Published in final edited form as:

Br J Dermatol. 2010 September ; 163(3): 564–571. doi:10.1111/j.1365-2133.2010.09812.x.

Malignant T cells in cutaneous T-cell lymphoma lesions contain decreased levels of the antiapoptotic protein Ku70

K. Ferenczi*, J. Ohtola*, P. Aubert*, M. Kessler*, H. Sugiyama*, A.K. Somani*, A.C. Gilliam*, J.Z. Chen†, I. Yeh†, S. Matsuyama†, T.S. McCormick*, and K.D. Cooper*,‡

*Department of Dermatology, University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, OH 44106, U.S.A

†Division of Haematology and Oncology, Department of Medicine, University Hospitals Case Medical Center, Cleveland, OH, U.S.A

‡Administration Medical Center, Cleveland, OH, U.S.A

Summary

Background—Malignant T cells in primary cutaneous T-cell lymphoma (CTCL) are genetically unstable and exhibit prolonged lifespans potentially explained by dysregulation of apoptosis, yet are responsive to apoptosis-inducing therapies. The heterodimeric protein Ku70/80 is known to play a role in DNA repair (Ku70 and Ku80) and inhibition of apoptosis (Ku70 only).

Objectives—To investigate the expression of Ku70/80 in CD3+ T cells derived from skin and blood in patients with CTCL and normal samples, as well as benign dermatoses.

Methods—Normal (n = 10), CTCL (n = 9) and benign dermatoses (n = 13) skin samples were stained for confocal imaging of Ku70/80 and CD3 and analysed using imaging software. Circulating CD4+ T cells in normal and CTCL peripheral blood were analysed by flow cytometry and Western blot for Ku70/80 expression (n = 6).

Results—Ku70 and Ku80 were significantly diminished in T cells of CTCL lesions relative to T cells of control skin. Decreased T-cell Ku70 expression was not a feature of the benign dermatoses psoriasis and contact dermatitis, suggesting that loss of Ku70/80 in CTCL is not simply the result of cutaneous inflammation. Reduced Ku70 was also noted in circulating CD4+ T cells in patients with CTCL with peripheral blood involvement.

Conclusions—Deficient expression or lack of Ku70/80 may result in genomic instability and play a role in tumorigenesis, as well as account for the increased susceptibility of malignant T cells to apoptosis-inducing treatment modalities in the setting of intrinsic resistance to apoptosis.

Keywords

apoptosis; cutaneous T-cell lymphoma; Ku70

Primary cutaneous T-cell lymphomas (CTCLs) represent a heterogeneous group of non-Hodgkin lymphomas characterized by clonal proliferation of neoplastic T cells and strikingly increased epidermal homing mechanism.^{1–3} Primary CTCLs such as mycosis fungoides (MF), the most common form of CTCL, typically have a long and protracted clinical course.³ The clinically indolent and chronic nature characteristic of most primary

CTCLs has been postulated to be mediated by gradual accumulation of the malignant T-cell clone in the skin, slow proliferative rate and lack of or dysregulation of apoptosis.^{4,5} Intrinsic resistance to apoptosis, a characteristic common to the pathology of several lymphomas, appears to be a prominent feature of malignant T cells in CTCL as well. Indeed, apoptosis, a mechanism that would allow elimination of cancer cells, is very rarely detected in MF skin lesions.⁶

Underlying mechanisms postulated to be involved in dysregulation of apoptosis in cutaneous lymphoma include defective Fas/Fas ligand expression and/or impaired signalling,^{7–10} constitutive STAT activation^{11,12} and increased expression of the antiapoptotic members of the bcl-2 family, including bcl-2 itself.¹³

