Cucurbitacin I Inhibits Stat3 and Induces Apoptosis in Sézary Cells

Marloes S. van Kester[1], Jacoba J. Out-Luiting[1], Peter A. von dem Borne[1,2], Rein Willemze[1], Cornelis P. Tensen[1] and Maarten H. Vermeer[1]

Sézary syndrome (Sz) is an aggressive cutaneous $CD4^+$ T-cell lymphoma with tumor cells (Sz cells) localized in the skin, lymph nodes, and peripheral blood. Using western blotting, we demonstrate the expression of phosphorylated (P)-Stat3 in the Sz-derived cell line Seax, and in freshly isolated tumor cells from Sz patients ($n=6$). *In Vitro* overnight culture without exogenous cytokines results in decreased expression of P-Stat3 ($n=3$), indicating that Stat3 is not constitutively activated. Incubation of the Seax cell line with the Jak/Stat3 inhibitor Cucurbitacin I resulted in a time- and concentration-dependent decrease of P-Stat3 and Stat3. In freshly isolated Sz cells ($n=3$), Cucurbitacin I induced a concentration-dependent decrease in Stat3 expression whereas P-Stat3 was undetectable. Finally, incubation of freshly isolated Sz cells ($n=4$) with 30 μM Cucurbitacin I for 6 hours induced apoptosis in the large majority (73–91%) of tumor cells. These data strengthen the notion that activation of Stat3 plays an essential part in the malignant transformation of Sz and provide further rationale for the therapeutical targeting of Stat3 in Sz.

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INTRODUCTION

Sézary syndrome (Sz) is an aggressive lymphoma/leukemia of skin homing, $CD4^+$ memory T cells characterized by the presence of neoplastic T cells (Sz cells) in the skin, lymph nodes, and peripheral blood. Patients present with pruritic erythroderma and generalized lymphadenopathy. Ectropion, alopecia, palmoplantar keratoderma, and generalized immunosuppression are common associated features (Willemze *et al.*, 2005). Although Sz can be treated with low dose prednisone and chlorambucil or immunomodulating therapies (extracorporeal photophoresis, IFN-α), the prognosis is poor, with a median survival between 2 and 4 years after diagnosis and a disease-specific 5-year survival around 20%.

The Stat proteins have dual roles as cytoplasmic signaling proteins and nuclear transcription factors. Following activation by receptors with tyrosinase kinase activity, phosphorylated Stats dimerize and translocate to the nucleus to induce expression of critical genes essential in normal physiological cellular events. Persistent activation of Stats, particularly Stat3, has been implicated in the pathogenesis of a whole spectrum of malignancies, including T-cell lymphoma (Darnell Jr *et al.*, 1994).

[1]*Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands and* [2]*Department of Hematology, Leiden University Medical Center, Leiden, the Netherlands*

Correspondence: Dr Cornelis P. Tensen, Department of Dermatology, Leiden University Medical Center, Room T2-003, Einthovenweg 20, Leiden 2333 ZC, the Netherlands. E-mail: c.p.tensen@lumc.nl

Abbreviations: Sz, Sézary syndrome; PBMC, peripheral blood mononuclear cell

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In Sz-derived cell lines as well as in Sz cells isolated from peripheral blood, expression of phosphorylated (P)-Stat3 and P-Stat5 has been reported. Recent studies have shown that in Sz autocrine/paracrine signaling of IL-2, IL-7, and IL-15 cytokines results in phosphorylation of Stat3 and Stat5 (Foss *et al.*, 1994; Zhang *et al.*, 1996; Eriksen *et al.*, 2001; Qin *et al.*, 2001; Zhang *et al.*, 2005). Therefore, activation of Stat3 and Stat5 through persistent cytokine signaling may play a crucial role in the pathogenesis of Sz.

Recently, a new series of selective and potent Jak/Stat3 inhibitors was developed. Of these, Cucurbitacin I specifically decreased the levels of P-Stat3 in various human cancer cell lines (Blaskovich *et al.*, 2003; Sun *et al.*, 2005; Shi *et al.*, 2006) and most importantly, inhibited tumor growth in mice models (Blaskovich *et al.*, 2003).

