The ITK-SYK Fusion Oncogene Induces a T-Cell Lymphoproliferative Disease in Mice Mimicking Human Disease

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Abstract

Peripheral T-cell lymphomas (PTCL) constitute a major treatment problem with high mortality rates due to the minimal effectiveness of conventional chemotherapy. Recent findings identified ITK-SYK as the first recurrent translocation in 17% of unspecified PTCLs and showed the overexpression of SYK in more than 90% of PTCLs. Here, we show that the expression of ITK-SYK in the bone marrow of BALB/c mice causes a T-cell lymphoproliferative disease in all transplanted mice within 8 weeks after transplantation. The disease was characterized by the infiltration of spleen, lymph nodes, bone marrow, and skin with CD3+CD4+CD8− and CD3+CD4−CD8− ITK-SYK-positive T-cells accompanied by a systemic inflammatory reaction with upregulation of interleukin 5 and INF-γ. ITK-SYK-positive T-cells showed enhanced apoptosis resistance and INF-γ production in vitro. The disease was serially transplantable, inducing clonal T-cell expansion in secondary recipients. The action of ITK-SYK in vivo was dependent on SYK kinase activity and disease development could be inhibited by the treatment of mice with SYK inhibitors. Interestingly, the translocation of ITK-SYK from the membrane to the cytoplasm, using a point mutation in the pleckstrin homology domain (ITK-SYK R29C), did not abolish, but rather, enhanced disease development in transplanted mice. CBL binding was strongly enhanced in membrane-associated ITK-SYK E42K and was causative for delayed disease development. Our results show that ITK-SYK causes a T-cell lymphoproliferative disease in mice, supporting its role in T-cell lymphoma development in humans. Therefore, pharmacologic inhibition of SYK in patients with U-PTCLs carrying the ITK-SYK fusion protein might be an effective treatment strategy. Cancer Res; 70(15): 6193–204. ©2010 AACR.

Introduction

Fusion proteins with constitutive activation of tyrosine kinases, such as BCR-ABL, are a common cause for cancer development (1–3). The invention of the ABL kinase inhibitor imatinib was a breakthrough towards a new strategy of targeted anticancer therapies, which directly inhibit the activity of kinase oncogenes causing malignancies (4). Consequently, the identification of kinase fusions in human malignancies and their characterization is an important way to define targets in oncology. ITK-SYK was identified as the first recurrent gene fusion in peripheral T-cell lymphomas (PTCL; ref. 5). PTCLs arise from various stages of T-cell development and characteristically infiltrate skin, spleen, lymph nodes, and other organs (6). Seventeen percent of patients with unspecified PTCLs carried the t(5;9)(q33;q22) translocation, resulting in an ITK-SYK fusion transcript (5). ITK-SYK represents the first-described kinase-to-kinase fusion and does not contain any dimerization or oligomerization motif, so it is unclear if and how the fusion may lead to T-cell transformation. The NH2-terminal part, provided by ITK, contains the Tec homology domain and a pleckstrin homology domain, which could facilitate the interaction with membrane-bound phosphatidylinositol(3,4,5)P3 (7). ITK itself is a tyrosine kinase which is primarily expressed in natural killer cells and T cells throughout all stages of T-cell development (8). The COOH-terminal part comprises the kinase domain of SYK and several auto- and trans-phosphorylation sites which are important for the regulation of the kinase activity of SYK and its binding to phospholipase C-γ1 (PLC-γ1; refs. 9–12). SYK belongs to the SYK/ZAP-70 family of nonreceptor tyrosine kinases and mediates Fcγ receptor signaling in
macrophages, FccRII signaling in mast cells, as well as B-cell receptor signaling (13–15). In T-cell antigen receptor (TCR) signaling, the SRC-related kinases LCK and FYN initiate TCR-triggered signal transduction by phosphorylating the CD3 and ζ subunits of the TCR complex. This modification permits the recruitment of ZAP-70 and SYK, which amplify the TCR-triggered signal by phosphorylating additional intracellular proteins (16, 17). Although ZAP-70 is expressed in all T cells, SYK is mainly present in immature T-cell populations and is downregulated in peripheral circulating CD4+ and CD8+ T-cells (18–20). Overexpression of SYK was shown in more than 90% of PTCLs (21).

Here, we investigated the role of ITK-SYK fusion in the development of T-cell malignancies in mice using a bone marrow transplantation model. We characterized the transforming activity of the fusion and investigated its dependency on its kinase activity, membrane binding, and association with the ubiquitin ligase Cbl in vivo. Finally, we show evidence that SYK kinase inhibition could be a new treatment option for patients with T-cell lymphomas carrying the translocation.