Interestingly, whereas malignant CTCL T cells are resistant to apoptosis, they appear to be responsive to apoptosis-inducing treatment modalities; skin-targeted therapies in early-stage disease can lead to durable remission. Most, if not all, therapies employed in the management of CTCL such as narrow-band ultraviolet (UV) B, psoralen plus UVA (PUVA), steroids, oral retinoids, extracorporeal photopheresis (ECP) and histone deacetylase inhibitors, such as vorinostat, rely upon induction of T-cell apoptosis to achieve efficacy and remission.^{4,5,14–20} The susceptibility to UV-induced apoptosis of malignant T-cell clones has been variably reported as similar or increased compared with normal T cells.^{16,18} Neoplastic CTCL lymphocytes, however, appear to have an enhanced susceptibility to apoptosis induction by vorinostat treatment.²⁰ Enhanced propensity for treatment-induced apoptosis, characteristic of malignant T cells, in the setting of a baseline intrinsic resistance to apoptosis is an interesting conundrum.

Ku70 is a recently described key inhibitor of apoptosis belonging to the bcl-2 family. Ku70 has been shown to be a target for apoptosis induction by UV radiation and histone deacetylase inhibitors.^{21,22} Ku70 was originally identified as a protein involved in the nonhomologous end joining (NHEJ) of DNA double-strand break (DSB) repair.^{23,24} Ku70 also plays a pivotal role in inhibition of apoptosis by blocking the translocation of proapoptotic Bax to the mitochondria.^{25,26} While Ku is a heterodimeric protein consisting of two subunits, Ku70 and Ku80, the antiapoptotic function of Ku70 via suppression of Bax-mediated apoptosis occurs independently of Ku80.^{21,25}

Previous reports suggest that deficient or low Ku70 expression may lead to genomic instability and tumorigenesis, and that Ku70 may act as a tumour suppressor.²⁷ Ku70 inactivation has been shown to result in susceptibility to malignant transformation.²⁷ Ku70 knockout (Ku70^{-/-}) mice have a profound deficiency in DNA DSB repair and are hypersensitive to ionizing radiation and agents that promote apoptosis. These animals develop thymic and disseminated T-cell lymphomas at an early age.^{27,28}

In this study, we assessed the expression of Ku70/80 in CTCL lesions and normal skin and showed that both Ku70 and Ku80 levels were significantly downregulated in lesional T cells in patients with CTCL compared with T cells derived from normal skin and benign dermatoses. Decreased Ku70 expression was also noted among circulating malignant T cells in patients with peripheral blood involvement compared with normal T cells.

Materials and methods

Patients

All studies involving human subjects were approved by the institutional review boards of Case Western Reserve University and University Hospitals Case Medical Center. Archival paraffin sections stored in the University Hospitals Case Medical Center (Cleveland, OH,

U.S.A.) Dermatopathology Laboratory (from 2000 to 2008) were used for this study. Tissue samples were stained from nine patients with histologically proven CTCL (all stages from IA to IVA), three with contact dermatitis, four with graft-versus-host disease (GVHD), six with psoriasis and 10 normal skin samples. Of the nine CTCL cases that were analysed for cutaneous Ku70 expression, eight patients fell into the category of either MF or Sézary syndrome, and one expressed the aggressive CD8+ T-cell phenotype. Of the nine patients, six had MF in various stages of the disease (stages IA, IB, IIA), and two patients had Sézary syndrome with clinical erythroderma (T4) and evidence of peripheral blood involvement that was in conformity with the recent criteria.^{3,29} Evidence for peripheral blood involvement in one of the patients with Sézary syndrome was based on flow cytometric detection of 99% of T cells with a CD7- CD4+ phenotype and a CD4/CD8 ratio of 17.6. Peripheral blood T cells in the other patient with Sézary syndrome included in this study had a significantly elevated CD7- CD4+ T-cell count (over 90%), and 98% of these circulating T cells were found to uniformly express the T-cell receptor V β 22 using flow cytometry. This finding was consistent with the presence of a large clonal T-cell population and near absence of normal T cells in the circulation.

Ku70 analysis in the peripheral blood was performed on peripheral blood mononuclear cells (PBMC) isolated from six patients with CTCL with peripheral blood involvement and 10 healthy controls. The selection of patients with CTCL with blood involvement, as noted previously, was based on one of the following criteria: CD4/CD8 ratio > 10, percentage of T cells with a CD7- CD4+ phenotype > 20% or detection of a T-cell clone in peripheral blood by polymerase chain reaction (PCR).

