). Curcumin and its analog have been shown to inhibit phosphorylation of Akt in prostate cancer cells but the effect of curcumin on Akt in most cancers susceptible to curcumin so far has not been investigated (14). We found that curcumin inhibited phosphorylation of Akt at serine 473 (pAkt) as early as 30 min in a dose-dependent manner and the Akt inhibition persisted even at 24 h (Fig. 3A). Similar results were obtained when an Ab to phosphothreonine 308 was used to determine Akt phosphorylation (data not shown). Total Akt levels were not changed by curcumin treatment.

Curcumin inhibits phosphorylation of Akt substrates Bad and GSK3-β

Bad is a proapoptotic protein which binds to antiapoptotic proteins like Bcl-2 and Bcl-xL and inhibits their prosurvival activity. Akt is known to promote cell survival by phosphorylation of Bad and its subsequent degradation by the proteasomes (17). Western Blot analysis showed that the levels of phosphorylated Bad (pBad) were almost undetectable in OCI-Ly10 (DLBCL) cells treated with curcumin supporting the notion that down-regulation of Akt phosphorylation also leads to a reduction in its activity (Fig. 3B). Akt phosphorylates GSK3-β, another substrate, whose activity is inhibited by phosphorylation. GSK3-β facilitates glucose to glycogen conversion in the cells. There is a dose- and time-dependent decrease in phosphorylation of GSK-3β in curcumin-treated OCI-Ly10 cells further confirming that curcumin inhibited Akt activity (Fig. 3C).

Ectopic expression of a constitutively active form of Akt (MyrAkt) overcomes curcumin-induced apoptosis in B lymphoma cells

Because curcumin inhibits Akt phosphorylation, we sought to determine whether introduction of a constitutively active form of Akt (MyrAkt) can overcome curcumin-induced apoptosis in B lymphoma cells. MyrAkt has been used extensively as a way to overexpress active Akt (33–35). In the presence of 5 and 10 μM concentrations of curcumin, WEHI-231 B lymphoma cells transiently transfected with MyrAkt are resistant to apoptosis compared with cells transfected with vector alone at both 24 and 48 h time points (Fig. 4A or Tables II and Table IV). Expression of MyrAkt was confirmed by Western blot analysis (Fig. 4B). These findings demonstrate that inhibition of Akt pathway is critical for curcumin to induce apoptosis in B lymphoma cells.

In vitro Akt is not a direct target of curcumin and curcumin directly inhibits phosphorylation of Syk, a critical regulator of Akt activity

To see whether Akt is a direct target of curcumin, we performed an in vitro kinase assay using GSK3-β as substrate and immunoprecipitated Akt. Surprisingly, curcumin did not inhibit Akt-mediated GSK3-β phosphorylation in vitro suggesting that it acts upstream of Akt (Fig. 5A). Preliminary experiments suggested that curcumin did not affect the activity of phosphoinositide-dependent kinase-1 (PDK1) also an upstream regulator of Akt (data not shown). Recently, it has been shown that mTOR-riotor complex is involved in Akt activation (36). Using immobilized anti-mTOR Ab to isolate mTOR and its associated complex from B lymphoma cells, we studied mTOR-mediated phosphorylation of rAkt in vitro and found that curcumin had no effect in this assay also (Fig. 5B). In B cells, Akt activation has been shown to be dependent on Syk, as Syk-negative B lymphoma cells have a dramatic reduction in Akt phosphorylation (22, 37, 38). Activated Syk has been shown to activate PI3K either directly or indirectly through adaptors proteins like B cell adaptor protein (BCAP) (39). Active PI3K generates phosphatidylinositol 3,4,5-trisphosphate, a lipid necessary for membrane recruitment of pleckstrin homology (PH) domain containing proteins like Akt. Membrane recruitment of Akt is absolutely essential for its activation by kinases like PDK1 and integrin linked kinase-1 (ILK1) and for its autophosphorylation (36, 40).