Materials and Methods

Animal experiments

BALB/c males were injected with 5-fluorouracil (150 mg/kg) i.p. and sacrificed after 4 days. Bone marrow cells (BMC) were harvested from the leg bones, followed by RBC lysis and cultivation in prestimulation medium [DME/h with 10% fetal bovine serum, SCF, interleukin (IL)-6, and IL-3]. Spin infections with retroviral pMSCV/ires/GFP control virus or ITK-SYK constructs (Supplementary Fig. S1) were performed twice and cells were transfected by i.v. injection into irradiated (900 cGy) recipient BALB/c females. Disease development was monitored by weekly weight measurements, biweekly blood cell counts, and other signs of disease such as tissue necrosis of the ears and tails. Mice were sacrificed when moribund and spleen, liver, lymph nodes, thymus, and other organs were extracted for weight comparison, formalin fixation, or direct analysis by flow cytometry.

For retransplantation experiments, BMCs and spleen cells were extracted from diseased mice or control mice and 2 × 10⁶ cells were retransplanted into sublethally irradiated BALB/c females. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Genomics Institute of the Novartis Research Foundation and were in accordance with the U.S. NIH Statement of Compliance with Standards for Humane Care and Use of Laboratory Animals.

Proliferation assays

Whole cells were isolated from the spleen of control mice or diseased ITK-SYK-positive mice using tissue filters. RBC were lysed, washed twice in PBS, and stained with a cocktail of biotinylated non–T-cell antibodies against B220, CD49b, CD11b, and Ter-119 for 15 minutes at 4°C. Anti-Biotin Micro Beads were added for 20 minutes at room temperature and magnetically labeled non–T-cells were separated using the Auto Macs system, leaving untouched T-cells for further experiments.

Normal T-cells (5 × 10⁵) or ITK-SYK-positive T-cells were added to each 96-well plate. The number of viable cells was determined using trypan blue exclusion daily for 7 days.

Immunohistochemistry and immunofluorescence staining

Mouse tissue was fixed for 48 hours in formalin and paraffin-embedded tissues were generated using standard procedures. Single-color 3,3′-diaminobenzidine immunoperoxidase staining was performed on paraffin sections using antibodies to CD3 (BD-PharMingen) according to the recommendations of the manufacturer. To determine the localization of the different ITK-SYK mutants, pMSCV/ITK-SYK wild-type (wt), pMSCV/ITK-SYK E42K, and pMSCV/ITK-SYK R29C were transfected into 293T cells using Fugene. After 48 hours, cells were fixed and permeabilized and immunofluorescence staining was performed using the ITK antibody (2F12) from Cell Signaling Technology.

Western blots and immunoprecipitation

Western blots and immunoprecipitations were performed using standard methods. Primary and secondary antibodies were purchased from Cell Signaling Technology: AKT (11E7) no. 4691, phosphorylated AKT Ser473 (D9E) no. 4060, PLC-γ1 no. 2822, phosphorylated PLC-γ1 no. 2871, signal transducers and activators of transcription 3 (STAT3; 12H6) no. 9139, phosphorylated STAT3 Ser727 no. 9134, STAT5, phosphorylated STAT5 Tyr414 (C11C5) no. 9359, p44/p42 mitogen-activated protein kinase (3A7) no. 9107, phosphorylated p44/42 mitogen-activated protein kinase (3A7) no. 9107, phosphorylated p44/p42 mitogen-activated protein kinase Thr202/Tyr204 (197G2), SYK no. 2712, phosphorylated SYK Tyr324 no. 2715, phosphorylated SYK Tyr352 no. 2701, ITK (2F12) no. 2380, and c-CBL no. 2747.

Detection of cytokine levels

Blood was taken from mice at different time points after transplantation, centrifuged, and serum was diluted 1:4 in PBS. For in vitro experiments, supernatants of cultured malignant and normal T-cells were diluted 1:4 in PBS. Cytokine levels were detected using the mouse Th1/Th2 cytokine kit (IL-2, IL-4, IL-5, IFN-γ, TNF) from BD Bioscience.