The histological criteria that were used in the diagnosis of CTCL included a combination of features: the presence of single lymphocytes lining up along the dermoepidermal junction, halos around lymphocytes, epidermotropism, disproportionate exocytosis in the absence of spongiosis, presence of hyperconvoluted lymphocyte nuclei and Pautrier's microabscesses and papillary dermal fibrosis.

Additional immunohistochemical stains using antibodies to CD3, CD4, CD8 and CD7 were included as an adjunctive aid in the diagnosis of cutaneous lymphoma to demonstrate a skewed CD4/CD8 ratio and loss of CD7 within the skin-infiltrating lymphocytes. PCR studies for detection of clonality in skin lesions were done and were positive in eight of the nine patients with CTCL.

This study was conducted in accordance with the Declaration of Helsinki Principles.

Reagents and antibodies

Mouse monoclonal Ku70 (clone N3H10; Abcam, Cambridge, MA, U.S.A.), mouse monoclonal Ku80 (clone B-1; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), mouse kappa monoclonal IgG2b (Abcam) and mouse monoclonal IgG1 (DakoCytomation, Carpinteria, CA, U.S.A.) antibodies were used for immunofluorescence, immunocytochemistry and flow cytometry experiments. Goat polyclonal R-phycoerythrin secondary antibody (DakoCytomation), CD3PerCP, IgG1PerCP, CD4FITC, CD7FITC and IgG1FITC (BD Biosciences, San Jose, CA, U.S.A.) antibodies were used for flow cytometry studies. Hoechst 33258 (Sigma-Aldrich, St Louis, MO, U.S.A.), rabbit monoclonal antibody to CD3 (clone SP7; Abcam), normal rabbit IgG (Santa Cruz Biotechnology), goat antimouse IgG Alexa568 and donkey antirabbit IgG Alexa488 (Invitrogen, Carlsbad, CA, U.S.A.) were used for confocal imaging in paraffin-embedded normal and CTCL tissue and for immunocytochemistry. In confocal imaging of PBMC by cytopspin, mouse monoclonal CD3 and IgG1 conjugated to Alexa488 (Caltag Laboratories/Invitrogen) were used to identify T cells.

Immunofluorescence for confocal microscopy

Antigen retrieval was performed using near boiling citrate buffer (DakoCytomation) for 20 min prior to the application of antibodies. Samples were blocked with 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA, U.S.A.), then incubated with either Ku70 or Ku80 primary antibody or IgG2b or IgG1 isotype controls, respectively (all $8 \mu\text{g mL}^{-1}$), followed by incubation with goat antimouse Alexa568 secondary antibody ($5 \mu\text{g mL}^{-1}$). Sections were also blocked with 10% normal donkey serum (Jackson ImmunoResearch), then incubated with CD3 primary antibody or IgG isotype control (both 1: 100), then incubated with donkey antirabbit Alexa488 secondary antibody ($5 \mu\text{g mL}^{-1}$), followed by Hoechst 33258 staining ($2 \mu\text{g mL}^{-1}$).

Analysis of confocal staining

Quantitative assessment of Ku70 and Ku80 expression in CD3+ T cells in CTCL and normal skin was performed using Metamorph imaging software (Version 7.1.0.0; Molecular Devices, Downingtown, PA, U.S.A.). Images were collected from a Zeiss LSM 510 META confocal microscope. During analysis, values that exceed a threshold of 11.0 were considered CD3 positive while cells below that value were considered negative. CD3+ cells were then further analysed for Ku70 and Ku80 staining intensity using the image of the cell nuclei to locate and define a given cell. A nuclear dilation algorithm was used to expand the size of each nucleus to incorporate both cytoplasmic and plasma membrane Ku expression. All data were exported to Microsoft Excel sheets to aid in sorting of positive and negative cells.

