Nfe2l3 (Nrf3) deficiency predisposes mice to T-cell lymphoblastic lymphoma

Grégory Chevillard,1,2 Marilene Paquet,4 and Volker Blank1-3*

1Lady Davis Institute for Medical Research and Sir Mortimer B. Davis Jewish General Hospital;

2Department of Medicine; 3Department of Physiology, McGill University, Montreal, QC, Canada; and

4Veterinary Comparative Pathology Services, Comparative Medicine & Animal Resources Centre, McGill University, Montreal, QC, Canada

*Corresponding author: Volker Blank, Lady Davis Institute for Medical Research, Department of Medicine, McGill University, 3755 Cote Sainte-Catherine Rd, Montreal, Quebec H3T 1E2, Canada; Phone: +1 514 340 8260 ext. 4984; Fax: +1 514 340 7573; Email: volker.blank@mcgill.ca

Running head: ROLE OF NFE2L3 (NRF3) IN LYMPHOMAGENESIS

Journal section category: Brief Report

Scientific category: LYMPHOID NEOPLASIA

Footnote: The online version of the article contains a data supplement.
Abstract

We have previously generated mice deficient for Nrf3 (NF-E2 p45 related factor 3), a member of the cap’n’collar (CNC) family of basic-leucine zipper transcription factors. To examine whether Nrf3 is involved in chemical-induced carcinogenesis, we exposed the mice to benzo[a]pyrene (B[a]P), a carcinogen found in cigarette smoke. Contrary to wild-type mice, Nrf3-null animals are highly susceptible to B[a]P exhibiting significantly increased mortality. Pathology analysis of affected tissue sections revealed a high incidence of T-cell lymphoblastic lymphoma in B[a]P-treated Nrf3−/− mice. Lymphoblastic lymphoma occasionally metastasized into the lung as demonstrated by perivascular malignant lymphocytic infiltration. Together, our studies show that absence of Nrf3 predisposes mice to lymphoma development, suggesting a protective role of this transcription factor in hematopoietic malignancies. Our data demonstrate the first in vivo function of Nrf3 and its link to tumor development. Nrf3-deficient mice may serve as a preclinical mouse model to study carcinogen-induced lymphomagenesis.
Introduction

The polycyclic hydrocarbon benzo[a]pyrene (B[a]P) is an environmental pollutant, a major component of cigarette smoke, and a well characterized rodent and human carcinogen.\(^1\) For instance, B[a]P can induce lymphoma in different genetically modified mouse models.\(^2,3\) Biotransformation of B[a]P is a requisite for its detoxification and excretion. The first step is catalyzed by cytochrome P450-dependent monooxygenases (phase I) and their products are subsequently coupled to endogenous metabolites (phase II).\(^4\) However, certain reactive intermediates interact covalently with DNA to form adducts that ultimately result in mutagenicity and/or carcinogenicity. It has been reported that absence of the Cap ‘n’ collar (CNC) factor Nrf2 renders mice more susceptible to tumorigenesis caused by B[a]P\(^5,6\) most likely due to incapacity to detoxify the carcinogen. These data provided a link between CNC factor-mediated induction of phase II and antioxidant enzymes and the susceptibility to carcinogens.

The CNC family includes p45 NF-E2, Nrf1, Nrf2, Nrf3, Bach1 and Bach2 proteins and can form heterodimers with small Maf proteins. We and others previously identified Nrf3 as an endoplasmic reticulum-associated protein that is Asn-glycosylated.\(^7,8\) We showed that Nrf3 gene expression is induced by butylated hydroxytoluene in the lung of mice.\(^9\) Recently, Pepe et al. showed a role for Nrf3 in smooth muscle cell differentiation.\(^10\) We have generated mice lacking a functional Nrf3 and found that these mice do not show any obvious abnormalities under non-challenging conditions.\(^11\)

Thus, to investigate whether the mice deficient for Nrf3 are tumor-prone, we challenged the mice with the carcinogen B[a]P. Our studies revealed a novel role for Nrf3 in the protection of mice against carcinogen-induced lymphomagenesis.
Study design