Flow cytometry analysis

Flow cytometry stainings for hematologic cell types were performed with antibodies CD3-PECy7, CD4-PE, CD8-APC, CD11b-PECy7, and B220-APC (BD PharMingen) and analyzed using the LSR2 flow cytometer (Becton Dickinson). Regulatory T-cells were detected using CD4-APC, CD25-PE (BioLegend), and intracellular Foxp3-eFluor450 (eBioscience) staining. For in vitro SYK inhibition, spleen cells from diseased ITK-SYK+ mice were treated with curcumin (Calbiochem), piceatannol (Tocris Bioscience), or Syk II inhibitor (Calbiochem) for 24 hours, and apoptosis was measured using the Annexin V-PE/7-AAD kit from BD Bioscience.

TCR Vβ spectratyping

RNA was extracted from splenic single-cell suspensions using RNeasy Mini Kit (Qiagen) and reverse-transcribed into
expression construct carrying the ITK-SYK fusion gene detected (5). T-cell lymphoproliferative disease in mice described (22), but using the Cb primer 5’-CCAAGCACAAGGGTACGCT-3’ for the initial PCR amplification and a newly designed “run-off” primer 5’-CTTAGTGGAGGTCACTTCTC-3’ for the primer extension reactions. Aliquots of the run-off reactions were analyzed on an ABI 3130 XL capillary sequencer (Applied Biosystems).

Constructs

The ITK-SYK plasmid constructs used in this study are shown in Supplementary Fig. S1. The human ITK-SYK fusion gene described by Streubel and colleagues was cloned from ITK and SYK human cDNA. The 495 bp ITK fragment was amplified using the following primer sequences: 5’-ATGAA-CACTTCTCCGAGGAA (f) and 3’-CTGTGTCGACGGAGTGGAG (r). The SYK fragment was amplified using the following primers: 5’-TCTCCCGAGGCTCAGGAACCGGCAA (f) and 3’-TTAGTCACCAGCTGATAGTAATT (r). PCR products were ligated and then the fused ITK-SYK construct was reamplified using an XhoI-ITK-primer and an EcoRI-SYK-r-primer. The XhoI-ITK-SYK-EcoRI PCR product was cloned into a pMSCV/IRES/GFP vector using the XhoI and EcoRI restriction sites. The ITK-SYK point mutants (R29C, E42K, Y323F, and K402R) were generated using the QuikChange II site-directed mutagenesis kit (Stratagene). Sequences from all constructs are shown in Supplementary Fig. S2.

Results

Expression of ITK-SYK in the bone marrow causes a T-cell lymphoproliferative disease in mice

To evaluate the role of ITK-SYK in the development of t(5;9)-positive T-cell lymphomas, we created a retroviral expression construct carrying the ITK-SYK fusion gene described in patients (pMSCV ITK-SYK/IRES/GFP; ref. 5). Bone marrow from 5-fluorouracil–pretreated donor mice was infected, infected with either control virus (pMSCV/IRES/GFP) or ITK-SYK virus (pMSCV ITK-SYK/IRES/GFP) and then transplanted into sublethally irradiated BALB/c females. Cells originally infected contained 68% CD11b+ myeloid cells, 13% B220+ B-cells, and 9.2% T cells (2.1% CD3+CD4−CD8−, 4.2% CD4+, 2.9% CD8+), 9% erythroid precursors and 0.3% HSCs (Lin-Kit+Sca+; Supplementary Table S1). Four weeks after transplantation, all ITK-SYK+ positive mice started to lose weight compared with the control group (Supplementary Fig. S3). Four weeks after transplantation, all ITK-SYK+ positive mice started to lose weight compared with the control group (Supplementary Fig. S3). The mice developed a systemic inflammatory disease with red eyes, swelling of the nose and tail, followed by visible skin infiltrates at the ears and tails resulting in focal skin necrosis (Fig. 1A). Inflammatory cytokines IL-5 and INF-γ were strongly upregulated in the serum of diseased mice, whereas IL-2, IL-4, and TNF remained normal (Fig. 1B). Characteristic changes in the peripheral blood included anemia, loss of peripheral lymphocytes, and elevation of ITK-SYK–negative neutrophils (Fig. 1C). GFP-positive cells in the peripheral blood were only between 1% and 2%, suggesting a subleukemic disease. Six to 8 weeks after transplantation, most of the mice had lost more than 20% of their body weight and were sacrificed. Spleen weights were elevated in ITK-SYK+ mice compared with control mice (mean control spleen weight, 95 mg; mean ITK-SYK spleen weight, 174 mg; Fig. 1D). Lymph nodes were slightly enlarged (Supplementary Fig. S4), although thymus size remained normal.

