ACCELERATED PUBLICATION

An inactivating caspase 11 passenger mutation originating from the 129 murine strain in mice targeted for c-IAP1

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INTRODUCTION

ES (embryonic stem) cell lines from the murine 129 strain are frequently used to derive genetically modified mice [1]. A recent report examining the role of caspase 1 in an endotoxin-induced murine model of sepsis revealed that the resistance to sepsis observed in caspase-1-targeted mice is largely due to a naturally occurring inactivating mutation within the caspase 11 gene (Casp4) locus [Kayagaki, Warming, Lamkanfi, Vande Walle, Louie, Dong, Newton, Qu, Liu, Heldens, Zhang, Lee, Roose-Girma and Dixit (2011) Nature 479, 117–121]. Thus, if 129 ES cells are used to target genes closely linked to caspase 11, the resulting mice might also carry the caspase 11 deficiency as a passenger mutation. In the present study, we examined the genetic loci of mice targeted for the closely linked c-IAP (cellular inhibitor of apoptosis) genes, which were generated in 129 ES cells, and found that, despite extensive backcrossing into a C57BL/6 background, c-IAP1−/− animals are also deficient in caspase 11. Consequently, data obtained from these mice should be re-evaluated in this new context.

Key words: 129 mouse strain, caspase 1, caspase 11, inhibitor of apoptosis protein (IAP), lipopolysaccharide (LPS), sepsis.
and found that, despite extensive backcrossing, c-IAP1-targeted mice, but not c-IAP2-deficient animals, have the reported caspase 11 intronic inactivating deletion. We also show that this was not due to differences in the 129 ES lines used to create the c-IAP-targeted mice, because the caspase 11 mutation was found to be penetrant in an extensive panel of 129 ES lines tested, which included the lineages used in the targeting of both the c-IAP1 and c-IAP2 genes. These data suggest first that data obtained from c-IAP1−/− mice may need further evaluation, and secondly that a selective pressure may exist for the maintenance of a wild-type caspase 11 gene during normal development.

EXPERIMENTAL

Cells

C57BL/6 and 129 ES cell lines were maintained in high glucose Dulbecco’s minimal essential medium (Invitrogen) supplemented with 15% (v/v) fetal bovine serum (Harlan), 4 mM glutamine, 1 μM 2-mercaptoethanol, 1% MEM non-essential amino acids, 50 IU/ml penicillin, 50 μg/ml streptomycin and 1000 IU/ml ESGRO (Millipore) on FVB/N mouse embryonic feeder cells mitotically inactivated by irradiation. Before DNA extraction and genotyping, ES cell lines were passaged twice on gelatin-coated dishes to eliminate feeders from the cultures as described in [15].

MEF (mouse embryonic fibroblast) cells were cultured in Dulbecco’s modified Eagle’s medium (Mediatech) supplemented with 10% (v/v) fetal bovine serum (Mediatech) and 2 mM glutamine (Gibco) at 37 °C with 5% CO2. Matched c-IAP1 wild-type and c-IAP1−/− MEFs, prepared from littermate controls, have been described previously [16].

Immunoblotting

Cell lysates from MEFs were prepared with Nonidet P40 lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 5 mM NaF and 1 mM Na2VO4) supplemented with protease inhibitors for 30 min on ice to ensure complete lysis. Protein quantification was determined using the Bradford assay (Bio-Rad Laboratories). Cell lysates of equal protein concentrations were prepared in lithium dodecyl sulfate sample buffer (Invitrogen) separated on denaturing NuPAGE 4–12% polyacrylamide gradient gels, and transferred on to 0.2-μm-pore-size nitrocellulose membranes (Invitrogen). Membranes were blocked in 2% (w/v) non-fat dried milk powder in TBS-T (Tris-buffered saline: 20 mM Tris/HCl and 135 mM NaCl, pH 7.6 with 0.1% Tween 20), followed by incubation overnight at 4 °C with the following antibodies against the following: caspase 11 (17D9, Novus Biologicals), c-IAP1 (1E1-1-10, Enzo Life Sciences) and β-actin (AC-74, Sigma). After washing with TBS-T, membranes were incubated with secondary antibodies for 1 h at room temperature (20 °C). Enhanced chemiluminescence (Pierce) was used to visualize the blots on HyBlot CL Autoradiography film (Denville Scientific).

RT (reverse transcription)–PCR

c-IAP1 wild-type or c-IAP1−/− MEFs were treated as indicated with LPS, and cells were then washed with PBS. Total RNA was isolated using the RNeasy minikit (Qiagen) following the manufacturer’s instructions. RT with random hexamer primers and MultiScribe™ reverse transcriptase (Applied Biosystems) was performed on 1 μg of total RNA. Full-length caspase 11 cDNA was amplified using the primers 5′-ATGGCTGAAAACAAACACCCT-3′ and 5′-TCAGTTGCCAGG-3′.

RESULTS

The genes encoding c-IAP1 and c-IAP2 lie in close proximity (~2.5 Mb) to that encoding caspase 11 on mouse chromosome 9 (Figure 1A). Since both c-IAP genes have been targeted previously using ES cell lines derived from 129 mice, we sought to examine the caspase 11 status of these mice. Sequence analysis from genomic DNA revealed that c-IAP1−/− mice also contain the mutant caspase 11 locus, despite the fact that these mice have been extensively backcrossed into the caspase 11 wild-type mouse strain, C57BL/6 (Figure 1B). In contrast, even with the close proximity of the c-IAP2 and caspase 11 genes, the
Whole-cell lysates were prepared and Western blot analysis was conducted for the levels of caspase 11, c-IAP1 or β-actin. Molecular masses are indicated in kDa. Casp, caspase.

