Brief report

Classical Hodgkin lymphoma is characterized by high constitutive expression of activating transcription factor 3 (ATF3), which promotes viability of Hodgkin/Reed-Sternberg cells

Martin Janz, Michael Hummel, Matthias Truss, Brigitte Wollert-Wulf, Stephan Mathas, Korinna Jöhrens, Christian Hagemeier, Kurt Bommert, Harald Stein, Bernd Dörken, and Ralf C. Bargou

Hodgkin/Reed-Sternberg (HRS) cells of classical Hodgkin lymphoma (cHL) display unique characteristics that discriminate cHL from other B-cell lymphomas and normal B cells. Therefore, comparative gene expression profiling of Hodgkin and non-Hodgkin B cells could lead to the identification of candidate genes that are critical for the pathogenesis of cHL. We performed microarray analysis of Hodgkin and non-Hodgkin cell lines and identified activating transcription factor 3 (ATF3), a member of the cyclic AMP response element binding protein (CREB)/ATF family, as a differentially expressed candidate gene. Extensive analysis of a large panel of cell lines, primary tumor samples, and normal tissues revealed that high expression of ATF3 is found in nearly all cases of cHL and is almost exclusively restricted to it. Selective knock-down of ATF3 by RNA interference suppressed proliferation and strongly reduced viability of Hodgkin cells. Thus, overexpression of ATF3 is a molecular hallmark of cHL that contributes to the malignant growth of HRS cells. (Blood. 2006;107:2536-2539)

© 2006 by The American Society of Hematology

Introduction

Classical Hodgkin lymphoma (cHL) is a common malignant lymphoma characterized by the presence of typical malignant cells, the mononuclear Hodgkin and multinuclear Reed-Sternberg (HRS) cells.1 HRS cells, which represent only a minor cellular fraction of the affected lymphoid tissue, are thought to be derived from germinal or postgerminatal center B cells.2,3 Although the precise molecular mechanisms underlying the malignant transformation of HRS cells are only partially understood, several observations indicate that defects in transcriptional regulation play a pivotal role in the pathogenesis of cHL. In line with this idea, we have previously identified high constitutive activity of the transcription factor nuclear factor (NF)-κB as a common characteristic of HRS cells that contributes to their survival and proliferation.4,5 In nonmalignant cells, NF-κB is only transiently activated, particularly by stress signals and in immune and inflammatory signaling events.6 Another hallmark of cHL is the aberrant expression of the AP-1 transcription factor family members c-Jun and JunB,7 which are usually activated in response to mitogenic stimuli and cellular stress signals.

HRS cells display unique morphologic and molecular characteristics that clearly discriminate cHL from B-cell non-Hodgkin lymphomas and normal B cells.8 Therefore, gene expression profiling is a promising approach to identify genes that contribute to malignant growth and survival in cHL. We performed oligonucleotide microarray analysis of Hodgkin and non-Hodgkin cell lines and identified activating transcription factor 3 (ATF3) as a differentially expressed candidate gene with high expression in HRS cells. ATF3 is a member of the ATF/CREB family of basic region leucine zipper transcription factors, which is involved in the cellular stress response.9 ATF3 expression is strongly induced by cellular damage in a variety of organ systems, including liver, heart, and neurons as well as in cultured cells following exposure to UV light, ionizing radiation, proteasome inhibitors, or interference with protein synthesis. Furthermore, ATF3 has been reported to be associated with cellular proliferation and survival.10-12 We therefore decided to analyze the role of ATF3 in cHL in more detail.

Study design

Cell lines, electroporation, and purification of transfected cells

Cell lines, culture conditions, and electroporation have been described previously.7 Where indicated, an additional purification step following
transfection was included, employing either fluorescence-activated cell sorting (FACS) or magnetic cell sorting (MACS) as detailed in Mathas et al and Chatterjee et al.

Oligonucleotide microarray analysis

RNA processing and hybridization to U95A GeneChip microarrays was performed according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). Primary data files were analyzed with the Microarray Suite software (Affymetrix). Hierarchical clustering analysis was carried out with the Cluster and TreeView software (M. Eisen, Berkeley, CA; http://rana.lbl.gov/EisenSoftware.htm).

Northern and Western blot analysis

Northern and Western blotting were performed according to standard protocols as described previously. The following primary antibodies were used: anti-ATF3 (sc-188; Santa Cruz Biotechnology, Santa Cruz, CA), anti–E2F-4 (clone 4E2F04; Neomarkers, Fremont, CA), and anti–α-tubulin (MCA78A; Serotec, München, Germany).