Histopathologic findings and immunophenotyping of malignant T cells

Immunohistochemical stainings for CD3 revealed the infiltration of various organs with CD3+ T-cells (Fig. 2A). Although the CD3+ T-cells in the normal spleen are mainly localized in the pericapillary zones and between the white and red pulpa, ITK-SYK+–positive mice showed infiltration of the whole lymph follicle including the germinal center with CD3+ T-cells. Furthermore, clusters of T-cells were detected in the red pulp and during the progression of the disease, those areas increased (Supplementary Fig. S5). Interestingly, this particular pattern of colonization of lymphoid follicles with malignant T-cells was also described for some of the human T-cell lymphomas expressing the ITK-SYK fusion protein (5). Also, non-lymphoid organs like skin, lung, and colon were infiltrated with CD3+ T-cells, which might be the reason for the diarrhea and dramatic weight loss observed in ITK-SYK+–positive mice.

Further characterization of ITK-SYK+ cells isolated from spleens, lymph nodes, and bone marrow of diseased mice revealed CD3 expression in the majority of GFP+ cells (Fig. 2B). Approximately 50% to 60% of the GFP+/CD3+ cells were CD3+CD5+CD4−CD8− T-cells and ~40% of the cells were CD3+CD5+CD4+CD8− T-cells (Fig. 2B), including regulatory T-cells (CD4+CD25+Foxp3+; Supplementary Fig. S6). Interestingly, in ITK-SYK+–positive PTCL patients, the phenotype was also described as CD3+CD5+CD4+CD8− and in one patient as CD3+CD5+CD4−CD8−. In contrast with lymph nodes, bone marrow, and spleens, we identified ITK-SYK+CD4+CD8+ double positive T-cells in the thymus, whereas no ITK-SYK+/CD8+ cytotoxic T-cells could be detected. CD10 (present in human disease) was expressed in 8% to 12% of all ITK-SYK+–positive T-cells. Unlike in human disease, we could not detect Bcl6 expression (data not shown).

In contrast with ITK-SYK/GFP+ T-cells, regular GFP-negative T-cells were suppressed in the spleen and bone marrow of ITK-SYK+ mice compared with control mice (spleen, 11% versus 41%, respectively; bone marrow, 9% versus 19%, respectively; Supplementary Fig. S7). The reduction of GFP-negative T-cells was due to a reduction of all T-cell subsets (Supplementary Fig. S8) besides the number of regulatory T-cells (CD4+CD25+Foxp3+; Supplementary Fig. S6). Therefore, the expansion of ITK-SYK+ T-cells is caused intrinsically by the ITK-SYK fusion oncogene and not due to a general increase in T-cells caused by uncontrolled cytokine secretion. The number of B-cells (B220+) and myeloid cells within the ITK-SYK/GFP+ population was suppressed to 10% and 11%, respectively (Supplementary Fig. S9), indicating that the ITK-SYK fusion preferentially induces expansion of the T-cell population. Regular ITK-SYK−negative B-cells were not affected even in terminally diseased mice (Supplementary Fig. S9).
The level of malignancy was assessed using various methods such as histology, clonality assays (TCR Vβ-spectratyping), and retransplantability of the disease into secondary recipients. Retransplantation of $2 \times 10^6$ BMCs or spleen cells from diseased mice into sublethally irradiated secondary recipients caused the same T-cell lymphoproliferative disease within 4 to 6 weeks after transplantation (Supplementary Fig. S10). For clonality analysis, we analyzed the T-cell receptor repertoire within the malignant population using TCR Vβ spectratyping. Similar to other bone marrow transplantation models, using retrovirally overexpressed oncogenes such as BCR-ABL or FLT3-ITD, the primary recipients developed a polyclonal disease. In contrast, we observed a monoclonal/biclonal disease in three out of four secondary recipients (Supplementary Fig. S11).