**DISCUSSION**

The findings described above suggest that the mice created by targeting c-IAP1 in a 129-derived ES cell line [12] contain an added layer of complexity if used to investigate roles of c-IAP1 in innate immunity and programmed cell death, because of a concealed defect in the caspase 11 gene (Figure 1B). It should be noted that, in that original study, the primary phenotype observed in c-IAP1−/− cells was a marked stabilization of c-IAP2, a result confirmed subsequently by Darding et al. [19].

**Figure 2** c-IAP1−/− MEFs are unable to induce caspase 11 in response to LPS

(A) c-IAP1+/+ or c-IAP1−/− MEFs were exposed to 0.1 mg/ml LPS (+) or 1 mg/ml LPS (++) for 6 h before lysis. Whole-cell lysates were prepared and Western blot analysis was conducted for the levels of caspase 11, c-IAP1 or β-actin. Molecular masses are indicated in kDa. The asterisk (*) indicates a non-specific protein recognized by the anti-c-IAP1 antibody.

(B) c-IAP1+/+ or c-IAP1−/− MEFs were treated as in (A). RNA was extracted and subjected to RT–PCR analysis for full-length caspase 11. Sizes are indicated in bp. wt, wild-type.

(C) Wild-type MEFs were pre-treated with the Smac (second mitochondrial-derived activator of caspase) mimetic AEG40730 (50 nM) for 30 min (SM), before being exposed to LPS for 6 h. Whole-cell lysates were prepared and Western blot analysis was conducted for the levels of caspase 11, c-IAP1 or β-actin. Molecular masses are indicated in kDa. Casp, caspase.
and references therein), indicating that this effect is independent of caspase 11 status. However, a role for c-IAP1 in the regulation of the innate immunity is not ruled out. Indeed, in Drosophila, an IAP orthologue termed DIA2 (Drosophila IAP2) is critical for activation of innate immune responses mediated by IMD (immune deficiency), the Drosophila orthologue of mammalian RIPK1 (receptor-interacting serine/threonine protein kinase 1) [20–24]. Furthermore, the syntenic nature of the c-IAP, caspase 1 and caspase 11 genes may represent a functional gene cluster. This synteny is also conserved in humans with caspases 4 and 5, the human orthologues of murine caspase 11, closely linked to the c-IAP genes.

Intriguingly, the caspase 11 defect was not detected in the c-IAP2−/− mice, despite the close proximity of the genes encoding caspase 11, c-IAP1 and c-IAP2 on mouse chromosome 9. This was not due to differences in the cell lines used in their construction, since ES cells derived from both 129 P2 and the 129 X1 mice, which were used to target c-IAP1 and c-IAP2 genes respectively, contain the mutation in the caspase 11 gene (Figure 1C). This suggests that in c-IAP2−/− mice, the caspase 11 and c-IAP2 alleles have recombined over successive crossovers into the C57BL/6 background. It is intriguing that all of the c-IAP2−/− mice are invariably homozygous for wild-type caspase 11, suggesting that the crossover event must have occurred at least twice within the population to become the predominant genotype in the c-IAP2−/− colony. These observations suggest that there may be a selective advantage for expression of the wild-type caspase 11 gene during murine development. Since the c-IAP1 locus is closer to that of caspase 11 (Figure 1A), the likelihood of this crossover event may be more rare, or the selective pressure for wild-type caspase 11 may only exist in the context of the c-IAP2 deletion.

Although the c-IAP2-deficient mice tested in the present study were found to be wild-type for caspase 11, the point at which the c-IAP2−/− and mutant caspase 11 loci separated is unknown. Clearly, in vivo studies to elucidate the role of c-IAP1 in the innate immune response, or indeed to study cell death, should ideally be performed in a background that is wild-type for caspase 11. Fortunately, this model currently exists. An elegant murine model has been described recently that facilitates independent or simultaneous inactivation of the linked c-IAP1 and c-IAP2 genes [25]. Importantly, these mice were generated using the C57BL/6 ES cell line Bruc4, which we have demonstrated does not carry the caspase 11 mutation (Figure 1C).

The present study has focused on the linkage between c-IAP1, c-IAP2 and the caspase 11 mutation carried from the constructed 129 ES cells. However, it is important to note that other genetic knockouts derived from the 129 ES cells may be inadvertently carrying the concealed mutation within the caspase 11 locus. Since the gene for caspase 12 (Casp12) is in close proximity to that of caspase 11 on mouse chromosome 9 (Figure 1A), it might be helpful to test mice experimentally targeted for caspase 12 constructed using a 129-derived ES cell line for the caspase 11 mutation [26]. Caspase 12 has subsequently been targeted in a C57BL/6-derived ES cell line [27], and this strain may be more appropriate for studying caspase 12 function as the wild-type status of caspase 11 has been confirmed [27].

**AUTHOR CONTRIBUTION**
Niall Kenneth, J. Michael Younger and Colin Duckett designed the research. Niall Kenneth and J. Michael Younger performed the research. Elizabeth Hughes and Thomas Saunders prepared and provided ES cell lines. Danielle Marcotte and Philip Barker prepared and provided genomic DNA from IAP-deficient mice. All authors analysed the data. Niall Kenneth and Colin Duckett wrote the paper.

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