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Monoclonal B-cell hyperplasia and leukocyte imbalance precede development of B-cell malignancies in uracil-DNA glycosylase deficient mice

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Abstract

Ung-deficient mice have reduced class switch recombination, skewed somatic hypermutation, lymphatic hyperplasia and a 22-fold increased risk of developing B-cell lymphomas. We find that lymphomas are of follicular (FL) and diffuse large B-cell type (DLBCL). All FLs and 75% of the DLBCLs were monoclonal while 25% were biclonal. Monoclonality was also observed in hyperplasia, and could represent an early stage of lymphoma development. Lymphoid hyperplasia occurs very early in otherwise healthy Ung-deficient mice, observed as a significant increase of splenic B-cells. Furthermore, loss of Ung also causes a significant reduction of T-helper cells, and 50% of the young $Ung^{-/-}$ mice investigated have no detectable NK/NKT-cell population in their spleen. The immunological imbalance is confirmed in experiments with spleen cells where the production of the cytokines interferon γ, interleukin 6 and interleukin 2 is clearly different in wild type and in Ung-deficient mice. This suggests that Ung-proteins, directly or indirectly, have important functions in the immune system, not only in the process of antibody maturation, but also for production and functions of immunologically important cell types. The immunological imbalances shown here in the Ung-deficient mice may be central in the development of lymphomas in a background of generalised lymphoid hyperplasia.

Keywords: Lymphoma; Uracil-DNA glycosylase; Immunological imbalance

1. Introduction

Uracil in DNA may originate from spontaneous or enzymatic deamination of cytosine, or by misincorporation of dUTP instead of dTTP during replication. Deamination results in U:G mismatches that need to be repaired before replication in order to prevent G:C to A:T transitions. Misincorporated uracil is not directly mutagenic, but may have undesirable effects by changing the affinity of DNA-binding factors [1].

Uracil-DNA glycosylases (UDGs) are a group of enzymes that remove uracil from DNA. Several mammalian enzymes with UDG activity have been characterised; UNG (UNG1 in mitochondria, UNG2 in nuclei), SMUG1, TDG, MBD4 and NEIL1/NEIL2. UNG2 is the quantitatively dominating uracil DNA glycosylases activity in the nucleus [2]. The combined action of these enzymes, together with dUTPases, ensures a low level of uracil in the genome. Loss of UNG has been shown to cause significantly elevated levels of uracil in humans [3] as well as mice [4,5], where it correlates with replicative status [5]. The global spontaneous mutation frequency in the Ung-deficient mice is only modestly increased [4], consistent with Smug1 acting as a backup enzyme repairing premutagenic U:G mismatches [6]. However, the high uracil level in $Ung^{-/-}$ mice demonstrates that neither Smug1 nor any other UDG enzyme can fully compensate for loss of Ung activity.

It has recently become evident that generation and subsequent removal of uracil are pivotal events in the mutagenic

Abbreviations: UNG, uracil-DNA glycosylase from the *UNG* gene; SMUG1, single strand selective monofunctional uracil-DNA glycosylase; TDG, thymine DNA glycosylase; MBD4, methyl-binding domain protein 4; NEIL, Nei-like; AID, activation-induced cytidine deaminase; CSR, class switch recombination; SHM, somatic hypermutation; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma

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process of affinity maturation and class switch recombination of immunoglobulin genes. Antibody diversification depends on expression of activation-induced cytidine deaminase (AID). According to current models, AID generates dU:dG mispairs that are further processed to obtain a normal spectrum of mutations and to obtain the switch from IgM to IgG, IgA or IgE (reviewed in [7]). The importance of UNG activity in these processes is evident in the Ung-deficient mice [8] and in patients suffering from hyper-IgM syndrome (HIGM) [9], that show an altered pattern of somatic hypermutation and severely compromised capacity for class switch recombination. B-cells from human HIGM patients have significantly increased levels of uracil in their genome, and, interestingly, they have a greatly reduced ability to remove uracil from single-stranded DNA [3].

UDG enzymes belong to a large group of DNA glycosylases that participate in base excision repair, which is probably the quantitatively most important mechanism of DNA repair [5]. Several DNA glycosylase-deficient knockout mice have been generated, but the majorities are not associated with a cancer-prone phenotype unless assayed in cancer-susceptible background [10–13]. The Ung-deficient mice, however, develop a lymphoproliferative disorder characterised by lymphoid hyperplasia, infiltration of lymphoid cells in non-lymphoid tissues and a 22-fold increased risk of developing lymphomas [14]. These lymphomas develop late in life and have been characterised as follicular B-cell lymphomas [14]. The underlying mechanism for the development of B-cell lymphomas in Ung-deficient mice is still unknown, but it has been suggested that the abnormalities in somatic hypermutation (SHM) and class switch recombination (CSR) may contribute to lymphomagenesis [15]. Although the number of mice subjected to macroscopic inspection in the previous report was high, the number of mice subjected to stringent histopathological analysis was fairly low (10 mice), and clonal analysis was not performed on the tumours [14]. Hence, a closer examination of a larger number of lymphomas from $Ung^{-/-}$ mice is required in order to give a more detailed characterisation. Here, we have characterised 22 tumours by hematoxylin–eosin–saffron staining and immunohistochemistry. Importantly, we used the same material for clonality analysis. We analysed very young animals (8–12 weeks) for pathology of the organs affected by tumourigenesis in later life and found clear indications of an early lymphoproliferative disorder affecting the B-cell population, and a general leukocyte imbalance accompanied by perturbed cytokine production at a stage preceding tumour formation.