Statistical analyses

Statistical significance ($P < 0.05$) of Ku70/80 mean staining intensity in CTCL and normal skin T cells was determined using the Mann–Whitney U-test.

Flow cytometry staining

PBMC from patients with CTCL ($n = 6$) with peripheral blood involvement and healthy controls ($n = 10$) were isolated using Ficoll gradient centrifugation. For cell surface staining, 5×10^5 cells per $100 \mu\text{L}$ were incubated with Ku70 or Ku80 primary antibody or IgG2b or IgG1, respectively, isotype control (all $1 \mu\text{g mL}^{-1}$). This was followed by incubation with the goat polyclonal R-phycoerythrin secondary antibody (1: 200) and surface T-cell marker fluorochrome-labelled CD3, CD4, CD7 and IgG1 antibodies (1: 20). Cells were then fixed with 2% formaldehyde. For intracellular staining, cell surface staining was first performed by incubation with the conjugated antibodies (1: 20), followed by a permeabilization step using Cytofix/Cytoperm kit (BD Biosciences) as per manufacturer's instructions and Ku70 or Ku80 staining followed by goat polyclonal R-phycoerythrin secondary antibody. Fluorescence intensity was measured on a BD LSRII flow cytometer (BD Biosciences) and analysed using Winlist Version 6.0 (Verity Software House, Topsham, ME, U.S.A.).

Cytospin preparation and immunocytochemistry

CD4+ T cells from two patients with CTCL with Sézary syndrome and two healthy controls were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA, U.S.A.), followed by incubation with CD3 and IgG2a antibodies conjugated to Alexa488 (1: 100). Cells ($3\text{--}5 \times 10^5$) were then spun on to ringed cytology glass slides using a Shandon Cytospin 3 Centrifuge (both Fisher Scientific) for 8 min at 40 g and allowed to dry to permeabilize cells. For intracellular staining, cells were blocked with 10% normal goat serum, then incubated with either Ku70 primary antibody or IgG2b isotype control (both $4 \mu\text{g mL}^{-1}$), followed by incubation with goat antimouse Alexa568 secondary antibody ($5 \mu\text{g mL}^{-1}$). In addition, samples were counterstained with Hoechst 33258 ($1 \mu\text{g mL}^{-1}$).

Western blot

Normal and CTCL peripheral CD4⁺ T cells were lysed in 100× dilution of protease inhibitor cocktail (Sigma-Aldrich) and 1 mmol L⁻¹ phenylmethylsulphonyl fluoride. Cell lysates were precleared by centrifugation (14 000 r.p.m., 20 min, 4 °C) to remove any insoluble fractions and cleared cell lysates were used for Western blot analysis. Samples containing 10 µg of protein were used to detect Ku70 and β-actin for each analysis. Primary antibodies used for this analysis were mouse monoclonal Ku70 antibody (1: 1000, A9; Santa Cruz Biotechnology) and mouse monoclonal β-actin (1: 1000, A5441; Sigma-Aldrich) and the secondary antibody was horse-radish peroxidase-conjugated goat antimouse antibody (1: 2500; Bio-Rad, Hercules, CA, U.S.A.). Densitometric analysis was performed using a Bio-Rad Gel Doc and Quantity One 4.5.1 software from Bio-Rad.

Results

Ku70/80 is constitutively expressed by a majority of normal skin-infiltrating lymphocytes

To study the expression pattern of Ku70/80 in normal skin, confocal imaging for Ku70/80 and CD3 was performed on 10 normal skin samples. As shown in Figure 1(a, c), normal keratinocytes constitutively expressed Ku70 and Ku80, respectively. While prominent nuclear Ku70 and Ku80 staining was seen in most epidermal layers, diminished or absent expression was noted in the stratum corneum. Confocal double-label imaging also revealed constitutive nuclear Ku70 (white arrows, Fig. 1b) and Ku80 (arrows, Fig. 1d) expression by a significant proportion of T cells (green) in normal skin. Image analysis revealed Ku70 expression in a mean of 77% of the total normal T-cell population, whereas only 23% of dermal T cells lacked detectable Ku70 expression (Fig. 1b). Similarly, Ku80 expression was detected in 77.5% of T cells and was absent in 22.4% of skin T cells (Fig. 1d). Interestingly, very strong, predominantly cytoplasmic Ku70 staining was noted in the dermis of normal skin by large CD3⁻ mononuclear cells, most likely monocytes/macrophages (yellow arrow, Fig. 1b).