Animals and treatments

Male WT and Nrf3 deficient mice\(^{11}\) (129S6/SvEvTac, 8 weeks old) were treated weekly for 4 consecutive weeks by gavage (150 µl) with benzo[a]pyrene (Sigma-Aldrich) at a dose of 100 mg per kg body weight dispersed into corn oil as vehicle. Mice were weighed and monitored weekly and sacrificed 30 weeks after the first administration of B[a]P or earlier if they showed signs of distress. Upon necropsy, tissues were excised, weighed and stored for further analysis. Procedures involving animals and their care were conducted according to McGill University guidelines, which are set by the Canadian Council on Animal Care. Mice were kept at 22\(^{\circ}\)C with equal periods of darkness. Water and food were available \textit{ad libitum}.

Analysis of clonality by Southern blotting

Genomic DNA isolation and Southern blotting were performed as previously described.\(^{11}\) Details are provided in Supplemental Methods.

Histology and immunohistochemistry

Mouse tissue processing, staining of hematoxylin-eosin (H&E) slides and immunohistochemistry were performed according to standard procedures. Details are provided in Supplemental Methods. All sections were examined by a board certified veterinary pathologist (MP).
Statistical analysis

Statistical analysis was performed using Graph Pad Prism (Graph Pad software). Kaplan-Meier test was used to calculate the survival curves, and the log-rank test was used for evaluation of significance. A P value of <0.05 was considered statistically different (*P < 0.05, **P < 0.01, ***P < 0.001).
Results & Discussion

High sensitivity of Nrf3 deficient mice to benzo[a]pyrene exposure

Although elevated Nrf3 transcript levels have been found in many different types of human cancers \(^{12-20}\), no strong evidence exists of a link between Nrf3 and tumor development. Here, we examined whether the mice deficient for the \(Nrf3\) gene are susceptible to exposure to the carcinogen B[a]P.

We treated wild-type and Nrf3 deficient mice weekly for 4 consecutive weeks with B[a]P (100mg/kg) and monitored the mice for tumor formation and survival until terminal sacrifice at week 30. We did not observe any change with respect to body weight of mice upon B[a]P exposure (data not shown) corroborating previously reported results.\(^6\) Only one out of sixteen (6%) B[a]P-treated wild-type mice died before week 30 (Figure 1A). In contrast, six out of nineteen (32%) Nrf3-deficient mice succumbed early starting from the 15\(^{th}\) week following B[a]P-treatment (Figure 1B). This clearly indicated a high sensitivity of Nrf3-null mice to carcinogen exposure.

High incidence of T-cell lymphoblastic lymphomas in benzo[a]pyrene-treated Nrf3-null mice