2. Materials and methods

2.1. $Ung^{-/-}$ mice

The generation of Ung-deficient mice has been described previously [4]. The mice were kept in a conventional animal facility until they spontaneously developed clear signs of disease and/or discomfort. Sacrificed animals were examined by a veterinarian and organs from 22 mice with macroscopic characteristics of lymphoma were fixed in 10% buffered formalin, paraffin embedded and used for hematoxylin–eosin–saffron staining, immunohistochemistry and clonality analysis.

2.2. Antibodies for immunohistochemistry

Rat anti-mouse CD3 (pan T-cell marker) and rabbit anti-Ki-67 (proliferation marker) were purchased from Novo Castra (Newcastle, UK), rat anti-mouse CD45R/B220 (pan B-cell marker) was provided by BD Pharmingen (Oxford, UK), rabbit anti-TdT (to stain lymphoblasts) was purchased from DakoCytomation (Glostrup, Denmark) and rabbit anti-Bcl-6 (stains cells derived from germinal centres) was provided from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal rabbit-anti-rat secondary antibody and the EnVision + TM detection system were purchased from DakoCytomation.

2.3. Immunohistochemistry

Serial sections (3–4 μm) of formalin fixed, paraffin embedded tissues were used for HES staining (hematoxylin–eosin–saffron) and immunohistochemistry. Sections were treated according to standard protocols for paraffin removal before staining, and epitopes were unmasked using 1 mM EDTA pH 8 and microwave boiling (for the Bcl-6 Ab we used 10 mM Tris, 1 mM EDTA, pH 9). Endogenous peroxidase was blocked with 0.3% H_2O_2 for 5 min. Primary antibodies were diluted (CD3, 1:200; CD45R, 1:50; TdT, 1:50; Ki-67, 1:1500; Bcl-6, 1:30) in PBS with 1% BSA and incubated at RT for 25–60 min (CD3: 25 min, CD45: 30 min, others: 60 min). After washing in PBS (2× 5 min) sections with rabbit primary antibodies were stained using the Envision + TM detection system (DakoCytomation) in accordance with the manufacturer's protocol. Sections with rat primary antibodies were incubated with rabbit-anti-rat (1:1500, 30 min, RT) before washing and detection.

2.4. Isolation of DNA from paraffin-embedded tissues

Paraffin-embedded material was stamped using a 18 Gy needle. Paraffin was removed using xylene treatment followed by ethanol washing and drying. The tissue was treated with proteinase K (1 mg/ml) for 3 h—overnight at 56 °C, and the supernatant was transferred to a BioRobot EZ1 for isolation of DNA.

2.5. Clonality analysis using multiplex PCR

In early stages of lymphoma development, microscopic and IHC evaluations may be insufficient, especially since follicular lymphoma and follicular hyperplasia potentially are difficult to distinguish. In order to distinguish between hyperplasia and malignancy, it is of interest to perform clonality analysis. We examined all samples using multiplex PCR to amplify the variable–joining (V_H–J_H) region of the IgH gene. All V_H-primers were specific for the framework 3 (FR3) regions of the variable genes and were designed from published genomic sequences of the variable heavy 1 (V1; VhJ558) and 5 (V5; Vh7183) gene families with focus on sequences from the C57Bl/6 strain. Four V1 primers, three V5 primers and four J-primers labelled with the fluorescent dyes FAM or HEX were used in multiplex PCR reactions. Four different combinations of primers (Table 1) were used; reaction 1; all V1 primers + J1 and J2, reaction 2; all V1 primers + J3 and J4, reaction 3; all V5 primers + J1 and J2 and reaction 4; all V5 primers + J3 and J4. Each DNA sample was analysed (in duplicate) with these four primer combinations. Each multiplex PCR reactions had a final volume of 25 μl containing 10 ng DNA, 1.5 mM Mg^{2+}, 0.04 units AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 0.38 μM of each V-primer and 0.11 μM of each J-primer. All amplifications included a 15 min initial denaturation step at 95 °C, a final extension period for 15 min at 72 °C and 35 cycles with 30 s denaturation (94 °C), 1 min annealing (55 °C) and 1 min extension (72 °C). The size of the PCR products was analysed using an ABI PRISM® 3100 Genetic Analyzer in accordance with the manufacturer's protocol. Monoclonal and polyclonal PCR products were identified based on the distribution of PCR fragments of varying lengths. B-cell lines were used as controls for optimising PCR conditions, and the amplification of IgH DNA was verified by sequencing PCR products.

2.6. Antibodies for flow cytometry

Rat anti-mouse CD4 TRI-COLOR (TC) and rat anti-mouse CD8 PE were obtained from Caltag (Burlingame, CA), while rat anti-mouse CD19 PE and mouse anti-mouseNK-1.1 FITC were obtained from Pharmingen (Oxford,