Ku70/80 expression is significantly reduced in cutaneous T-cell lymphoma skin lesions

Ku70/80 expression was analysed in nine patients with CTCL, stages IA, IB, II and IV, with biopsy-proven disease. At the time of biopsy, seven patients with CTCL included in this study were on no systemic therapies, one was treated with narrowband UVB and bexarotene and two were on twice monthly ECP therapy. Ku70/80 staining intensity was compared with that seen in normal skin samples (n = 10) and benign dermatoses (n = 13).

Representative examples of Ku70 staining in CTCL epidermis and dermis in a patient with Sézary syndrome are shown in Figure 1(e, f). In contrast to constitutive Ku70 expression noted in normal dermal T cells (white arrows, Fig. 1b), Ku70 staining was diminished to nearly absent in cutaneous dermal and epidermal CTCL T cells despite increases in the number of CD3⁺ infiltrating lymphocytes (Fig. 1e and arrows, f). Only 37% of lesional T cells in CTCL expressed detectable levels of Ku70 compared with 76.6% in normal skin T cells. Quantitative analysis of total T-cell Ku70 expression in normal and diseased skin revealed that Ku70 was significantly downregulated in lesional T cells in patients with CTCL relative to control (mean fluorescence intensity of 649 vs. 1355, respectively; $P = 0.0076$) (Fig. 2). Ku70 mean staining intensity was also analysed separately in epidermal and dermal CTCL T cells, revealing a slight decrease in Ku70 expression by epidermal T cells when compared with the dermis (579 vs. 684, respectively) (data not shown). However, the difference was not significant ($P = 0.5457$), which may be explained by the fact that malignant T cells are not restricted to the epidermal compartment but are present in the dermis as well as mixed with reactive, so-called 'bystander' T cells. A summary of Ku70 expression in T cells from all patients with CTCL and normal controls included in this study

is given in Table 1. As shown in Table 1, no correlation between the stage of the disease and Ku70 expression was noted. In order to determine whether diminished Ku70 detected in the skin lesions from patients with CTCL is not due to skewed results from decreased Ku70 expression noted in one patient with the unusual CD8+ phenotype, the P-value was also calculated without this patient's data included ($P = 0.0155$). This result further confirmed that Ku70 is significantly diminished in patients with MF and Sézary syndrome.

Similar to Ku70, Ku80 expression was also found to be significantly diminished in CTCL skin lesions relative to normal skin T cells (mean staining intensity 721 vs. 1238, respectively, $P = 0.0175$) (data not shown). On average, 60.7% of T cells in CTCL skin lesions expressed detectable levels of Ku80 compared with 77.6% in normal skin T cells. This difference was again significant when the CTCL patient with the CD8+ phenotype was excluded from the analysis ($P = 0.0140$).

Quantitative analysis of epidermal keratinocyte nuclear Ku70 levels showed a mean staining intensity of 890 and 966 in CTCL and normal skin, respectively (data not shown). Interestingly, while in most cases low levels of Ku70 were noted in CTCL keratinocytes (Fig. 1e), in two patients with CTCL Ku70 expression was 2- or 3-fold higher compared with that observed in normal keratinocytes (Fig. S1b; see Supporting Information). Similar findings were noted when Ku80 expression was analysed in normal and CTCL skin (data not shown).