**In vitro characterization of ITK-SYK-positive T-cells**

Naive T-cells were isolated from the spleens of ITK-SYK+ or control mice by negative selection and cultured in 96-well plates with RPMI 1640 and 10% fetal bovine serum. Although...
Figure 2. Immunophenotyping and in vitro characterization of ITK-SYK-positive T-cells. A, immunohistochemistry for CD3 in paraffin-embedded tissues from ITK-SYK versus control mice. B, flow cytometric characterization of GFP-positive T-cells for CD3 (PE-Cy7), CD4 (PerCP), and CD8 (APC). C, cultivation of normal splenic T-cells and ITK-SYK+ T-cells in vitro over 7 d. Left, number of viable CD3+ T-cells (×10^4) assessed daily by trypan blue exclusion (one of three experiments). Right, spontaneous apoptosis measured by Annexin V/7-AAD staining of normal T-cells (CD4+ and CD3+CD4−CD8+) or ITK-SYK+ T-cells after 3 d of in vitro cultivation (n = 3 spleens, ±SD). D, INF-γ production from in vitro cultivated CD4+ T-cells assessed by flow cytometry using the mouse Th1/Th2 cytokine detection kit (n = 3, ±SD).
the number of viable control T-cells decreased to <20% within 3 days (Fig. 2C), the number of ITK-SYK–positive T-cells decreased by only 40% within 7 days. Both ITK-SYK–positive T-cell subsets (CD4+ and CD3+CD4–CD8–) showed reduced apoptotic cell death (Annexin V) compared with control T-cells under basic culture conditions (Fig. 2C), indicating a considerable degree of resistance to intrinsic apoptosis in vitro.

To determine, whether increased INF-γ and IL-5 levels in diseased mice are produced by ITK-SYK+ T-cells, supernatant was taken from in vitro–cultured ITK-SYK+ CD4+ T-cells or control CD4+ T-cells. Although INF-γ production was increased 10-fold in supernatant from ITK-SYK+ CD4+ T-cells (Fig. 2D), IL-5 production remained normal (data not shown). Under physiologic conditions, IL-5 is produced by CD4+ T-cells in TH2 response, whereas INF-γ is produced by CD4+ T-cells in TH1 response. The lack of increased IL-5 production in ITK-SYK+ cells in vitro indicates that IL-5 production might be mediated by specific stimuli only present under in vivo conditions or in the presence of other cells producing IL-5, which are also involved in disease.

The transformation of T-cells by ITK-SYK is kinase-dependent

A point mutation was inserted into the SYK kinase domain of ITK-SYK (Fig. 3A; Supplementary Fig. S1), that abolishes its kinase activity (ITK-SYK K402R). ITK-SYK wt and ITK-SYK K402R were transfected into 293T cells and their phosphorylation status was tested using three different phosphorylated SYK-specific antibodies. Y323 in the linker region of SYK is an autophosphorylation site and functions in its phosphorylated state as the binding site for the ubiquitin ligase c-CBL (23, 24). Y325 is localized in the activation loop of SYK, is an autophosphorylation site and its phosphorylation augments, but is not essential for the kinase activity of SYK (9). Y332 is not essential for SYK kinase activity, but mediates SYK binding to PLC-γ (9). Expression of ITK-SYK wt in 293Ts resulted in the phosphorylation of all three tyrosine residues

![Figure 3. Transforming activity of ITK-SYK is kinase-dependent and involves the activation of STAT5 and PLC-γ1. A, kinase-deficient ITK-SYK K402R. B, expression of control vector, ITK-SYK wt, and kinase-deficient ITK-SYK K402R in 293T cells. Western blot analysis for ITK, phosphorylated SYK Tyr323, phosphorylated SYK Tyr525, phosphorylated SYK Tyr332, phosphorylated SYK Tyr530, STAT5, phosphorylated STAT5, PLC-γ1, and phosphorylated PLC-γ1. C, average spleen weight from three mice per transplantation group 4 wk after treatment (n = 3, sSD). D, survival curve for mice expressing ITK-SYK wt or kinase-deficient ITK-SYK K402R.](image)
including both autophosphorylation sites, indicating constitutive activation of the SYK kinase (Fig. 3B). In contrast, both autophosphorylation sites were not phosphorylated in ITK-SYK K402R, but Tyr323 was phosphorylated independent of SYK kinase activity. The downstream targets STAT5 and PLC-γ were specifically phosphorylated in 293T cells transfected with ITK-SYK wt, but not in control 293T or 293T cells transfected with ITK-SYK K402R (Fig. 3B).

To verify the importance of the kinase activity of ITK-SYK for the induction of a T-cell lymphoproliferative disease in vivo, we transplanted ITK-SYK wt and ITK-SYK K402R expressing bone marrow into irradiated recipient mice. In marked contrast with ITK-SYK wt mice, ITK-SYK K402R mice did not develop any signs of disease (weight loss, inflammation) and no elevation in spleen weights were seen 4 weeks after transplantation (Fig. 3C). Nine mice were monitored continuously over a time period of 8 months and none of those mice developed any disease symptoms, whereas all ITK-SYK wt mice were sacrificed premoribund within 2 months after transplantation (Fig. 3D). Moreover, GFP-positive cells expressing kinase-deficient ITK-SYK K402R isolated from mice 8 weeks after transplantation showed similar numbers of total CD3+ T-cells and T-cell subsets compared with control mice, which indicates that ITK-SYK K402R expression does not interfere with the survival of regular T-cells in vivo (Supplementary Fig. S12).