Table IV. Apoptosis analysis of WEHI-231 B lymphoma cells (murine) treated with curcumin for 48 h

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MyrAkt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 ± 1</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Curcumin (5 μM)</td>
<td>10 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Curcumin (10 μM)</td>
<td>62 ± 1</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

*p = 0.05; values are presented as the percentage of cells in the sub-G1 phase of cell cycle. Phase of cell cycle was determined by flow cytomteric analysis of PI-stained cells. Experiments were performed two to three times with a similar outcome.
Ly10 (Fig. 5G, indeed curcumin strongly inhibited in vitro kinase activity by probing for tyrosine-phosphorylated Syk using a p-tyr Ab. As shown in Fig. 5E, the presence of varying concentrations of curcumin in DLBCL cells and B lymphoma cell lines with little or no phosphorylation in resting splenic B cells and PBMC B cells from healthy donors (Fig. 5, C and D).

Curcumin did not inhibit Lyn activity or phosphorylation of Igα, a component of the BCR-signaling complex

Because BCR cross-linking leads to activation of Syk in a Lyn- and Igα-dependent manner, we investigated whether curcumin-mediated down-regulation of Syk is due to targeting of Lyn or Igα activity. Interestingly, curcumin did not inhibit phosphorylation of Lyn as shown in Fig. 5E. Moreover, the phosphorylation of the tyrosine motifs of the Igα cytoplasmic tail remains unaffected in the presence of varying concentrations of curcumin in DLBCL cells (Fig. 5F).

To test whether Syk is a direct target of curcumin, we immunoprecipitated Syk and measured its autophosphorylation activity by probing for tyrosine-phosphorylated Syk using a p-tyr Ab. As shown in Fig. 5G, indeed curcumin strongly inhibited in vitro kinase activity of Syk immunoprecipitated from the DLBCL line Ly10 (Fig. 5G) or from the mouse B lymphoma line CH31 in a dose-dependent manner (data not shown). When BKS-2 (mouse) and OCI-Ly10 (human DLBCL) B lymphoma cell lines were treated with piceatannol, a Syk inhibitor, there was a dose-dependent reduction in the basal proliferation compared with vehicle-treated cells (Fig. 6A). Partially knocking down Syk with a specific siRNA decreases the growth of SUDHL-4 (DLBCL) B lymphoma cells accompanied by a decrease in the phosphorylation of Akt and a decrease in Bcl-xL, an NF-κB target gene (Fig. 6B). These results demonstrate that constitutively active Syk is required for B lymphoma growth and that blocking its activity by multiple agents including curcumin, piceatannol, and siRNA decreases lymphoma growth.

Curcumin inhibits B lymphoma growth in vivo

Because all the studies on the growth inhibitory effects on B lymphoma have been in vitro, we investigated the in vivo effectiveness of curcumin using our mouse model of B lymphoma, BKS-2. There was a significant reduction in the tumor burden of curcumin-treated but not vehicle (DMSO)-treated mice carrying the BKS-2 B lymphoma as measured by the total splenocyte numbers (p < 0.01) (Fig. 7A). Representative spleens from both vehicle and curcumin-treated mice are shown in Fig. 7B.

Discussion

NHL poses a serious challenge in terms of cure and recovery. Current therapies include chemotherapy and a mAb that targets CD20 Ag expressed by B cells leading to depletion of B cells from the patients (42, 43). Naturally occurring compounds derived from plants have been long shown to have antitumorigenic and anti-inflammatory properties. Curcumin, a spice used in Asian foods, has strong antitumor properties both in vitro and in vivo with little or no toxicity to normal cells at a wide range of doses (2, 44). We report here that curcumin induces a dose- and time-dependent apoptosis in B lymphoma cells of both murine and human origin in vitro and murine B lymphoma in vivo.