Based on these observations, inhibition of Stat3 signaling is a promising therapeutic target in Sz but functional experiments to validate this hypothesis have not been performed thus far. The objectives of this study were (1) to determine the expression of (P)-Stat3 in both freshly isolated Sz cells and the Sz-derived Seax cell line, (2) to establish the type of activation (constitutive vs non-constitutive), and (3) to study the effects of the Stat3 inhibitor Cucurbitacin I on (P)-Stat3 levels and the ability to trigger apoptosis in Sz cells.

RESULTS

Expression of activated Stat3(P-Stat3) by malignant cells in patients with Sz

To determine the activation status of Stat3 by malignant cells in Sézary patients, 10 μg protein extracted by Trizol was loaded on gel. As shown in Figure 1a, in all Sz patients both expression of P-Stat3 and Stat3 was detected. In contrast, in

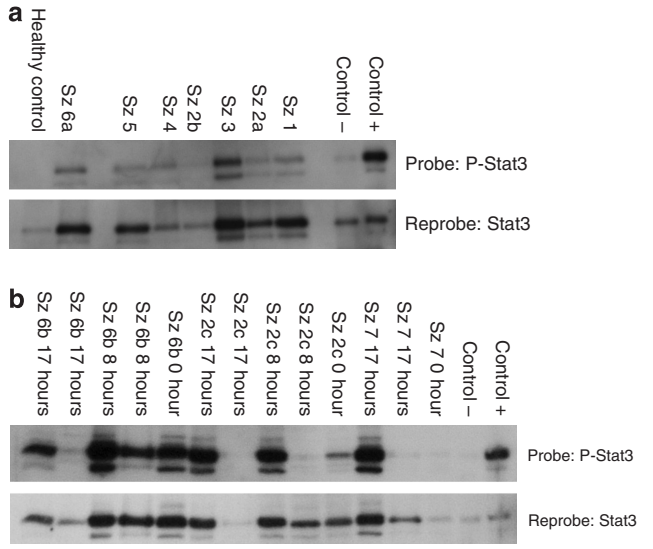

Figure 1. Stat3 is consistently expressed, but not constitutively activated in patient-derived Sz cells. Western blot analysis of proteins isolated (Trizol extraction) from 5×10^6 PBMCs of six Sz patients (**a**) or CD4$^+$ T cells ($n = 3$) are cultured in the presence (*c*) and absence (*s*) of cytokines (10% T-cell extract) for 17 hours (**b**) 10 μg protein was loaded on gel and subjected to western blotting using P-Stat3 specific antibody. The blot was stripped and reprobed with Stat3 antibody.