Ku70 and Ku80 expression is not reduced in benign dermatoses, suggesting that diminished Ku70 is not secondary to inflammation

To determine whether detection of significantly lower levels of Ku70/80 expression in CTCL skin lesions was a nonspecific finding secondary to inflammation, we studied Ku70 and Ku80 expression in a variety of inflammatory dermatoses, such as involved psoriasis ($n = 6$), contact dermatitis ($n = 3$) and GVHD ($n = 4$). As shown in Figure 1(g, h), Ku70 expression in dermal T cells of contact dermatitis and psoriatic skin lesions showed a staining pattern similar to that in normal dermis with the majority of T cells constitutively expressing Ku70 (Fig. 1b). The mean Ku70 staining intensity in these dermatoses was comparable with that observed in normal skin (contact dermatitis: 1448, psoriasis: 1438 vs. 1355, respectively, $P = 0.7197$) (Fig. 2). Epidermal keratinocyte Ku70 expression in patients with contact dermatitis and psoriasis was also similar to levels in normal keratinocytes (Fig. S1; see Supporting Information).

Ku70 staining evaluated in uninvolved skin from one patient with psoriasis revealed very high levels of Ku70 expression compared with involved skin (2763 vs. 1438) (data not shown). Expression of Ku70 was variable in four patients with GVHD studied: two demonstrated diminished levels of Ku70 compared with normal, one far exceeded normal levels and one was similar to the normal mean. A scatter plot summarizing the results of the mean Ku70 staining intensity in CTCL vs. normal skin and benign dermatoses is shown in Figure 2.

Baseline Ku70 expression in circulating malignant T cells is also diminished

To investigate any differences in Ku70 levels in peripheral blood, normal and CD7⁺ CD4⁺ T cells in patients with CTCL with advanced stage/Sézary syndrome were analysed using flow cytometry ($n = 6$) and confocal imaging ($n = 2$). Circulating CD7⁺ CD4⁺ T cells represented more than 50% of the total T-cell population, gating on CD3⁺ cells. Flow cytometry staining for Ku70 and Ku80 revealed that the majority (> 70%) of normal peripheral blood T cells expressed intracellular Ku70 and Ku80 constitutively (Fig. 3a, b). Interestingly, we found that a small subset of peripheral blood T cells also expressed the

membrane-bound form of Ku70 (Fig. S2; see Supporting Information). Previous reports have shown that although Ku70 expression is predominantly nuclear, it can also be detected on the cell surface.^{30,31} In addition, we found that although Ku70 expression is heterogeneous in both normal peripheral blood T cells as well as malignant T cells, decreased Ku70 was found in patients with CTCL with peripheral blood involvement (arrows, Fig. 3a, b). Flow cytometric analysis revealed that whereas 65% of benign CD4+ T cells expressed intracellular Ku70, only 36% of the CD7- CD4+ T cells appeared to be Ku70 positive (Fig. 3c).

Western blot analysis of cell lysates from CD4+ T cells obtained from patients with CTCL (n = 6) with peripheral blood involvement and healthy controls (n = 6) showed diminished Ku70 expression in five of the six patients with CTCL compared with healthy control T cells (Fig. 3d). Densitometry analysis revealed that this difference was significant (P = 0.0411). In contrast, the level of Ku80 protein in peripheral blood T cells in patients with CTCL was similar to or slightly diminished (n = 3) compared with normal circulating T cells.

Discussion

In this study, we demonstrate that Ku70/80, a critical component of the DNA DSB repair and key inhibitor of apoptosis, is significantly decreased in T cells infiltrating CTCL lesions when compared with normal skin lymphocytes. Decreased T-cell Ku70/80 level was not a feature of psoriasis and contact dermatitis, suggesting that diminished Ku70/80 expression in CTCL lesions is not secondary to an inflammatory cytokine milieu, but rather a feature of malignant T cells or the cutaneous microenvironment in CTCL. Deficient expression of Ku70/80 in CTCL would have two major consequences: impairment in NHEJ/DSB repair, which would influence both genomic stability and suppression of translocations, and enhanced apoptosis.