Previous studies from our laboratory demonstrated that curcumin induced both apoptosis and growth arrest (12). Current studies using caspase inhibitor and the Bcl-2 overexpressing cell line suggest that apoptosis is the primary mechanism of curcumin-induced growth inhibition. The proximal signaling pathways that are targeted by curcumin are poorly understood. One of the well-characterized targets for curcumin is the NF-κB pathway (10). Curcumin was shown to inhibit IκB kinase (IKK), an upstream component of the NF-κB pathway. Here, we showed that in B lymphoma cells curcumin inhibited the NF-κB pathway as evident by down-regulation of Bcl-xL, an antiapoptotic protein regulated by NF-κB. Moreover, overexpression of Bcl-xL partially overcame curcumin-induced apoptosis in B lymphoma cells.

The upstream signaling components that regulate NF-κB pathways are fairly well-characterized. Two such components are Akt and Syk. Akt is a serine/threonine kinase which was shown to promote survival and proliferation of a variety of cell types by modulating the activity or expression of a number of downstream effectors including Bad, p27, caspase-9, and Bcl-xL. Akt is shown to be dysregulated in a variety of tumors due to its overexpression, mutation of the oncogene Ras and inactivation of a key phosphatase, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (45). Two independent studies demonstrated that Akt regulates NF-κB in an IKK-dependent manner in cells treated with platelet-derived growth factor (PDGF) or TNF-α (46, 47). We observed a dose-dependent reduction in constitutive Akt phosphorylation in B lymphoma cells treated with curcumin. Inhibition of Akt is essential for curcumin-mediated growth inhibition of B lymphoma as overexpression of a constitutive form of Akt (MyrAkt) overcomes curcumin-induced apoptosis of lymphoma cells. This is consistent with a previous report where curcumin was shown to block TNF-induced Akt activation in a myeloid leukemia cell line (48). Moreover, we showed that phosphorylation of Bad and GSK-3β, two well-defined substrates of Akt were down-regulated in curcumin-treated B lymphoma cells. Thus, curcumin induces apoptosis of B lymphoma cells by inhibiting activation of a key survival kinase Akt.

Although curcumin-induced apoptosis is Akt dependent, surprisingly, curcumin did not directly inhibit Akt (in vitro kinase assay) or its recently identified key regulator mTOR/riCTOR complex. So, we hypothesized that curcumin-mediated inhibition of Akt is indirect. A variety of cytoplasmic tyrosine kinases are activated in response to BCR cross-linking and growth factor stimulation in B cells. Syk is a nonreceptor tyrosine kinase expressed by all hemopoietic cells including B, T, and NK cells (49). In the absence of Syk, immature B cells can be detected in the T cell zones of the spleen but fail to undergo maturation into recirculating B cells (50, 51). These results suggest that Syk is an essential transducer of BCR signals required for the transition of immature into mature recirculating B cells. Syk is shown to regulate Akt activation through activation of PI3K (20, 22, 52, 53). PI3K generates phosphatidylinositol 3,4,5-trisphosphate, a lipid necessary for membrane recruitment of Akt. Presently, the role of Syk in B lymphoma growth is not known. Curcumin inhibited constitutive Syk phosphorylation of both murine and human B lymphoma cell lines in a dose-dependent manner and this process is direct as shown by the in vitro kinase assay. Previous studies suggested the IKK complex as a target for curcumin in cancer cells (10, 11, 54). But none of these studies showed that curcumin directly affects the target as in these experiments lysates from curcumin-treated cells were analyzed for the target protein change. Hence, it is unclear whether IKK is inhibited directly by curcumin or was inhibited due.
to the effect of curcumin on an upstream component. It has been shown that IKK is phosphorylated and NF-κB is activated in an Syk- and Akt-dependent manner in response to BCR cross-linking, TNF stimulation, and oxidative stress (22, 37, 38, 46, 47, 55, 56). These findings suggest a critical link between the upstream kinases and the NF-κB pathway. For the first time, here we demonstrate under in vitro conditions, curcumin can directly inhibit phosphorylation of Syk and thus identify Syk as a novel target for curcumin in B lymphoma cells. Consistent with the concept that Syk is a direct target of curcumin, Lyn activity and Ig phosphorylation, which are upstream of Syk activation, were not affected by curcumin in B lymphoma cells.