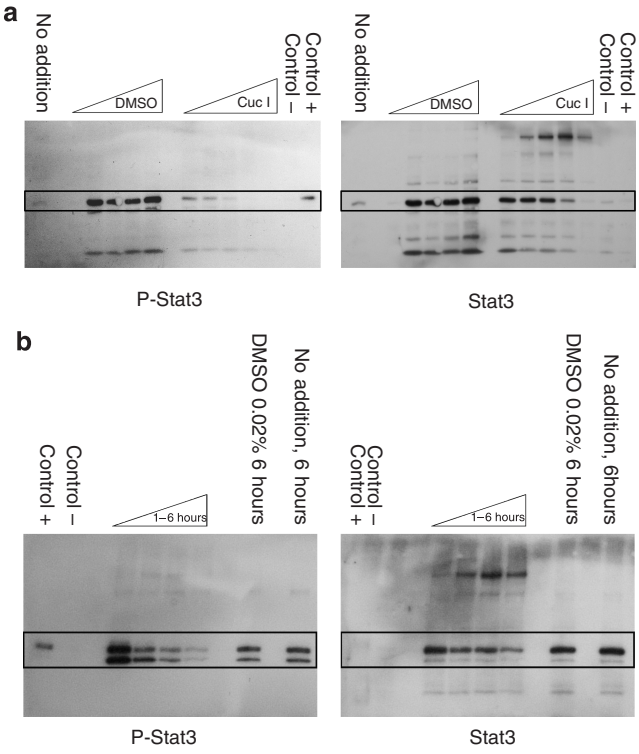

Figure 2. Cucurbitacin I inhibits P-Stat3 and Stat3 expression in Seax cells in a concentration- and time-dependent manner. (**a**) Cucurbitacin I (0.3, 1, 3, 10, and 30 μM), a volume equivalent of DMSO (solvent), or nothing was added to 2×10^5 Seax cells for 6 hours. In (**b**), 10 μM Cucurbitacin (1, 2, 4, or 6 hours), a volume equivalent of DMSO (6 hours), or nothing (6 hours) was added. Cells were lysed with lysis buffer and 1/6 of the total cell lysate was subjected to western blot analysis using specific antibody against P-Stat3. The blot was stripped and reprobed with antibody against Stat3.

peripheral blood mononuclear cells (PBMCs) from a healthy control no expression of P-Stat3 and low levels of Stat3 were observed. *In Vitro* overnight culture without exogenous cytokines resulted in decreased expression of P-Stat3 ($n = 3$), whereas in the presence of exogenous cytokines P-stat3 expression increased or remained unaltered (Figure 1b).

Cucurbitacin I inhibits P-Stat3 and Stat3 in Seax cells

To determine whether Cucurbitacin I is capable of suppressing Stat3 phosphorylation, time course experiments and dose-escalating studies were performed using the Seax cell line. Results from western blotting demonstrated that Cucurbitacin I induced a dose-dependent decrease of both P-Stat3 and Stat3 in Seax cells. In contrast, no decrease in P-Stat3 or Stat3 expression was observed in negative controls (Figure 2a).

Time course experiments demonstrated a time-dependent decrease in expression for both P-Stat3 and Stat3 (Figure 2b). This decrease in expression could already be detected after 1 hour of incubation. The decrease was more pronounced for P-Stat3 than for the total amount of Stat3 protein. Again, no decrease in expression of P-Stat3 or Stat3 expression was observed in the negative control (Figure 2b).

Cucurbitacin I inhibits Stat3 in Sézary cells

In a dose-escalating study, 3×10^5 freshly isolated CD4$^+$ T cells from two Sézary patients (Sz 2 and 5) were incubated for 6 hours with various concentrations of Cucurbitacin I or volume equivalents of DMSO as negative controls. After incubation with Cucurbitacin I, cells were washed twice with phosphate-buffered saline and subjected to lysis with lysis buffer. A dose-dependent decrease in the total amount of Stat3 (Figure 3a and b) was visible, while P-Stat3 was undetectable in all samples tested (data not shown). To test whether the absence of P-Stat3 was due to a limited amount of protein, the number of Sézary cells was increased to 5×10^6 CD4$^+$ in a follow-up experiment (Sz 6a) and the cells were subjected to Trizol protein extraction. On each lane, 5 μg protein was loaded to normalize for the protein content. However, P-Stat3 was still undetectable (data not shown), whereas a similar dose-dependent decrease in total Stat3 level was observed (Figure 3c).

Cucurbitacin I-mediated inhibition of Stat3 induces apoptosis in Sézary cells

To examine whether apoptosis is induced in Sézary cells by inhibition of Stat3, freshly isolated CD4$^+$ T cells (five samples from four Sézary patients Sz 2c, 5, 6a, 6b, and 7) were incubated with 30 μM Cucurbitacin I for 6 hours. After incubation with Cucurbitacin I, 73–91% apoptotic cells were detected by flow cytometry analysis with Annexin-V and propidium iodide staining (Figure 4). Incubation with medium alone or medium containing volume equivalents of DMSO resulted in a low percentage (5–19%) of apoptotic cells. Incubation of freshly isolated CD4$^+$ T cells from healthy controls ($n = 3$) with Cucurbitacin I for 6 hours

