Molecular Immunology 45 (2008) 3085–3095

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Cooperation of Gata3, c-Myc and Notch in malignant transformation of double positive thymocytes

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ARTICLE INFO

Article history:
Received 30 January 2008
Received in revised form 7 March 2008
Accepted 10 March 2008
Available online 8 May 2008

Keywords:
Lymphoma
c-Myc
Gata3
T cell
Expression profiling

ABSTRACT

Gata transcription factors are critical regulators of proliferation and differentiation implicated in various human cancers, but specific genes activated by Gata proteins remain to be identified. We previously reported that enforced expression of Gata3 during T cell development in CD2-Gata3 transgenic mice induced $CD4^+CD8^+$ double-positive (DP) T cell lymphoma. Here, we show that the presence of the DO11.10 T-cell receptor transgene, which directs DP cells towards the CD4 lineage, resulted in enhanced lymphoma development and a dramatic increase in thymocyte cell size in CD2-Gata3 transgenic mice. CD2-Gata3 DP cells expressed high levels of the proto-oncogene c-Myc but the Notch1 signaling pathway, which is known to induce c-Myc, was not activated. Gene expression profiling showed that in CD2-Gata3 lymphoma cells transcription of c-Myc and its target genes was further increased. A substantial fraction of CD2-Gata3 lymphomas had trisomy of chromosome 15, leading to an increased *c-Myc* gene dose. Interestingly, most lymphomas showed high expression of the Notch targets Deltex1 and Hes1, often due to activating Notch1 PEST domain mutations. Therefore, we conclude that enforced Gata3 expression converts DP thymocytes into a pre-malignant state, characterized by high c-Myc expression, whereby subsequent induction of Notch1 signaling cooperates to establish malignant transformation. The finding that Gata3 regulates c-Myc expression levels, in a direct or indirect fashion, may explain the parallel phenotypes of mice with overexpression or deficiency of either of the two transcription factors.

1. Introduction

Gata transcription factors, which bind DNA at a consensus sequence (A/T)GATA(A/G) through conserved zinc-finger domains (Evans et al., 1988), are important regulators of cellular proliferation and differentiation. Gata1 and Gata2 are expressed in hematopoietic cells, while Gata4-6 are expressed in non-hematopoietic organs, such as heart, lung and intestine (Patient and McGhee, 2002). Gata3 is present in T cells but also in non-hematopoietic tissues, including kidney, central nervous system, skin and mammary gland (Ho et al., 1989).

The embryonic lethality of *Gata3*$^{-/-}$ mutant mice at day 11 of gestation demonstrated its crucial developmental function (Pandolfi et al., 1995). Gene targeting experiments showed that Gata3 is essential in early T cell development (Hendriks et al., 1999; Ting et al., 1996). Gata3 is already expressed in the earliest T cell progenitors in the thymus, the $CD4^-CD8^-$ double negative (DN) cells. In these DN cells, gene segments coding for the T-cell receptor (TCR) β chain undergo V(D)J recombination, whereby only cells with a functional TCRβ gene rearrangement are selected for further maturation. This β-selection process is characterized by cell size increase and induction of proliferation, followed by upregulation of CD4 and CD8 expression. Analysis of a Gata3-lacZ reporter mouse showed that Gata3 is induced in those cells that have passed β-selection (Hendriks et al., 1999). Conditional deletion of the *Gata3* gene in DN cells revealed that Gata3-deficient thymocytes fail to undergo β-selection, indicating that Gata3 is essential for this process (Pai et al., 2003). At the $CD4^+CD8^+$ double-positive (DP) stage, functional TCRα gene rearrangement results in expression of a complete αβ TCR, which has the capacity to recognize peptide antigens pre-

Abbreviations: bHLH, basic-helix-loop-helix; DN, double negative; DP, double positive; SP, single positive; T-ALL, T cell acute lymphoblastic leukemia; TCR, T-cell receptor.

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doi:10.1016/j.molimm.2008.03.018

sented by major histocompatibility complex (MHC) class I and class II molecules. Upon engagement by self-MHC peptide complexes, low- to intermediate-avidity interactions rescue DP thymocytes from death by neglect, resulting in positive selection to either CD8 single positive (SP) cells in the context of MHC class I or CD4 SP cells in the context of MHC class II (Bosselut, 2004; Kappes and He, 2005). By contrast, strong TCR signals trigger apoptosis, leading to negative selection. Gata3 expression is induced in DP cells after TCR stimulation by MHC class II and probably acts in developing CD4 SP cells in a positive feedback loop to upregulate TCR expression (Hendriks et al., 1999; Hernandez-Hoyos et al., 2003; Ling et al., 2007). By contrast, during development of CD8 SP cells Gata3 is downregulated. In effector T cells, Gata3 acts a master regulator of Th2 differentiation, which is essential for transcriptional regulation of the Th2 cytokine locus and inhibits the differentiation of Th1 and regulatory T cells (Mantel et al., 2007; Ouyang et al., 1998; Zheng and Flavell, 1997).

