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Possible pitfalls investigating cell death responses in genetically engineered mouse models and derived cell lines

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ABSTRACT

Genetically engineered mouse models are frequently used to identify pathophysiological consequences of deregulated cell death. Targeting pro-apoptotic or anti-apoptotic proteins of the extrinsic or intrinsic apoptotic signalling cascade is state of the art since more than two decades. Such animal models have been increasingly made use of over the past years to study loss- or gain-of-function consequences of one or more components of the molecular machinery leading to cell death. These studies have helped to separate redundant from non-redundant functions of apoptosis-related proteins in normal physiology and sometimes unravelled unexpected phenotypes. However, correct interpretation of data derived from knockout mice or derived cells and cell lines is often flawed by the comparison of cells originating from different inbred or mixed genetic backgrounds. Here we want to highlight some basic problems associated with genetic background-based modulation of cell death sensitivity and describe some methods that we use to investigate cell death responses in hematopoietic and non-hematopoietic cells. Thereby, we show that hematopoietic cells derived from wild type mice on a C57BL/6:129/SvJ recombinant mixed genetic background are significantly more resistant to spontaneous cell death or DNA-damage induced apoptosis *in vitro* than cells derived from inbred C57BL/6 mice. Furthermore, we show as an example that C57BL/6 mice are more susceptible to γ-irradiation induced cell death after whole body irradiation *in vivo* and subsequent T cell lymphomagenesis.

1. Introduction

Programmed cell death is critical for the correct development of all metazoans. The most prominent mode of programmed cell death in vertebrate cells is referred to as the "intrinsic" or "mitochondrial" pathway of apoptosis, initiated by loss of mitochondrial integrity, a process also known as mitochondrial outer membrane permeabilization (MOMP). This process is controlled by the complex interplay of different members of the B cell lymphoma-2 (BCL-2) protein family, that all orchestrate activation of the largely redundant pro-apoptotic effector proteins BAX and BAK. As a consequence of MOMP, members of a second protein family, essential for apoptosis, the cysteine-driven aspartate-specific proteases (caspases), i.e. caspase-9 and caspase-3, -6, -7 are activated and lead to the processing of downstream targets, ending in and mediating morphological features of apoptotic cell death (see for review [1]). The second apoptotic cell death pathway, the "extrinsic" pathway or death receptor (DR) pathway involves ligand-dependent activation of membrane receptors of the tumor necrosis factor receptor 1 (TNF-R1) superfamily, which in turn triggers the formation of the death inducing signalling complex (DISC), activating caspase-8 and in humans also caspase-10. Both pathways meet at the level of caspase-3 activation [2]. Not surprisingly, the activation of caspases is carefully controlled to avoid unwanted induction of apoptosis and deregulation of programmed cell death contributes to the pathogenesis of several human diseases including autoimmunity and cancer [3]. Accordingly, genetically engineered mouse models have been used increasingly to investigate the molecular mechanisms of apoptotic cell death and to study consequences of defective apoptosis in different disease settings, e.g., to gain new insights into carcinogenesis and the effectiveness of antitumor therapies and the molecular basis of drug-resistance.

Targeting proteins critical for cell death, including caspases, death receptors or BCL-2 family proteins in genetically engineered mouse model has been commonly used to study the pathophysiological consequences of impaired apoptosis at the level of the whole organism, but often also in cell lines, most prominently MEF or primary cells such as lymphocytes, hepatocytes or neurons derived from such mouse mutants. So far, mouse models targeting all known murine caspases (caspase-1, -2, -3, -6, -7, -8 and -9, -11, -12; see for review [4–7] and mouse models targeting members of

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the BCL-2 family have been generated and are still intensively used today [8–11].

2. Impact of genetic background on cell death susceptibility

The number of mouse strains used to establish embryonic stem cell lines was initially limited and the most commonly used embryonic stem cell lines were derived from the 129/SvJ inbred strain [12]. Chimeras are usually produced by injection of manipulated ES cells into blastocystes derived from C57BL/6 mice that are finally implanted into pseudopregnant outbred foster mothers. Thus, the resulting pups have tissues build up from cells derived from different genetic origins, i.e. from the embryonic stem cells of 129/SvJ mice and the inner cell mass of the C57BL/6 donor blastocyst. Male chimaeras are subsequently crossed with C57BL/6 (B6) females to screen for germ line transmission of the mutated allele in *Agouti* coloured offspring (F1). Usually heterozygous mutant mice should be backcrossed onto one of the two possible genetic backgrounds (usually C57BL/6) for at least eight to ten generations to avoid comparison of mice with different contribution of either background in F1 offspring that give rise to a recombinant inbred strain in F2 and all their possible descendants (F3, F4...). However this is not always performed vigorously enough to save time. In such recombinant inbred strains, the contribution of each individual background can vary significantly between siblings and litters, affecting all subsequent analysis. A number of reports have been published in the past demonstrating the strong influence of genetic background onto the results from transgenic or knockout mouse models. For example, caspase-3 deficient mice were described to die at an age of 1–5 weeks, were born at lower Mendelian frequency and of smaller size on a mixed C57BL/6:129/SvJ background [13,14] while *Casp3*$^{-/-}$ mice on a pure C57BL/6 background reach adulthood, are fertile and do not show the dramatic exencephalic phenotype noted on the mixed genetic background ([15] and own unpublished observations).