Altersations in ITK-SYK membrane binding can change T-cell lymphoma development, but cannot induce B-cell transformation

A different SYK fusion, TEL-SYK, has previously been identified in a patient with myelodysplastic syndrome (25). TEL-SYK was shown to transform BaF/3 cells (26) and induces B-cell lymphomas/leukemias in vivo (27). In contrast with TEL-SYK, ITK-SYK was not able to transform BaF/3 cells (pre-B cell line) in vitro (data not shown) and no B-cell lymphomas could be detected in ITK-SYK+ mice. Whereas TEL-SYK is expressed in the cytoplasm of cells (26), we observed that ITK-SYK was mainly localized at the plasma membrane of transfected 293T cells (Fig. 4A; ref. 28).

To investigate whether the localization of ITK-SYK plays a role in B-cell versus T-cell transformation, we inserted point mutations into the NH2-terminal pleckstrin homology domain to alter its membrane-binding ability. Both mutants were designed homologous to mutations described for the Tec kinase BTK (ref. 29; Supplementary Fig. S1). Accordingly, the point mutant ITK-SYK R29C was predominantly localized in the cytoplasm and nucleus of 293T cells, whereas ITK-SYK E42K was mainly associated with the membrane (Fig. 4A). BMCs expressing those mutants or ITK-SYK wt were transplanted into irradiated recipient mice. Previous experiments in 3T3 fibroblasts had suggested that membrane localization of ITK-SYK would be essential for its transforming potential (28). In contrast with those findings, the cytoplasmically localized ITK-SYK R29C even enhanced T-cell lymphoproliferative disease as shown by lymphopenia (Fig. 4B), spleen weight (Fig. 4C), signs of inflammation (Supplementary Fig. S13), and reduced survival time of the transplanted mice (Fig. 4D).

Furthermore, ITK-SYK E42K (enhanced membrane localization) reduced disease development in mice and the average survival time was nearly doubled compared with ITK-SYK R29C mice (Fig. 4D). Our data indicates that cytoplasmic localization of ITK-SYK enhances T-cell lymphoproliferative disease in mice, whereas forced membrane binding reduces disease development. Similarly, overexpression of membrane localized SYK (chimeric construct that contains the c-Src myristoylation sequence at the NH2 terminus of SYK) in SYK-deficient mast cells results in reduced downstream propagation of intracellular signals (30). Although ITK-SYK R29C such as TEL-SYK is localized in the cytoplasm, we did not detect B-cell lymphoma development as described for TEL-SYK, which indicates that factors other than the localization of the activated SYK kinase are decisive between T-cell or B-cell lymphoproliferative disease induced by those fusions.

Enhanced binding of ITK-SYK to CBL reduces disease development in mice

Further characterization of ITK-SYK R29C (cytoplasm) and ITK-SYK E42K (membrane) showed an identical phosphorylation status for Tyr323 and Tyr525. However, we detected enhanced phosphorylation of Tyr323 in membrane-bound ITK-SYK E42K compared with ITK-SYK wt and ITK-SYK R29C in three independent experiments (Fig. 5A). Phosphorylated Tyr323 is the binding site for the ubiquitin ligase c-CBL, which polyubiquitinylates proteins and therefore causes their degradation. To support the theory that hyperphosphorylation of Tyr323 and the resulting increase in CBL binding to ITK-SYK E42K might be the cause of the reduced disease development, we performed immuno-precipitations to detect ITK-SYK CBL binding. Indeed the ITK-SYK E42K mutant showed a more than 10-fold increase in CBL binding compared with ITK-SYK wt and ITK-SYK R29C (Fig. 5B). To prove that CBL binding to ITK-SYK could indeed change the onset of T-cell lymphoma development, we inserted a second point mutation into the ITK-SYK E42K mutant which replaces the Tyr323 by phenylalanine (ITK-SYK E42K Y323F) and therefore reduces CBL binding (Fig. 5B). BMCs expressing ITK-SYK E42K or ITK-SYK E42K Y323F were transplanted into irradiated recipients. Indeed, we detected a significantly faster onset of disease and a shorter survival time in ITK-SYK E42K Y323F mice (no CBL binding) compared with ITK-SYK wt and ITK-SYK R29C (Fig. 5B). To prove that CBL binding to ITK-SYK could indeed change the onset of T-cell lymphoma development, we inserted a second point mutation into the ITK-SYK E42K mutant which replaces the Tyr323 by phenylalanine (ITK-SYK E42K Y323F) and therefore reduces CBL binding (Fig. 5B). BMCs expressing ITK-SYK E42K or ITK-SYK E42K Y323F were transplanted into irradiated recipients. Indeed, we detected a significantly faster onset of disease and a shorter survival time in ITK-SYK E42K Y323F mice (no CBL binding) compared with ITK-SYK wt and ITK-SYK R29C (Fig. 5B).