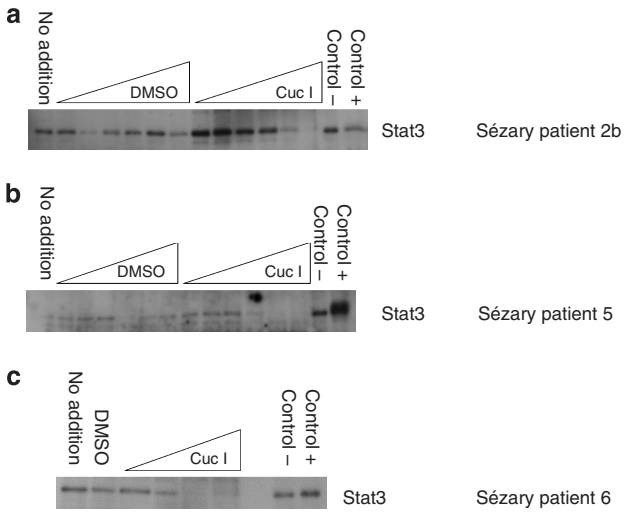

Figure 3. Cucurbitacin I decreases Stat3 expression in Sézary cells. (**a**, **b**) Cucurbitacin I (0.1, 0.3, 1, 3, 10, and 30 μM), a volume equivalent of DMSO (solvent), or nothing was added to 3×10^5 $CD4^+$ T cells for 6 hours. In (**c**), Cucurbitacin I (0.3, 1, 10, and 30 μM) was added to 5×10^6 $CD4^+$ T cells of a single Sz patient for 6 hours and a single DMSO control was used (corresponding to the highest concentration inhibitor). (**a**, **b**) Cells were lysed with lysis buffer or (**c**) protein was extracted with Trizol. (**a**, **b**) Cell lysates, 1/6 of total, or (**c**) 5 μg protein was analyzed by western blotting using antibody against Stat3.

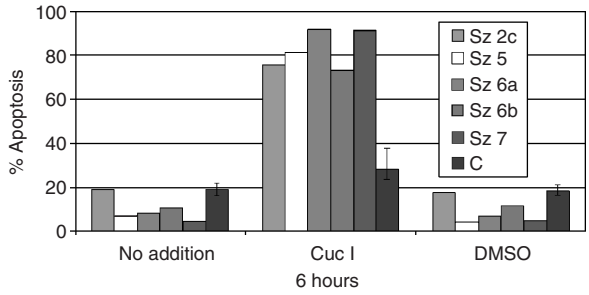

Figure 4. Apoptosis in $CD4^+$ T cells of four different Sz patients and three healthy donors. Sz cells and healthy $CD4^+$ T cells were cultured with or without 30 μM Cucurbitacin I or with a volume equivalent of DMSO (solvent) for 6 hours. Cells were subjected to propidium/Annexin V labeling and apoptotic cells were quantified by FACS analysis. Healthy donors (indicated with *C*): no addition 6 hours (mean 19% ± SEM 3%), Cucurbitacin I 6 hours (mean 28% ± SEM 10%), DMSO 6 hours (mean 19% ± SEM 3%).

resulted in apoptosis in 28% of the cells (mean, SEM 9.7%) (Figure 4).

DISCUSSION

In this study, we show expression of P-Stat3 in Sz cells and that inhibition of Stat3 by Cucurbitacin I results in decreased expression of Stat3 and apoptosis of Sz cells.

Previous studies showed expression of P-Stat3 in Sz-derived cell lines, whereas in freshly isolated Sz cells from peripheral blood, P-Stat3 was detected in only 4 of 13 samples (Zhang *et al.*, 1996). In this study, expression of P-Stat3 was a consistent finding in all Sz patients tested. Also in one patient in whom two samples taken with an interval of 14 months, expression of P-Stat3 was observed in both samples. This difference in expression of P-Stat3 might result from different clinical criteria used for patient selection or differences in protein extraction and detection techniques.

In contrast to a former study performed in Seax cells (Eriksen *et al.*, 2001), Stat3 is not constitutively expressed in primary patient material. After 8 hours, diminished expression of (P)-Stat3 is visible in cells cultured in the absence of exogenous cytokines compared to culture in the presence of cytokines or immediately after isolation. These observations suggest that paracrine cytokine stimulation is essential for activation of Stat3.

