Combined reduction in the expression of MCL-1 and BCL-2 reduces organismal size in mice

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Abstract
The intrinsic apoptotic pathway is controlled by the BCL-2 family of proteins, which exhibit either a pro-death or pro-survival function. Gene knockout studies revealed that different pro-survival BCL-2 proteins are critical for the survival of distinct cell types, although overlapping functions among such proteins have also been identified. In the process of studying mice lacking single alleles of Mcl-1 (Mcl-1+/−), Bcl-2 (Bcl-2+/−), or both in combination (Mcl-1+/−Bcl-2+/−), we observed that Mcl-1+/−Bcl-2+/− mice weighed less when compared with their wild-type littermates as they aged. Body composition analysis demonstrated that while fat mass was similar to wild-type controls, lean mass was significantly reduced in Mcl-1+/−, Bcl-2+/−, and, most strikingly in Mcl-1+/−Bcl-2+/− mice. The weights of several tissues including the heart, tibialis anterior, and kidney were likewise reduced in Mcl-1+/−Bcl-2+/− mice. When lean mass and specific tissue weights were expressed relative to body weight, these differences were no longer significant, indicating that Mcl-1+/−Bcl-2+/− mice, and to a lesser extent Mcl-1+/− and Bcl-2+/− mice, are smaller than their wild-type counterparts. Consistently, the anal-naso length was reduced in Mcl-1+/−Bcl-2+/− mice. While minor reductions in size were observed in female Mcl-1+/−Bcl-2+/− mice, these effects were most prominent in males. Notably, Mcl-1+/−Bcl-2+/− males had markedly smaller testes even after accounting for differences in body weight. Collectively, these data reveal that combined loss of a single allele of Mcl-1 and Bcl-2, while not overtly impairing organismal development, leads to a reduction in animal size.
through the study of single, as well as multiple gene knockout mice\textsuperscript{36}. Such studies revealed that systemic loss of either pro-survival MCL-1\textsuperscript{7} or BCL-XL\textsuperscript{8} resulted in embryonic lethality, while the loss of BCL-2 gave rise to runty animals that succumbed to polycystic kidney disease at a young age (4–7 weeks post-birth)\textsuperscript{9,10}. We examined the overlapping roles of different pro-survival BCL-2 family members by generating Mcl-1\textsuperscript{+/−}/Bcl-x\textsuperscript{+/−}, Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} and Bcl-x\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} mice. While the combined loss of single alleles of Mcl-1 and Bcl-x disrupted normal embryogenesis and gave rise to animals with severe craniofacial abnormalities, with most dying soon after or even before birth, these defects were prevented by the deletion of a single allele of the pro-apoptotic BH3-only protein, BIM\textsuperscript{7}. In contrast, Mcl1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} and Bcl-x\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} mice appeared grossly normal and were long-lived\textsuperscript{8}.

However, in the course of maintaining these colonies, we observed that double heterozygote Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} mice gained less weight as they aged in comparison to their wild-type (WT) counterparts. This reduced weight gain was due to reduced growth, with male Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} mice displaying lower lean mass, tissue weights, and a commensurate decrease in body length. However, their fat mass remained normal and no overt developmental anomalies were observed in comparison to WT animals.

**Results**

**Mice lacking a single allele of Mcl-1 and a single allele of Bcl-2 are smaller in size compared to WT animals**

Over the course of breeding Mcl-1\textsuperscript{+/−} and Bcl-2\textsuperscript{+/−} animals, we noticed that mice lacking single alleles of Mcl-1 or Bcl-2, and in particular the Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} double heterozygotes, gained less weight and were smaller than WT controls (Fig. 1a–c).

We initially considered that Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} mice may be protected from the development of age-related obesity. Therefore, to determine whether the observed weight differences could be attributed to alterations in fat mass, we assessed body composition by EchoMRI\textsuperscript{11} to measure body mass (Fig. 2a, b), fat mass (Fig. 2c, d), and lean mass (Fig. 2e, f) in an independent cohort of aged male and female WT, Mcl-1\textsuperscript{+/−}, Bcl-2\textsuperscript{+/−} and Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} animals. In contrast to our hypothesis, no differences in fat mass were observed between the genotypes examined (Fig. 2a, b). However, a striking difference in lean mass was evident between WT and Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} mice, which was more pronounced in males than females (compare Fig. 2e, f). Consistent with these data, we explored the overall size of the mice and noted a significant decrease in measured body length (naso-anal length) and tibia length in male Mcl-1\textsuperscript{+/−}/Bcl-2\textsuperscript{+/−} animals when compared with WT controls (Fig. 2g–j).