Immunohistochemistry

All cases were retrieved from the files of the Institute of Pathology, Charité, Campus Benjamin Franklin, Berlin, Germany. Diagnosis was established after histologic and immunohistologic analyses according to the criteria of the World Health Organization classification. ATF3 immunohistochemistry was performed with a polyclonal rabbit anti-ATF3 antibody (sc-188; Santa Cruz Biotechnology) after heat pretreatment of the deparaffinized tissue sections in a pressure cooker for 2 minutes in citrate buffer (10 mM). Subsequently, a mouse anti–rabbit antibody (DacoCytomation, Glostrup, Denmark) was applied followed by development employing alkaline phosphatase and fast red as substrate. Approximately two-thirds of the tissue sections were arranged in tissue microarrays, whereas the remaining cases have been stained as single tissue sections.

Construction of siRNA expression plasmids

Vectors for transient expression of small interfering (si) RNAs were derived from the pSUPER plasmid according to the guidelines described in Brummelkamp et al. The epimidal plasmid vector pRep-H1 (M.T., Uschi Luz, Reinhold Schafer, and C.H., manuscript in preparation) was constructed by insertion of an H1-promoter–based PolIII transcription unit into the vector pTIPHA1 × 3. EGF that contains a puromycin selectable marker, a truncated EBNA gene, and an Epstein-Barr virus (EBV) oriP sequence. The following target sequences were chosen: EGF 5′-GCGACGACGCTACTCTCAAG-3′, Luc (directed against firefly luciferase; pGL2 vectors; Promega, Madison, WI) 5′-CGTACGCGGAATACTTCGA-3′, E2F-4 5′-GGCGCGGATGTTACATTACGCATTT-3′, ATF3_1 5′-GAGCGTGAG-GTTGCCATCC-3′, ATF3_2 5′-GAGGCGACGAGAAAGAAT-3′, and ATF3_3 5′-GAAGAGGAGAAGCAGGAG-3′.

Proliferation assay and analysis of apoptosis

DNA synthesis was determined by [3H]-thymidine incorporation assays as described in Lentsch et al. To assess the percentage of viable and apoptotic cells, annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining was performed using a human annexin V–FITC kit (Bender MedSystems, Eching, Germany). Cells were processed as described in the manufacturer’s protocol and analyzed by flow cytometry.

Image acquisition

Images of the immunohistological stainings for ATF3 were captured with an Olympus AX70 microscope with plan-apochromatic objective lenses (4 × 0.13 numeric aperture [NA]; 10 × 0.40 NA; 20 × 0.70 NA; 40 ×/0.95 NA) coupled to a digital camera (KY-F40; JVC, Friedberg, Germany). The images were stored and processed with the Diskus Program (version 4.20; Hilgers Technisches Buero, Koenigswinter, Germany). Original magnifications for Figure 1B: top row, from left to right: ×160, ×400, ×160; bottom row, from left to right: ×250, ×250, ×25.

Results and discussion

To identify candidate genes that are involved in the pathogenesis of cHL, we performed oligonucleotide microarray analysis of Hodgkin and non-Hodgkin B-cell lines. Hierarchical clustering separated Hodgkin from non-Hodgkin cell lines of distinct B-cell differentiation stages. One of the most pronounced up-regulated genes in Hodgkin cell lines was the transcription factor ATF3 (Figure S1, available on the Blood website; see the Supplemental Figure link at the top of the online article). To validate the microarray data on ATF3 expression, Northern and Western blot analyses were carried out on a broad panel of cell lines. All Hodgkin cell lines demonstrated strong overexpression of ATF3 mRNA and protein compared with non-Hodgkin cell lines (Figure 1A). ATF3 expression in primary malignant and nonmalignant lymphoid tissue was determined by immunohistochemistry. A total of 415 lymphoma samples were immunostained for ATF3, comprising 71 cases of cHL and 26 cases of lymphocyte-predominant HL (LPHL) as well as 239 B-cell non-Hodgkin lymphomas, 46 T-cell non-Hodgkin lymphomas, and 33 anaplastic large cell lymphomas (ALCL). In addition, 20 specimens of reactive lymphoid tissue were included (Figure 1B and Table 1). Strong nuclear expression of ATF3 was detected in nearly all cases of cHL.
analyzed (69/71). Nuclear ATF3 staining was restricted to HRS cells and could not be detected in surrounding bystander cells (Figure 1B). In contrast, nuclear ATF3 immunoreactivity was observed in only a few samples of B- or T-cell non-Hodgkin lymphomas (6/239 and 2/46, respectively), including diffuse large B-cell lymphoma (2/80), plasmacytoma (4/20), and angioimmunoblastic T-cell lymphoma (2/10) (Table 1). In these cases, positive staining was found in only a varying, usually small proportion of the tumor cell population with generally weaker signal intensity compared with cHL and ALCL. In addition, no nuclear staining was observed in reactive lymphoid tissue (Figure 1B). Interestingly, ATF3 expression could not be demonstrated in the nuclei of LPHL tumor cells (Figure 1B). This is in agreement with the notion that cHL and LPHL represent independent entities that differ in biology and clinical course. On the other hand, besides cHL, a proportion of ALCCL cases demonstrated ATF3 staining (24/33; Figure 1B), which did not correlate with the expression of ALK fusion proteins (data not shown). ATF3 expression in cHL and ALCL is in line with the observation that these lymphomas share several features, including CD30 expression as well as high Notch-1 and AP-1 activity. It remains to be determined whether this pattern reflects common pathogenetic pathways in both lymphoma types. Taken together, our immunohistochemical analysis demonstrated that nuclear expression of ATF3 is almost exclusively restricted to HRS cells of cHL and to a fraction of ALCCL.