Loss of the tumor suppressor p53 on a 129/SvJ:Ola background manifests in a shorter latency period to tumor development and allows development of a small number of pituitary adenocarcinomas not seen on other genetic backgrounds [16]. Furthermore, on a mixed genetic background (129/SvJ × C57BL/6) approximately 9% of female p53$^{-/-}$ embryos show a defect in neuronal tube closure, compared to >90% penetrance of this phenotype on a pure C57BL/6 background [17–19]. In contrast, p53-deficency on inbred C57BL/6 background shows severe ocular abnormalities, in contrast to mice on the 129/SvJ background [20]. Bcl-2-deficient mice show a severe phenotype manifested in polycystic kidney disease and lymphopenia on a C57BL/6 background causing their death within the first 6 weeks of life, while *Bcl-2*$^{-/-}$ on a 129/SvJ background have a much milder phenotype [21]. Furthermore, mice lacking the BH3-only protein Bad on 129/SvJ background were reported to develop diffuse large B cell lymphomas with an incidence of about 20% but no such phenomenon was observed after backcrossing onto C57BL/6 [22,23] and the severe autoimmune pathology noted in Bim-deficient mice on a 129SvJ:B6 background [9,24] or Fas-deficiency in the *MRL* background is clearly strongly ameliorated upon backcrossing onto C57BL/6 [25]. Hence, the choice of a uniform genetic background and appropriate controls, if not littermates, is most critical to allow direct comparison of findings made in different mouse mutant strains. However, it has to be noted that even extensive back-crossing does not allow a completely clean transfer of a targeted allele onto the desired genetic background as genomic regions in close proximity of the targeted allele will maintain their original genetic identity, as proximity will prevent meiotic crossing over in these regions. In addition, extensive "in-house" breeding bares the danger of introducing genetic drifts into individual strains of mice kept in isolation without recurrent "infusion" of the original genetic background. However, given the extra time needed to "refresh" the original genetic background of nowadays often multi-allelic mouse mutant strains, this problem is often ignored.

To illustrate some of these problems, we noted a drastic difference in the mean lifespan of wild-type (wt) mice of different genetic backgrounds but also within the same tumor-prone mouse strain over time in our own colonies. A cohort of male mice maintained on an uncontrolled recombinant mixed C57BL/6:129/SvJ (1:1) background died due to age-related diseases with a median of 494 days ($n = 10$). Necropsy suggested severe inflammation of the urogenital tract ($n = 2$), hepatocellular carcinoma and/or kidney tumors ($n = 4$), lymphoma ($n = 1$), gastrointestinal tumor ($n = 1$) or stroke ($n = 2$), as cause of death, while male mice on a pure C57BL/6 background showed a mean disease-free survival of 730 days ($n = 7$), the defined experimental cut-off ($p = 0.001$; Logrank Mantel–Cox analysis; Fig. 1A). Yet, in a model of split-dose γ-irradiation induced thymic lymphomagenesis mice of mixed genetic background showed significantly delayed onset of disease (Fig. 1B). Briefly, 4-week old C57BL/6 wt mice were irradiated with a low dose of 1.75Gy 4-times in weekly intervals. Wild-type mice start to develop thymic lymphomas with a median onset of 183 days ($n = 10$; 7 female and 3 male animals), while mice on a mixed genetic background (C57BL/6:129/SvJ) showed a significantly longer ($p = 0.0017$) median tumor free survival of 260 days ($n = 11$; 8 female and 3 male; Fig. 1B) that was most prominent in female mice ($p = 0.0029$) but not significant in males, with the caveat of very low numbers. In relation to genetic drift caused by in-house breeding, we noted a significantly delayed onset of B cell lymphomagenesis in a cohort of C57BL/6J *Eμ-Myc* transgenic mice monitored in the years 2008–2010, (mean latency 202 days) when compared to earlier cohorts published with a mean latency of 138 days [26]. Strikingly, re-infusion of wt C57BL/6 background into the *Eμ-Myc* transgenic line reduced tumor latency again to 135 days in a new cohort monitored in 2011–2012 (Fig. 1C). Notably this genetic drift appeared to affect mainly male *Eμ-Myc* mice (Fig. 1D).

Analysing the distribution of immune cell subsets in bone marrow, thymus or spleen from wt mice on C57BL/6:129/SvJ (1:1) mixed genetic background showed significantly reduced total cellularity in the spleen compared to inbred C57BL/6 mice ($p = 0.047$). The difference observed was mainly due to lower numbers of B220$^+$ B cells, affecting all stages of development, including transitional type 1 (T1), type 2 (T2) and follicular (FO) B cells (Fig. 2A–D).

So far we did not further specify which type of C57BL/6 mice were used, as there are several substrains available that are not genetically identical and for which behavioural differences have been reported [27]. The two most commonly used substrains are C57BL/6J and C57BL/6N. The original Jax strain is C57BL/6J and the 6N substrain was derived from it at the NIH and separated in 1951 [28]. The major difference between these lines is deficiency for the nicotinamid nucleotide transhydrogenase (*Nnt*) gene in the C57BL/6J substrain. Nnt is a pyridine nucleotide transhydrogenase and integral inner mitochondrial membrane protein. It is part of the energy-transfer system of the respiratory chain and catalyzes the transfer of a hydride ion between nicotinamide adenine dinucleotide, NAD(H), and oxidized nicotinamide dinucleotide phosphate, NADP(H). A direct comparison of cell death sensitivity between C57BL/6J and C57BL/6N strains has not been performed but as the enzyme contributes to antioxidant defense, differences in tolerance to ROS can be expected. Loss of *Nnt* was recently described as a cause for familial glucocorticoid deficiency in humans and increased levels of active caspase-3 were found in the adrenal glands from C57BL/6J mice when compared to the C57BL/6N strain [29]. Knockdown of NNT in a human adrenal cell line also caused