About one third of Nrf3-deficient mice treated with B[a]P died before the end of the treatment with signs of respiratory distress. Upon necropsy, we found the thymus of these mice to be enlarged in most cases (67%, 4 out of 6) (Figure 2A) reducing space and compressing the lungs caudally against the diaphragm which corroborates with the breathing difficulties exhibited by these mice.\(^{21}\) Occasionally, we detected tumors in the mucosa of the forestomach of mice which are most likely due to B[a]P treatment\(^6\), but the number of these tumors in wild-type and Nrf3\(^{-/-}\) mice was not significantly different (data not shown). Histopathological examination of H&E-stained tissue sections revealed that all Nrf3\(^{-/-}\) mice which died prematurely developed lymphoma originating mostly from the thymus and few from the spleen. The malignant lymphocytes invaded and effaced
the normal thymic and splenic architecture (Figure 2B). In addition, we found the presence of metastatic lymphoma in the lungs of a majority of these animals. We also observed splenomegaly in about 50% of the Nrf3\textsuperscript{-/-} mice developing lymphoma (Figure 2A). Immunohistochemistry analysis of the affected tissues with immature lymphoid cell (Terminal deoxynucleotidyl transferase, TdT), T-cell specific (CD3) and B-cell specific (CD45) markers revealed that the most common type of lymphoma (67%, 4 out 6) found in Nrf3\textsuperscript{-/-} mice was of T-cell origin (Figure 2C). Since clonal population is a hallmark of malignancy, we assessed by Southern blot the rearrangement of T cell receptor (TCR), an important event in T cell ontogeny.\textsuperscript{22,23} Accordingly, we observed a rearrangement at the joining region J\textbeta\textsubscript{2} of the T cell receptor \beta locus in the tissues of mice developing T-cell lymphoblastic lymphoma (Figure S1). The second type of lymphoma observed in Nrf3\textsuperscript{-/-} mice does not originate from the thymus. This lymphoma, derived in the spleen from an immature lineage with TdT positive cells and is characterized by a probable leukemic phase visible on the lung tissue sections (Figure S2). In contrast to Nrf3-null mice, only one wild-type mouse out of 16 (6%) had a distinct type of lymphoma observed late at time of sacrifice (week 30)(Figure 2D). This different type of lymphoma was characterized as a splenic marginal zone lymphoma as indicated by both H&E staining and positive immunostaining of the spleen using an anti-immunoglobulin kappa (Ig\kappa) antibody. Clonal origin of this lymphoma was confirmed by an immunoglobulin H rearrangement present in spleen, lung and thymus of the mouse (data not shown). Together, our results clearly showed a distinct spectrum of lymphoma subtypes and a significant increase in the incidence of lymphoma in the carcinogen-treated Nrf3\textsuperscript{-/-} mice (32%) compared to their wild-type counterparts (6%) (Figure 2D). This result strongly suggests that Nrf3 protects mice from B[a]P-induced lymphoma formation, in particular T-cell lymphoblastic lymphoma. In accordance with these data, we had previously shown that Nrf3 transcripts are highly
expressed in the thymus.\textsuperscript{11} In addition, we did not find any compensatory regulation of the Nrf3 homologs Nrf1 and Nrf2 at the transcript level in the thymus of Nrf3\textsuperscript{−/−} mice compare to wild-type animals (data not shown).

Similarly to the effect described in the present paper, other mouse models have been described as highly susceptible to benzo[a]pyrene-induced lymphomagenesis including mice deficient for the genes \textit{Msh2} (mutS homolog 2)\textsuperscript{3} or \textit{XPA} (Xeroderma Pigmentosum complementation group A).\textsuperscript{2} Interestingly, both Msh2 and XPA proteins are involved in the mechanism of DNA repair and according to the present data one could speculate that \textit{Nrf3} gene might also be involved in this process.

Of interest, a series of laboratories have observed increased levels of human Nrf3 transcripts in Hodgkin lymphoma, in non-Hodgkin cell lineages as well as in Mantle cell lymphoma specimen using genechip arrays.\textsuperscript{13-17,20} Nevertheless, the functionality and modulation of Nrf3 proteins in these cells have not been demonstrated. Considering that Nrf3 acts as a tumor suppressor gene, one cannot exclude that these human lymphoma cells harbour a non-functional Nrf3.

In humans, T-cell lymphoblastic lymphoma is a rare but aggressive form of non-Hodgkin’s lymphoma mostly affecting children, adolescents and young adult males.\textsuperscript{24} Various genetic aberrations have been described for T-cell lymphoblastic lymphoma. In most cases, these translocations juxtapose promoter and enhancer elements of T-cell receptor (\textit{TCR}) genes located at chromosome 7 to transcription factors involved in T-cell differentiation.\textsuperscript{25} Interestingly, the Nrf3 locus maps to this region\textsuperscript{11} and further experiments will be required to determine whether the \textit{Nrf3} gene is affected in the translocations causing different lymphoblastic lymphomas.\textsuperscript{26-28}