To assess the functional significance of ATF3 overexpression in HRS cells, we selectively blocked ATF3 expression by RNA interference. Three independent ATF3 siRNA sequences were chosen that resulted in a significant down-regulation of ATF3 protein level after transfection into L428 and L540Cy Hodgkin cells (Figure 2A). Several control siRNA sequences for irrelevant targets had no effect on ATF3 expression (Figure 2A). Next, we asked whether siRNA-mediated knock-down of ATF3 in Hodgkin cells would affect proliferation and tumor cell viability. Therefore, we transiently transfected Hodgkin cells with ATF3-directed siRNA expression plasmids and performed [3H]-thymidine incorporation assays. Down-regulation of ATF3 resulted in a marked reduction of DNA synthesis compared with control-transfected cells (Figure 2B). To investigate the effects of ATF3 down-regulation over an extended period of time, we used vector-based siRNA constructs that are propagated by extrachromosomal replication and carry a puromycin resistance gene permitting the selection of siRNA-expressing cells. Transfection of L428 and L540Cy Hodgkin cells with ATF3-directed siRNA plasmids and subsequent antibiotic selection resulted in a significant and sustained loss of viable cells, as determined by annexin V–FITC/propidium iodide (PI) staining and flow cytometry (Figure 2C). This effect could be induced with different ATF3-targeting constructs, but not with control siRNA plasmids directed against irrelevant targets. Our observations on the role of ATF3 in HRS cells are in good accordance with previous reports that describe a function of ATF3 in the control of cell cycle progression and apoptosis resistance in other cellular systems. Ectopic expression of ATF3 in hepatocytes stimulates cyclin D1 expression and proliferation. In addition, ATF3 has been shown to have anti-apoptotic functions in cardiomyocytes and neuronal cells. In line with its cell cycle–promoting and anti-apoptotic properties, ATF3 has been shown to partially transform chicken embryo fibroblasts, indicating an intrinsic oncogenic potential. Thus, in conjunction with previously published observations on its role in proliferation and survival, our data suggest an important oncogenic role of constitutive ATF3 expression in cHL.

## Acknowledgment

We are indebted to Reuven Agami (Amsterdam, The Netherlands) for the gift of the pSUPER siRNA expression plasmid.

### Table 1. ATF immunostaining in primary malignant and nonmalignant lymphoid tissue

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>ATF3 Positive</th>
<th>ATF3 Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical Hodgkin lymphoma</td>
<td>69</td>
<td>2</td>
</tr>
<tr>
<td>Lymphocyte-predominant Hodgkin lymphoma</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>B-NHL</td>
<td>6*</td>
<td>233</td>
</tr>
<tr>
<td>T-NHL</td>
<td>21</td>
<td>44</td>
</tr>
<tr>
<td>Anaplastic large cell lymphoma (ALCL)</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>Reactive lymphatic tissue</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

**Summary of ATF3 immunohistochemistry results.** The group of B-cell non-Hodgkin lymphomas (B-NHLs) includes 21 mantle cell lymphomas (MCLs), 27 B-cell chronic lymphocytic leukemias (B-CLLs), 72 follicular lymphomas (FLs), 80 diffuse large B-cell lymphomas (DLBCLs, including 6 primary mediastinal B-cell lymphomas), 19 Burkitt lymphomas (BLs), and 20 plasmacytomas. T-cell non-Hodgkin lymphomas (T-NHLs) comprise 20 peripheral T-NHLs (PTCLs), 10 angioimmunoblastic T-NHLs (AITLs), 5 enteropathy-associated T-NHLs, 4 NK/T-cell lymphomas, and 7 cases of mycosis fungoides. *Two cases of DBCL (one centrolobular variant and one primary mediastinal B cell lymphoma), 4 cases of plasmacytoma. †Two cases of angioimmunoblastic T-cell lymphoma (AITL), nuclear ATF3 staining in malignant T cells.
References


Classical Hodgkin lymphoma is characterized by high constitutive expression of activating transcription factor 3 (ATF3), which promotes viability of Hodgkin/Reed-Sternberg cells

Martin Janz, Michael Hummel, Matthias Truss, Brigitte Wollert-Wulf, Stephan Mathas, Korinna Jöhrens, Christian Hagemeier, Kurt Bommert, Harald Stein, Bernd Dörken and Ralf C. Bargou