Gata factors have been implicated in various human cancers. Acquired missense mutations in the Gata1 gene cause acute megakaryoblastic leukemia in humans with Down's syndrome (Wechsler et al., 2002). Gata2 is located near 3q21 breakpoints in acute myeloid leukemia, which is accompanied by increased Gata2 expression. Reciprocal changes in the expression levels of Gata4 and Gata6 have been associated with adrenocortical tumor formation (Kiiveri et al., 1999; Vuorenoja et al., 2007; Wieser et al., 2000). Evidence for the involvement of Gata3 in human cancers came from its aberrant expression in pancreatic cancer (Gulbinas et al., 2006) and its expression in association with estrogen α receptor in breast cancer (Hoch et al., 1999). But also somatic mutations resulting in loss of Gata3 function may contribute to breast cancer tumorigenesis (Usary et al., 2004). Gata3 is expressed in human T cell acute lymphoblastic leukemias (T-ALL) (Matsuo et al., 2004; Minegishi et al., 1997) and capable to form a complex with the transcription factors LIM-only domain protein Lmo2 and the basic-helix-loop-helix (bHLH) protein Tal1, which are often aberrantly expressed in human T-ALL (Ono et al., 1998).

Direct evidence for a role of Gata3 in T-ALL came from our finding of thymic lymphomas in transgenic mice with enforced Gata3 expression throughout T cell development, driven by the human CD2 promoter and locus control region (Nawijn et al., 2001a,b). These CD2-Gata3 mice developed monoclonal thymic lymphomas, which were mostly $CD3^+CD4^+CD8^{+/low}$, suggesting a DP origin. However, downstream targets of Gata3 at the DP cell stage that may explain the oncogenic potential of Gata3 have not been identified yet. It is also not known how Gata3 relates to other genes known to play an important role in T ALL development in human and mice, such as the bHLH proteins c-Myc, Tal1 and E2A, the LIM-only domain proteins Lmo1/Lmo2 and the heterodimeric transmembrane receptor Notch1 (O'Neil and Look, 2007). In this report, we characterized the DP compartment of CD2-Gata3 transgenic mice. We found that enforced Gata3 expression converts DP cells into a pre-malignant state characterized by increased cell size and enhanced c-Myc expression. DP lymphomas from CD2-Gata3 transgenic mice manifested a further increase of c-Myc and showed induction of Notch1 signaling, showing cooperation of Gata3, c-Myc and Notch1 in lymphomagenesis.

2. Materials and methods

2.1. Mice

CD2-Gata3/FVB (Nawijn et al., 2001b), CD2-Gata3;DO11.10 (BALB/c) and CD2-Gata3;HY;$Rag2^{-/-}$ (C57BL/6) mice have been described (Ling et al., 2007). Mice were kept under pathogen-free conditions in the Erasmus MC animal care facility. In survival analyses only mice that developed a lymphoma were marked as a case. Mice that died because of other reasons or were used for experiments were marked as censored. Kaplan–Meier analysis was performed using SPSS 11.0.1 statistical software.

2.2. Flow cytometry, antibodies and cell sorting

The generation of single-cell suspensions and four-color flow cytometry have been described previously (Nawijn et al., 2001b). Antibodies were purchased from BD Bioscience (San Diego, CA), samples were acquired on a FACSCalibur™ flow cytometer; data were analyzed by CellQuest™ software (BD Bioscience). FACS sorting of $CD4^+CD8^+7AAD^-$ thymocytes was performed with a FACSVantage VE, equipped with Diva Option and BD FACSdiva software. Purity of fractions was >99%.

2.3. Spectral karyotyping (SKY)

Cells were treated with 10 ng/ml colcemid (GIBCO-BRL, KaryoMAX Colcemid solution) for 15 min to arrest cells at metaphase and subsequently treated with 75 mM KCl and fixed with methanol/acetic acid (3:1). SKY was performed using the Applied Spectral Imaging system (ASI, Migdal Ha'Emek, Israel) following manufacturer's protocols. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) containing DABCO/Vectashield. Chromosomes were analyzed by Zeiss Axioplan 2 microscope equipped with the Spectra Cube system (ASI). Over 10 metaphases from each sample were analyzed, using Skyview analysis software (ASI).

2.4. Preparation of probes, microarray hybridization and data analysis

Oligonucleotide arrays printed with the Operon Mouse Genome Oligo Set version 3.0 (32K mouse) were obtained from the Netherlands Cancer Institute central microarray facility (NKI-CMF, Amsterdam, The Netherlands). Protocols for sample preparation and array hybridization were supplied by NKI-CMF (http://microarrays.nki.nl). In brief, total RNA was isolated using the GeneElute mammalian total RNA miniprep system (Sigma). The quantity and quality of RNA was determined using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE). Samples with a 260/280 nm optical density ratio >1.8 were used. Two micrograms of total RNA was used for amplification using T7 MEGAscript Kit (Ambion, Austin, TX), whereby aminoallyl-UTP (Ambion) was incorporated into amplified RNA (aRNA). Subsequently, Cy5- or Cy3-dye (Amersham, GE Healthcare, Piscataway, NJ) was coupled to the aminoallyl-modified aRNA. Labeled aRNA was purified and concentrated using Microcon YM30 columns (Millipore, Billerica, MA). Oligonucleotide arrays were co-hybridized with purified probes of CD2-Gata3 lymphoma and control pooled wild-type DP T cells and scanned with a Scanarray Express HT scanner (PerkinElmer, Boston, MA). Data were extracted using Imagene software 6.0 (Biodiscovery, CA, USA). Each experiment consisted of 2 oligonucleotide arrays, whereby dyes were reversed between aRNA from CD2-Gata3 lymphoma and the control wild-type DP T cells pool.

Obtained array data were uploaded in the NKI-CMF database, analyzed using NKI-CMF software (http://dexter.nki.nl) and normalized per subarray using the Lowess normalization method. Normalized data from dye-reversed hybridizations were combined by means of a weighted average. Experiments were combined and data were filtered for genes that were differentially expressed in experiments with $p < 0.01$. Hierarchical clustering of genes and experiments was performed using Genesis 1.5.0 (Sturn et al., 2002).