Whereas our findings show that both Ku70 and Ku80 are diminished in CTCL lesions compared with normal skin T cells, we noted that Ku70, but not Ku80, was decreased in circulating T cells of patients with CTCL with advanced disease and peripheral blood involvement. As decreased or depleted Ku70 renders cells more sensitive to a variety of apoptotic stimuli,^{25,32} it is tempting to speculate that diminished Ku70 expression by neoplastic T cells in CTCL might account for increased susceptibility of the malignant clone to apoptosis-inducing stimuli and good clinical response to apoptosis-inducing treatment modalities. In early stages of CTCL, when most clonal lymphocytes reside in the skin, skin-targeted therapies that induce malignant T-cell apoptosis result in durable remission.¹ Due to its key role in both suppression of Bax translocation to the mitochondria and DNA repair, Ku70 participates in the regulation of apoptosis and survival and the level of Ku70 expression might be critical in determining cell fate upon apoptotic stimuli.

It has been suggested that Ku degradation is one of the steps necessary to activate Bax based on the observation that apoptotic stress stimulates Ku70 ubiquitination facilitating proteasome-dependent proteolysis of Ku.³³ While under physiological circumstances Ku70 plays a cytoprotective role, acetylation of Ku70 leads to impaired NHEJ, dissociation and release of Bax, eventually resulting in apoptosis.³⁴ Most apoptosis-inducing treatments used in the management of CTCL are known either to acetylate Ku70 (UV, vorinostat)^{21,34} and/or induce DNA DSB (psoralens in PUVA, nitrogen mustards),⁵ which would result in the inability of Ku70 to exert its cytoprotective role, such as suppression of apoptosis and participation in NHEJ/DSB repair.

Although activation of multiple apoptotic pathways is likely to be involved in apoptosis induction following various treatment modalities utilized in the management of CTCL,⁵

most, if not all, are known to target Ku70 acetylation and/or induce DNA DSB repair. As Ku70 has a crucial protective role in determining the fate of a cell upon apoptotic stimuli, it is possible that low Ku70 in malignant CTCL T cells results in a lack of defence and susceptibility to apoptosis induction following these treatments.

Our hypothesis is that decreased Ku70 might be responsible, at least in part, for enhanced susceptibility of CTCL T cells to apoptosis induction. Decreased Ku70 expression in the malignant T-cell clone may therefore influence response to treatment. We predict that patients with treatment-resistant CTCL should have higher Ku70 expression, thus providing higher protection from apoptosis-inducing therapies. In this context, levels of Ku70 expression may be useful as a predictor of response to therapy.

We propose that low Ku70 in malignant T cells might be the ‘Achilles heel’ of neoplastic T cells in CTCL, explaining the paradox that clonal T cells in CTCL have a baseline resistance to apoptosis, yet are responsive to apoptosis-inducing therapies. In conclusion, our studies show decreased baseline expression of the cytoprotective protein Ku70 in malignant cells in CTCL skin and blood. These studies suggest a critical link between the paradox of inherent resistance to apoptosis yet increased susceptibility to apoptosis-inducing signals characteristic of malignant T cells in CTCL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Michael Sramkoski (Case Comprehensive Cancer Center), Minh Lam (Case Department of Dermatology) and Scott Howell (Case Visual Sciences Research Center) for the excellent technical assistance, and Elma Baron, Christiane Sykes and Christi Malbasa (Case Skin Study Center), as well as Bernadette Jekutis (University Hospitals Ireland Cancer Centre) and Sean Carlson (University Hospitals Case Medical Center Department of Dermatology) for assistance with sample collection. This work was funded by National Institutes of Health grants R21CA115057 and P30AR39750 (to K.D.C.), the flow cytometry core facilities of the Case Comprehensive Cancer Center (P30CA43703) and the Department of Dermatology Resident Research and Education Fund.

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What's already known about this topic?

- Clonal T cells in cutaneous T-cell lymphoma (CTCL) have prolonged lifespans due to resistance to apoptosis, yet they are susceptible to apoptosis-inducing therapies.
- Expression of Ku70/Ku80, a protein with known cytoprotective functions, has not been addressed in CTCL.