Curcumin-mediated growth inhibition of B lymphoma cells via Syk led us to hypothesize that Syk may be constitutively active in primary lymphoma cells. Accordingly, we showed that a variety of B lymphoma cells (cell lines and primary tumor from Eµ-Myc transgenic mice) expressed constitutively active Syk with little or no active Syk in normal splenic and PBMC B cells. Also, we found that Syk is constitutively active in primary B lymphoma cells from human patients (67). Moreover, for the first time we demonstrate that the constitutively activated Syk is required for B lymphoma growth. Syk induces multiple signaling pathways including PI3K, NF-κB, phospholipase C-γ, and JNK (20, 57, 58). Curcumin has been shown to target NF-κB, Akt, JNK, JAK, STAT3, and Src kinases in tumor cells. In multiple myeloma cells, it was shown that IL-6-induced STAT3 phosphorylation is blocked by curcumin resulting in growth inhibition (10, 11). Similar observation was made in brain microglia (59). In addition, curcumin was shown to target Src activity in vitro in murine fibroblast cell lines (60). In light of these findings, we propose that curcumin-mediated targeting of some of the above-mentioned pathways is Syk dependent in B lymphoma cells. Thus, by targeting Syk, all downstream

FIGURE 5. Curcumin targets Syk but not Lyn or Igo. A, Akt was immunoprecipitated from Ly-10 B lymphoma cell lysates (DLBCL) and incubated with the substrate GSK-3β and ATP and different concentrations of curcumin or DMSO at room temperature for 30 min. The reaction was stopped by boiling the samples in SDS sample buffer and analyzed by immunoblotting with an Ab to phospho-GSK-3β. The blots were stripped and then probed again for total GSK3β.

B, Immunoprecipitated mTOR from Ly-10 B lymphoma cell lysates (DLBCL) was incubated with the substrate Akt (recombinant protein) and ATP and different concentrations of curcumin or DMSO at room temperature for 30 min. Reaction was stopped by boiling the samples in SDS sample buffer and analyzed by immunoblotting with an Ab to phospho-Akt. The blots were then stripped and reprobed for total Akt.

C, Ly-10 (top panel) DLBCL, WEHI-231 (middle panel, murine), and Ramos (bottom panel, Burkitt’s) B lymphoma cells were incubated with medium, 10 μM or 20 μM of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to phospho-Syk (Tyr525/526) and then stripped and probed for Syk.

D, Both B lymphoma cell lines and primary tumors from both murine and human origin but not normal splenic or human peripheral blood B cells express the phosphorylated form of Syk constitutively. Mouse primary tumors are B lymphomas isolated from Eµ-Myc transgenic mice. Experiments were performed two to four times with similar results. E, Ly-10 B lymphoma cells (DLBCL), incubated with medium or curcumin for the indicated time periods were lysed and analyzed by immunoblotting with an Ab to phospho-Lyn or β-actin (after stripping). F, Cell lysates from untreated or curcumin treated Ly-10 B lymphoma cells (DLBCL) were subjected to immunoprecipitation with anti-Igo as described in Materials and Methods and immunoprecipitated samples were analyzed by immunoblotting with an Ab to phosphotyrosine and then stripped and probed for Igo. G, Syk, isolated from Ly-10 B lymphoma cell lysates by immunoprecipitation was incubated with ATP and different concentrations of curcumin or DMSO (equivalent to the highest concentration of curcumin) at room temperature for 30 min. The reaction was stopped by boiling the samples in SDS sample buffer and analyzed by immunoblotting with an Ab to phosphotyrosine. Numbers indicate the ratio of pSyk to Syk in C, pLyn to β-actin in E and ptyr of Igo to Igo in F, ptyr of Syk to Syk in G.