To further assess the reduced lean mass phenotype observed, we explored the weights of several major organs.
matched mice of the different genotypes (Supplementary Fig. 1). Across all genotypes, we observed varying levels of fat in the liver along with perivascular infiltration, as well as an expansion of the lymphoid areas in the spleen in a proportion of the animals. However, no obvious differences were found between groups. Since the age of the cohort examined was over a year old, these observations were most likely attributable to aging.

Fig. 1 Mcl-1+/− Bcl-2−/− mice are smaller in size compared with WT mice. a, b Body weights of WT, Mcl-1+/−, Bcl-2−/− and Mcl-1+/− Bcl-2−/− male (n = 7–15 mice per genotype) and female (n = 10–13 mice per genotype). Mice were observed for over 300 days. Graphs depict the mean ± S.E.M. of mice weights over time and data were analysed using two-way ANOVA. Statistically significant differences between groups are indicated in the corresponding tables. c Representative image of age-matched male mice (280 days) from all 4 genotypes examined.
Fig. 2 Mcl-1<sup>−/−</sup>Bcl-2<sup>−/−</sup> mice are shorter in length and have a lower lean mass compared to WT controls. EchoMRI was used to determine the (a, b) body mass, (c, d) fat mass, and (e, f) lean mass of male (n = 11–14 animals per genotype, mean age of 509 days) and female (n = 8–14 animals per genotype, mean age of 517 days) mice. g, h Body length and (i, j) tibia length were also measured. Data represent mean ± S.E.M. and were analysed using one-way ANOVA comparing the mean of each group to every other mean. P ≤ 0.05 indicate statistical significance.
Fig. 3 Organ weights of male and female animals. Major organs including the (a, b) heart, (c, d) TA muscle, (e, f) kidneys, (g, h) spleen and (i, j) liver were weighed. Data represent mean ± S.E.M. of organ weights from male or female mice and were analysed by one-way ANOVA. *P* ≤ 0.05 indicate statistical significance.
WT, Mcl-1+/−, Bcl-2+/−, and Mcl-1+/−Bcl-2+/− mice display similar levels of serum testosterone and IGF-1 levels

The testes produce anabolic hormones, such as testosterone, that have a major role in muscle and bone growth. We considered that the markedly reduced testes weight, and potentially a concomitant reduction in testosterone production, may be a primary driver of the abnormally reduced growth in male Mcl-1+/−Bcl-2+/− mice. However, we observed no differences in serum testosterone levels between mice of any of the genotypes tested (Fig. 6a). Similarly, no differences in IGF1, another major anabolic hormone, were observed between mice of the different genotypes (Fig. 6b).

Discussion

We have shown that Mcl-1+/−Bcl-2+/− mice, and to a lesser extent Mcl-1+/− and Bcl-2+/− mice, are smaller than their WT counterparts. While this effect was apparent in females, it was markedly more pronounced in males. When we first noted that Mcl-1+/−Bcl-2+/− animals appeared smaller than single heterozygote and WT animals, we hypothesised that this may have been due to a reduction in fat mass. However, EchoMRI analysis revealed that fat mass was similar between WT, Mcl-1+/−, Bcl-2+/−, and Mcl-1+/−Bcl-2+/− mice, and the reduction in body weight was entirely attributable to lower lean mass. Consistently, we also observed reductions in the weights of numerous tissues in Mcl-1+/−Bcl-2+/− mice. Importantly, Mcl-1+/−Bcl-2+/− males had a lower naso-anal length and tibia length compared to WT controls. When expressed relative to body weight, differences in lean mass, as well as weights of the heart, kidney and TA muscle were not significant between the genotypes examined. Collectively, our data indicate that Mcl-1+/−Bcl-2+/− mice, and to
Fig. 5 Compound loss of one allele of Mcl-1 and Bcl-2 leads to a reduction in tissue weights compared with WT mice. The (a) TA muscle, (b) heart, (c) kidney, (d) lean mass, (e) liver, (f) spleen, and (g) testes weights of male mice were expressed in relation to their body weight. Note that testes weight measurements are obtained from a younger age-matched cohort (average age of 280 days) while other organs weights are obtained from older male mice (average age of 509 days). Data represent mean ± S.E.M. and were analysed using one-way ANOVA comparing the mean of each group to every other mean. Statistically significant differences (P ≤ 0.05) are indicated in each graph.
a lesser extent Mcl-1+/− and Bcl-2+/− mice have a reduced organismal size compared with their WT counterparts and this appears to be due to a reduction in cell numbers per tissue (rather than a decrease in cell size). Interestingly, the spleen and testes of Mcl-1+/−Bcl-2+/− mice remained significantly smaller compared to control animals even when expressed relative to overall body weight.