The importance of the consistent expression of activated Stat3 in Sz lies not only in a better understanding of the pathogenesis of Sz but also in the validation of Stat3 as a therapeutic target in the management of this disease. In this study, we demonstrate a dose-dependent decrease in the level of P-Stat3 expression using Cucurbitacin I. Of note, this decrease in P-Stat3 levels was achieved in the presence of crude T-cell factor, demonstrating that even in the presence of cytokine stimulation, Cucurbitacin I can inhibit phosphorylation of Stat3 in Seax cells.

In contrast to previous studies on human tumor cell lines (Blaskovich *et al.*, 2003; Sun *et al.*, 2005; Shi *et al.*, 2006), we observed a dose-dependent decrease in both P-Stat3 and total amount of Stat3 protein after Cucurbitacin I treatment of Seax cells.

Known targets of Stat3 are the antiapoptotic genes *BCL-X_L*, *MCL1*, and *Survivin*, and proliferation-inducing genes *MYC* and *cyclin D1* (Buettner *et al.*, 2002). Previous studies in ALK^+ ALCL, lungadenocarcinoma and breast cancer cell lines demonstrated that inhibition of P-Stat3 with Cucurbitacin I results in decreased expression of Stat3 target genes and apoptosis of these cells (Blaskovich *et al.*, 2003; Shi *et al.*, 2006). Moreover, using *in vivo*/murine models, Cucurbitacin I has also been shown to have antitumor effects on various types of tumors (Blaskovich *et al.*, 2003). Our results demonstrate that Cucurbitacin I induced a similar dose-dependent decrease in the expression of Stat3 in freshly isolated Sz cells as in Seax cells. In contrast, we could not detect P-Stat3 in freshly isolated Sz cells, which may be related to the sensitivity of the western blotting assay, because in this particular experiment only 5 μg protein was available, whereas for the other experiments 10 μg protein could be used. We demonstrated that incubation of freshly isolated Sz cells with 30 μM of Cucurbitacin I for 6 hours induced apoptosis in 73–91% of Sz cells. Normal $CD4^+$ T cells showed a much lower sensitivity to Cucurbitacin I-induced apoptosis supporting the potential of Stat3 inhibitors as therapeutic agents in Sz.

In conclusion, we show consistent expression of P-Stat3 in Sz cells and demonstrate that inhibition of Stat3 using Cucurbitacin I results in apoptosis of these cells. Based on our study, Stat3 inhibitors are a potential therapeutic agent for Sz.

MATERIALS AND METHODS

Cell lines

The Seax line (Kaltoft *et al.*, 1987) was cultured in RPMI 1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, penicillin

100 U ml^{-1}, streptomycin 100 μg ml^{-1} (all from Invitrogen, Breda, the Netherlands), and 10% T-cell extract (made from a buffy coat of a healthy individual after stimulation with phytohaemagglutinin (PHA) and IL-2).

Patients and healthy donors

Seven patients with Sz as defined by the World Health Organisation-European Organization for Research and Treatment of Cancer (WHO-EORTC) classification were selected for this study (Willemze et al., 2005). In six patients, PBMCs were isolated at diagnosis by Ficoll density centrifugation. In addition, in four of seven patients (Sz 2, 5, 6, and 7), CD4$^+$ T cells were isolated from PBMCs by negative selection using magnetic beads (CD4$^+$ T-cell isolation kit, Miltenyi Biotec, Bergisch Gladbach, Germany). From one patient (Sz 2), PBMCs were isolated both at diagnosis, after a 14-month follow-up and after an 11-month follow-up, during which the patient had been treated with low dose prednisone (10–40 mg daily). From another patient (Sz 6), CD4$^+$ T cells were isolated at diagnosis and after a 10-month follow-up, during which the patient had been treated with 20–30 mg prednisone and chlorambucil 2–4 mg daily. PBMCs isolated from a healthy donor served as normal control for western blotting. For apoptosis assays, CD4$^+$ T cells isolated from healthy donors ($n=3$) were used as normal controls. The freshly isolated CD4$^+$ T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin 100 U ml^{-1}, and streptomycin 100 μg ml^{-1} during the Stat3 inhibition assays and the apoptosis assays. Approval for these studies was obtained from the institutional review board of the LUMC. Informed consent was provided according to the Declaration of Helsinki Principles.