Inhibition of SYK kinase activity induces apoptosis in ITK-SYK–positive T-cells in vitro

Curcumin was recently described as an inhibitor of SYK kinase activity and was shown to inhibit B-cell lymphoma growth in mice (31). Indeed, curcumin treatment of ITK-SYK–expressing 293T cells resulted in the dephosphorylation of
autophosphorylation sites Tyr\(^{525}\) and Tyr\(^{323}\) in ITK-SYK and the dephosphorylation of STAT5 and PLC-\(\gamma\), indicating that curcumin inhibits the kinase activity of ITK-SYK (Fig. 6A). Furthermore, SYK inhibition by curcumin, SykII inhibitor, or piceatannol (32) in ITK-SYK–positive T cells isolated from the spleens of diseased mice resulted in a dose-dependent dephosphorylation of Tyr\(^{525}\) in ITK-SYK (Fig. 6B) and an induction of apoptosis (Fig. 6C).

Figure 4. Influence of the localization of ITK-SYK on its transforming activity. A, ITK fluorescence staining of 293T cells transfected with ITK-SYK wt or the mutants ITK-SYK E42K and ITK-SYK R29C. B, WBC counts from mice 4 wk after transplantation. C, average spleen weights 4 wk after transplantation (\(n = 3, \pm SD\)). D, left, average survival days after transplantation (\(n = 9, \pm SD\)). Right, survival curve after transplantation.
SYK inhibition could prevent ITK-SYK–induced T-cell lymphoproliferative disease in mice

ITK-SYK+ mice with similar cytokine levels for IL-5 and INF-γ 3 weeks after transplantation were chosen for treatment with curcumin (50 or 100 mg/kg b.i.d.) or vehicle control by i.p. administration. After 2 weeks of treatment, curcumin-treated mice showed reduced spleen weights compared with vehicle control (Fig. 6D). The number of ITK-SYK/GFP+ T-cells in the spleen were reduced by 48% (curcumin, 50 mg/kg b.i.d.) and 73% (curcumin, 100 mg/kg b.i.d.; Supplementary Fig. S14), and cytokine levels (IL-5, INF-γ) in the serum dropped to nearly normal levels (Fig. 6D). Furthermore, phosphorylation of Tyr525 in ITK-SYK–positive T-cells measured by flow cytometry (Supplementary Fig. S15) was strongly reduced, indicating that curcumin could block SYK kinase activity in vivo. In contrast, curcumin treatment of healthy BALB/c females did not reduce spleen weights or the number of T cells (Supplementary Fig. S16). Our results indicate that SYK inhibition could inhibit T-cell lymphoproliferative disease caused by ITK-SYK, and that SYK inhibitors might be a new treatment option for patients carrying the ITK-SYK fusion.

Discussion

Constitutive activation or altered expression patterns of kinases are a common cause for cancer development. Here, we show that the forced expression of ITK-SYK in the bone marrow causes a T-cell lymphoproliferative disease. Within the ITK-SYK–positive population, we detected an accumulation of CD4−CD8− T-cells, a complete loss of CD8+ T-cells and deregulation of CD4+ T-cells including enhanced apoptosis resistance and autonomous INF-γ production. T-cells in the peripheral blood were strongly suppressed and did not express GFP, which indicates that the expression of the fusion protein might interfere with regular T-cell migration and function. The expression of the protein kinase SYK is
tightly regulated throughout T-cell development and maturation. Mature CD8+ T-cells have no detectable SYK expression levels, whereas SYK is widely expressed in immature T-cells and tissue-specific CD4+ T-cells (18, 19). In contrast with this, ITK is expressed at all stages of T-cell development. Because the ITK promoter region controls the expression of the ITK-SYK fusion protein, normal regulation of SYK expression in T-cells is abolished and a lack of SYK downregulation might be responsible for the alterations in T-cell populations and function.