The anti-apoptotic roles of MCL-1 and BCL-2 in immune cell survival have been well documented, and it is therefore not unexpected that the spleen weights were reduced in Mcl-1+/− and Bcl-2+/− mice9,12, and further reduced in Mcl-1+/−Bcl-2+/− double heterozygotes (Fig. 3g). Interestingly, the effects of Mcl-1 and/or Bcl-2 deficiency on spleen weight was less pronounced in female mice (Fig. 3g). This may suggest that male hormones exacerbate the impairment in cell survival caused by the reduction in BCL-2 and/or MCL-1. Alternatively, female hormones could have a protective effect.

With regards to the reduction in testes weight observed in Mcl-1+/−Bcl-2+/− males, apoptosis is known to play a key role in spermatogenesis, with only 25% of germ cells successfully maturing and the remainder undergoing apoptosis in the early post-natal period13. These high rates of apoptosis are essential to establish the appropriate balance between germ cells and Sertoli cells, which support the survival, proliferation, and maturation of the former. Several BCL-2 family members are expressed in the testes and display distinct patterns of expression during testes development14. Moreover, previous studies have established important roles for BCL-2 family members in spermatogenesis and testes development. Mice deficient in the pro-apoptotic effector BAX, those lacking BH3-only proteins BIK and BIM, those lacking pro-survival BCL-W, or expressing a Bcl-2 transgene, all exhibit dysregulated apoptosis, disorganised spermatogenesis, and altered testes weight14–17. This suggests that both abnormally increased, as well as abnormally decreased apoptosis can cause defects in spermatogenesis. Our observations of significantly reduced testes weight in Mcl-1+/− Bcl-2+/− males are consistent with these prior findings and suggest an important role for MCL-1 and BCL-2 in testes development.

We have previously shown that the body weight of E19.5 Mcl-1+/− pups was significantly lower than WT littersmates6, and observed that Mcl-1+/− mice developed normally and survive into late adulthood6. These findings are consistent with the current observations and suggest that the reduction in MCL-1 either alone or in combination with a reduction in BCL-2 likely affects numerous aspects of organismal development and growth. This may be explained by an abnormal increase in apoptosis, leading to reduced cell numbers in early embryogenesis that will carry through to a reduction in overall body cellularity and thus body size. Importantly, with the exception of being smaller in size, Mcl-1+/−Bcl-2+/− double heterozygote mice do not display any overt developmental defects. Collectively, our results demonstrate rate limiting roles for MCL-1 and BCL-2 in organismal growth.

Methods

Mice

All experiments were approved by the animal ethics committees of the Walter and Eliza Hall Institute of Medical Research and the Baker Heart and Diabetes Institute and conducted in accordance with the Australian code for the care and use of laboratory animals. Mcl-1+/− mice were generated from Mcl-1+/+ mice18. The Mcl-1+/− and Bcl-2+/−10 were all maintained on a C57BL/6 background. All mice were bred and aged at the Walter and Eliza Hall Institute of Medical Research and were maintained in a 14-h light and 10-h dark cycle at 22 °C and fed ad libitum on standard
mouse chow. Once the cohorts had aged they were transferred to the Baker Heart and Diabetes Institute, where they were habituated in the new environment for ~2 weeks before assessment of body mass and body composition. Approximately 1 week after the assessment of body composition, mice were culled and body length, tissue weights, and tibia length determined.

**Body composition and mass**

Lean and fat mass were determined using a 4-in-1 EchoMRI body composition analyser (EchoMRI™, Houston, TX, USA), as previously described. Standard laboratory scales were used to determine total body mass (Mettler Toledo, Greifensee, Switzerland).

**Statistical analysis**

The data presented in Fig. 1 were analysed using a 2-way (genotype x time) analysis of variance (ANOVA) with repeated measures on the time factor. Where a significant interaction effect was observed, statistical analysis of specific pairwise comparisons was conducted using a post-hoc Tukey test. Data in Figs. 2–6 were analysed by 1-way ANOVA using the PRISM software. Where the ANOVA revealed a significant F value, statistical analysis of specific pairwise comparisons was conducted using the Tukey’s test, which adjusts P values for multiple comparisons. Where statistical significance was achieved, the adjusted P values are reported within the specific figures.

**Histology**

All mouse tissues were fixed in 10% buffered formalin solution and subsequently embedded in paraffin prior to sectioning. Slides were stained with haematoxylin and eosin, then examined and photographed using the Case-Viewer Software (3DHISTECH).

**ELISA**

ELISAs for testosterone (KGE010) and IGF1 (MG100) were obtained from R&D systems and performed according to the manufacturer’s instructions.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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