Cucurbitacin I

Cucurbitacin I (JSI-124) (National Cancer Institute NCI identifier: NSC 521777), purchased from Indofine Chemical Company (Hillsborough, NJ), was dissolved in DMSO to a concentration of 50 mM. During the experiments, Cucurbitacin I was diluted in culture medium to the final concentrations.

Stat3 activation assays

Protein from of 5×10^6 PBMCs (patient 1, 2a, 3, 2b, 4, 5, 6a) was extracted using Trizol (Invitrogen) to determine the level of P-Stat3 and Stat3. To establish the type of activation, CD4$^+$ T cells (patient 2c, 6b, and 7) were cultured in the presence and absence of 10% T-cell extract for 17 hours. The protein concentration of the samples was measured by a Nanodrop (ND-1000 Spectrophotomter, PEQLAB Biotechnologie GMBH, Erlangen, Germany) and 10 µg of protein was loaded on a 7% polyacrylamide gel.

Stat3 inhibition assays

In dose-escalating studies, 2×10^5 Seax or 3×10^5 Sz (patient 2b and 5) or 5×10^6 Sz (patient 6a) cells were treated with increasing concentrations of Cucurbitacin I for 6 hours. The Seax or Sézary cells maintained in medium with an equivalent volume of DMSO (solvent) added to the medium served as negative controls.

In time course experiments, Seax cells were maintained for 1–6 hours in 2 ml medium containing 10 μM Cucurbitacin I. Seax cells maintained in medium containing equivalent volume of DMSO was used as control.

Cells from Cucurbitacin I inhibition assays (Seax and patients 2b and 5) were washed twice with phosphate-buffered saline, lysed with lysis buffer containing Tris-buffered saline-NP40 1%, 1:100 phosphatase inhibitor cocktail A (sc-45044, Santa Cruz Biotechnologies, Heidelberg, Germany), 1:100 phosphatase inhibitor cocktail B (sc-45045, Santa Cruz Biotechnologies), and complete mini Protease Inhibitor cocktail (Roche, Penzberg, Germany). One-sixth of the lysate plus loading buffer was heated for 3 minutes at 100°C, spinned off and loaded on each lane of 7% polyacrylamide gel. The protein content of the cells of patient 6 was extracted by means of Trizol and measured by the Nanodrop, subsequently 5 µg was electrophoresed.

Western blotting

Electrophoresed protein was transblotted to polyvinylidine difluoride membranes (Amersham Biosciences, Buckinghamshire, England). After transblotting, the polyvinylidine difluoride membranes were blocked overnight at room temperature in 5% milk (Protifar plus, Nutricia, Zoetermeer, the Netherlands) in Tris-buffered saline containing 0.1% Tween 20. P-Stat3 and total Stat3 antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Goat anti-rabbit IgG HRP was used as secondary antibody (Santa Cruz Biotechnology). All antibodies were diluted in 5% milk in Tris-buffered saline containing 0.1% Tween 20.

Chemilluminescence (West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL) was used for visualization of the western blots. All blot experiments were performed in duplo. After the initial series of antibodies and substrates and recording on X-ray films, blots were stripped (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and reciprocally reprobed with the other antibody. Stat3 Control Cell Extracts (no. 9133) from Cell Signaling technology were used as positive and negative controls.

Apoptosis assays

Based on the results of the experiments on Seax cells (Figure 2), freshly isolated CD4$^+$ T cells were incubated with 30 µM Cucurbitacin I for 6 hours labeled with Annexin V and Propidium iodide (Annexin-V-Fluos Staining kit, Roche) and analyzed by flow cytometry using a Becton Dickenson FACS Scan. Results were evaluated with Cell Quest software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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