In summary, our studies demonstrate that absence of Nrf3 renders mice more susceptible to lymphomagenesis, particularly of T-cell origin, in response to chemical carcinogenesis. Our results
suggest that Nrf3 deficiency can predispose to development of hematologic malignancies. Additional studies at the molecular level will be needed to confirm the close link between lymphomagenesis and Nrf3 function in humans. We finally hypothesize that Nrf3 is a tumor suppressor gene whose function is deregulated by loss and/or mutation in lymphoma and its inactivation may contribute to lymphomagenesis.
Acknowledgments

The authors would like to thank Zaynab Nouhi and Anna Derjuga for outstanding mouse husbandry support, helpful discussions and critical reading of the manuscript. We thank Jadwiga Gasiorek for critical reading of the manuscript and Dr Monica Justice for helpful discussions. We are grateful to Julie Hinsinger, Micheline Fortin and Mélina Narlis of the histopathology facility (Institute for Research in Immunology and Cancer, Montreal) for the great help with immunohistochemistry. GC currently holds a postdoctoral fellowship of the Fonds de la recherche en santé du Québec (FRSQ). This work was supported by a grant from the Canadian Institute of Health Research (MOP-97932) to VB.

Authorship

Contribution: G.C. helped in the design and performed the majority of the experiments, analyzed the data and wrote the manuscript; M.P. provided histopathology expertise; V.B. designed and supervised the research and edited the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

Correspondence: Volker Blank, Lady Davis Institute for Medical Research, Department of Medicine, McGill University, 3755 Cote Sainte-Catherine Rd, Montreal, Quebec H3T 1E2, Canada; Phone: +1 514 340 8260 ext. 4984; Fax: +1 514 340 7573; Email: volker.blank@mcgill.ca
References

Figure legends

Figure 1. Reduced survival rate and hypersensitivity to lymphoma development in Nrf3-deficient mice treated with B[a]P compared to wild-type mice. (A-B) Kaplan-Meier survival curves are shown for wild-type mice (n=34) (A) and Nrf3-deficient mice (n=35) (B) treated or not with B[a]P. Control mice (open circle and triangle) and B[a]P-treated mice (filled circle and triangle) are shown. (C) Kaplan-Meier survival curves (lymphoma-free) are shown for wild-type mice (n=15) and Nrf3-deficient mice (n=17) treated with B[a]P.

Figure 2. Nrf3-null mice treated with B[a]P exhibit a high incidence of T-cell lymphoblastic lymphoma. (A) Thymus and spleen from a Nrf3−/− control mouse (left) and from a B[a]P-treated Nrf3−/− mouse with a T-cell lymphoblastic lymphoma accompanied by splenomegaly (right). (B) Histological sections of the thymus (top), spleen (middle) and lung (bottom) from a B[a]P-treated Nrf3−/− mouse with a T-cell lymphoblastic lymphoma were stained with haematoxylin and eosin (original magnification X200). Bars represent 100 μm. (C) Immunohistochemistry using anti-TdT, anti-CD3 and anti-CD45 antibodies demonstrates that the malignant lymphoid cells in the thymus (top) and in the lung metastases (bottom) of B[a]P-treated Nrf3−/− mice are of T-cell origin (original magnification X400). Bars represent 50 μm. (D) Spectrum of lymphoma subtypes in B[a]P-treated wild type mice vs B[a]P-treated Nrf3-null mice.
Figure 1
Figure 2

A

Nrf3-/ control

Nrf3-/ + B[a]P

thymus

spleen

1 cm

B

Nrf3-/ + B[a]P

thymus

spleen

lung

C

Nrf3-/ + B[a]P

<table>
<thead>
<tr>
<th></th>
<th>TdT</th>
<th>CD3</th>
<th>CD45</th>
</tr>
</thead>
<tbody>
<tr>
<td>thymus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lung</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D

WT + B[a]P

Nrf3--/ + B[a]P

- no lymphoma
- immature TdT+ lymphoma
- marginal zone lymphoma
- T-cell lymphoblastic lymphoma

Figure 2
Nfe2l3 (Nrf3) deficiency predisposes mice to T-cell lymphoblastic lymphoma

Grégory Chevillard, Marilene Paquet and Volker Blank