The transforming capacity of SYK has been shown before. TEL-SYK, a rare fusion identified in a patient with myelodysplastic syndrome, induces a B-ALL type disease in mice (27). In contrast, ITK-SYK exclusively caused T-cell lymphomas and no B-cell malignancies were observed in our studies. Interestingly, the ITK-SYK fusion also showed a lack of transforming potential in Baf/3 cells (pre-B cells), whereas TEL-SYK induces IL-3–independent growth of Baf/3 cells (27), indicating that there are significant differences in the transformation process caused by those fusions.

Figure 6. Curcumin inhibits SYK pathway activation and abolishes ITK-SYK induced T-cell lymphoproliferative disease. A, transfection of 293T cells with ITK-SYK wt and treatment with increased doses of curcumin for 30 min. Detection of the activation status of ITK-SYK using antibodies for ITK, SYK Tyr323 and SYK Tyr525, and the downstream targets phosphorylated STAT5 and phosphorylated PLC-γ1. B, treatment of ITK-SYK+ spleen cells with different concentrations of curcumin for 2 h in vitro. Measurement of phosphorylated SYK Tyr525 expression in the ITK-SYK/GFP-positive population by flow cytometry (one of three experiments). C, apoptosis induction measured by Annexin V staining of ITK-SYK/GFP+ T-cells treated with curcumin, piceatannol, or SykII inhibitor for 24 h in vitro. D, comparison of spleen weights, IL-5 and INF-γ levels from mice treated with curcumin or vehicle control for 14 d (n = 5). Differences between vehicle versus curcumin treatment 100 mg/kg b.i.d. are statistically significant (unpaired t test, P < 0.05).
Although TEL-SYK was shown to be predominantly localized in the cytoplasm, we found that the ITK-SYK fusion protein is primarily localized in the membrane. This difference might cause the activation of different downstream signaling cascades, but our studies using the cytoplasmically localized ITK-SYK mutant R29C showed that membrane binding is not a requirement for ITK-SYK to induce T-cell lymphomas and cytoplasmic localization of ITK-SYK did not induce B-cell lymphomas in the transplanted mice. Therefore, localization of the protein might not be the major differential effect between those fusions. Instead, dimerization and the resulting constitutive activation of the SYK kinase in the TEL-SYK fusion construct and differences in interaction partners, which bind either to the TEL or the ITK part of the fusions, might be more likely explanations for the difference between the initiation of either B-cell or T-cell lymphomas and will be investigated in further studies.

Several of our results point towards the role of ITK-SYK in the development of human T-cell lymphomas, although not all details reflect human disease. All ITK-SYK–positive mice developed a T-cell lymphoproliferative disease within a short latency (8 weeks) after transplantation. ITK-SYK–positive T-cells infiltrated the skin, spleen, lymph nodes, and other organs, which is concordant with the phenotype seen in human disease. The T-cell subsets involved in lymphoproliferative disease in mice (CD4+ and CD4–CD8– T-cells) were also described for human disease and also exclude the CD8– T-cell population. The infiltration phenotype of the germinal center in the spleen reflects the infiltration phenotype described for some of the patient samples. The disease is serially transplantable, inducing a clonal/biclonal disease in three of four of the examined secondary transplants. The T-cell subsets involved show enhanced apoptosis resistance. Differences between mouse and human disease concern the general inflammation observed in ITK-SYK–positive mice. This inflammation phenotype is not generally described for U-PTCLs in humans, but is more reminiscent of angioimmunoblastic T-cell lymphomas.

Our results and current findings that SYK is overexpressed in more than 90% of PTCLs in humans points towards SYK translocations, or more generally, the ectopic and unregulated expression of SYK in T-cells as an important event in the development of PTCLs. Moreover, our experiments show that SYK kinase inhibition could reverse the disease caused by ITK-SYK fusion protein in mice. Therefore, SYK inhibition in PTCL patients carrying the ITK-SYK translocation or other SYK fusions might be a new and successful treatment strategy for this disease.

Disclosures of Potential Conflicts of Interest

Markus Warmuth is shareholder of Novartis and also is employed with Novartis. Novartis does not have any SYK inhibitor in the clinic.

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References

22. Pannetier C, Cochet M, Darche S, et al. The sizes of the CDR3...


Correction: The ITK-SYK Fusion Oncogene Induces a T-Cell Lymphoproliferative Disease in Mice Mimicking Human Disease

In this article (Cancer Res 2010;70:6193–204), which was published in the August 1, 2010 issue of Cancer Research (1), the name of the 12th author is incorrect. The correct name should be Katja Zirlik.

Reference


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