FIGURE 6. Effects of Syk inhibitor on B lymphoma growth. A, BKS-2 (left panel, human DLBCL) B lymphoma cells were incubated with medium, different doses of Syk inhibitor (Piccatannol) or vehicle (DMSO) alone for 2 days and proliferation was measured as described in Materials and Methods. Results were expressed as mean ± SE of triplicate cultures. B, SUDHL-4 cells (human DLBCL) were transfected with control or Syk specific siRNA and cell cycle analysis was performed 48 h later by Hoechst staining (right panel). Left panel represents the Western blot showing levels of Syk, phospho-Akt and Bcl-xL, with the ratios (normalized to actin) of 1:0.5, 1:0.5, 1:0.6 for control vs Syk siRNA-treated cells respectively. Experiments were done two to three times with similar results. * p < 0.01 when response with Syk inhibitor is compared with the vehicle.

FIGURE 5. Curcumin targets Syk but not Lyn or Igo. A, Akt was immunoprecipitated from Ly-10 B lymphoma cell lysates (DLBCL) and incubated with the substrate GSK-3β and ATP and different concentrations of curcumin or DMSO at room temperature for 30 min. The reaction was stopped by boiling the samples in SDS sample buffer and analyzed by immunoblotting with an Ab to phospho-GSK-3β. The blots were stripped and then probed again for total GSK3β. B, Immunoprecipitated mTOR from Ly-10 B lymphoma cell lysates (DLBCL) was incubated with the substrate Akt (recombinant protein) and ATP and different concentrations of curcumin or DMSO at room temperature for 30 min. Reaction was stopped by boiling the samples in SDS sample buffer and analyzed by immunoblotting with an Ab to phospho-Akt. The blots were then stripped and reprobed for total Akt. C, Ly-10 (top panel) DLBCL, WEHI-231 (middle panel, murine), and Ramos (bottom panel, Burkitt’s) B lymphoma cells were incubated with medium, 10 μM or 20 μM of curcumin for the indicated time periods. Cell lysates were analyzed by immunoblotting with an Ab to phospho-Syk (Tyr525/526) and then stripped and probed for Syk. D, Both B lymphoma cell lines and primary tumors from both murine and human origin but not normal splenic or human peripheral blood B cells express the phosphorylated form of Syk constitutively. Mouse primary tumors are B lymphomas isolated from Eµ-Myc transgenic mice. Experiments were performed two to four times with similar results. E, Ly-10 B lymphoma cells (DLBCL), incubated with medium or curcumin for the indicated time periods were lysed and analyzed by immunoblotting with an Ab to phospho-Lyn or β-actin (after stripping). F, Cell lysates from untreated or curcumin treated Ly-10 B lymphoma cells (DLBCL) were subjected to immunoprecipitation with anti-Igo as described in Materials and Methods and immunoprecipitated samples were analyzed by immunoblotting with an Ab to phosphotyrosine and then stripped and probed for Igo. G, Syk, isolated from Ly-10 B lymphoma cell lysates by immunoprecipitation was incubated with ATP and different concentrations of curcumin or DMSO (equivalent to the highest concentration of curcumin) at room temperature for 30 min. The reaction was stopped by boiling the samples in SDS sample buffer and analyzed by immunoblotting with an Ab to phosphotyrosine. Numbers indicate the ratio of pSyk to Syk in C, pLyn to β-actin in E and ptyr of Igo to Igo in F, ptyr of Syk to Syk in G.
components are down-regulated which is a potential way to modulate pathways that are diversified from the proximal components. Using a Syk-selective inhibitor and by RNA interference, we also demonstrated that constitutive Syk activity is critical for B lymphoma growth. Unlike breast cancer cells, where Syk was shown to be a tumor suppressor, we show here that Syk has a prosurvival role in B lymphoma suggesting multiple roles for Syk depending on the cellular context. Loss of Syk expression in breast tumors as well as in patients suggests that curcumin may have some important therapeutic benefits in the treatment of B lymphomas as there is a significant reduction in splenic tumor burden in our in vivo mouse model.

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Disclosures

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