What does this study add?

- We found decreased T-cell Ku70/Ku80 in patients with CTCL relative to normal controls, which may result in genomic instability and play a role in tumorigenesis.

Fig 1.

Confocal imaging of normal, cutaneous T-cell lymphoma (CTCL) and benign dermatoses skin. Three-colour laser confocal microscopy staining of Ku70/80 (red), CD3 (green) and the nuclear dye, Hoechst 33258 (blue) in paraffin-embedded skin samples. (a, c) Note strong constitutive nuclear keratinocyte Ku70 and Ku80 expression in the epidermis of normal skin. Dashed yellow line shows dermoepidermal junction. (b, d) Positive Ku70 and Ku80 staining is seen in the vast majority of dermal T cells in normal skin (white arrows). Additionally, Ku70 expression was also found in the dermis of normal skin on CD3-mononuclear cells (yellow arrow). (e, f) Three-colour laser confocal microscopy image showing diminished to near absent Ku70 expression in lesional skin of a patient with CTCL with Sézary syndrome. Note lack of detectable Ku70 expression by epidermal and dermal T cells in CTCL skin lesion. (g, h) Confocal microscopy staining showing Ku70 expression in lesional skin from patients with (g) psoriasis and (h) contact dermatitis. Note that the majority of T cells in psoriasis and contact dermatitis expresses Ku70. (a–h) Bar = 20 μ m.

Fig 2.

Ku70 expression in cutaneous T-cell lymphoma (CTCL) vs. normal skin and benign dermatoses. Scatter plot displaying Ku70 staining intensity in skin-infiltrating T cells in patients with CTCL, normal skin and benign dermatoses, such as psoriasis and contact dermatitis. Horizontal lines indicate means. Note the significant difference in the mean T-cell Ku70 staining intensity in CTCL skin lesions compared with normal skin and benign dermatoses. Psoriasis and contact dermatitis samples were combined to increase the number of samples and calculate significance. * $P < 0.05$. GVHD, graft-versus-host disease.

Fig 3. Ku70 expression in peripheral blood CD4⁺ T cells. (a, b) Confocal microscopy staining of Ku70 (red), CD3 (green) and Hoechst 33258 (blue) in peripheral blood CD4⁺ T cells from (a) a healthy control and (b) a patient with cutaneous T-cell lymphoma (CTCL) with Sézary syndrome. CD4⁺ T cells were isolated with negative selection using magnetic bead separation. Note the very strong Ku70 staining in normal circulating T cells compared with lower Ku70 staining intensity observed in T cells from the patient with CTCL. (c) Flow cytometry analysis showing that 65% of normal peripheral blood T cells express Ku70 vs. Ku70 expression observed in 36% of malignant (CD7[−] CD4⁺) T cells. (d) Representative Western blot analysis of peripheral blood T-cell Ku70 expression in a healthy control compared with a patient with Sézary syndrome. Note diminished Ku70 in malignant T cells from the patient compared with normal lymphocytes. (a, b) Bar = 10 μm.

Table 1

Summary of Ku70 staining intensity in normal and cutaneous T-cell lymphoma (CTCL) T cells

Normal skin	Mean intensity	CTCL lesion	Disease stage	Mean intensity
1	832	1	IA (T1, N0, M0)	369
2	1392	2	IB (T1b, N0, M0)	2011
3	973	3	IB (T2, N0, M0) ^a	231
4	1221	4	IB (T2, N0, M0)	627
5	1400	5	IB (T1b, N0, M0)	1148
6	1639	6	IB (T1b, N0, M0)	569
7	895	7	IIA (T2, N1, M0)	178
8	1763	8	IVA (T4, N1, M0, B2)	107
9	1591	9	IVA (T4, N1, M0, B2)	607
10	1846			
Mean	1355	Mean		649

^aCD8+ aggressive CTCL. Note that the TNM staging system was used for CTCL lesions: T, tumour; N, lymph node; M, metastasis.