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Role of the Msh2 gene in genome maintenance and development in mouse fetuses

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ABSTRACT

In an attempt to evaluate the roles of the mismatch repair gene *Msh2* in genome maintenance and in development during the fetal stage, spontaneous mutations and several developmental indices were studied in Msh2-deficient lacZ-transgenic mouse fetuses. Mutation levels in fetuses were elevated at 9.5 dpc (days post coitum) when compared to wild-type mice, and the level of mutations continued to increase until the fetuses reached the newborn stage. The mutation levels in 4 different tissues of newborns showed similar magnitudes to those in the whole body. The levels remained similar after birth until 6 months of age. The molecular nature of the mutations examined in 12.5 dpc fetuses of $Msh2^{+/+}$ and $Msh2^{-/-}$ revealed unique spectra which reflect errors produced during the DNA replication process, and those corrected by a mismatch repair system. Most base substitutions and simple deletions were reduced by the presence of the Msh2 gene, whereas G:C to A:T changes at CpG sequences were not affected, suggesting that the latter change was not influenced by mismatch repair. On the other hand, analysis of developmental indices revealed that there was very little effect, including the presence of malformations, resulting from Msh2-deficiencies. These results indicate that elevated mutation levels have little effect on the development of the fetus, even if a mutator phenotype appears at the organogenesis stage.

1. Introduction

Although the importance of DNA mismatch repair (MMR) in genome maintenance and cancer prevention has been demonstrated in the human genetic disease hereditary non-polyposis colon cancer (HNPCC), as well as in MMR gene-deficient mice, many puzzling questions still remain concerning the role of MMR [1–3]. One of these questions concerns the fact that apparently the presence of high mutation levels (a mutator phenotype) resulting from an MMR-deficiency does not seem to interfere with normal fetal development, at least in mice. Knockout mice for the *Msh2* or *Mlh1* genes, the two essential genes active in MMR, show very high levels of spontaneous mutations in all of the adult tissues examined so far [4–13], and reveal high cancer incidence rates in some tissues [3]. Interestingly, the examination of newborn mice obtained from crossing MMR gene-heterozygotes showed little effect in their appearance or genotype distribution, which suggests that there might be no effect of MMR deficiency on the normal development of the fetus [14–16]. Although no direct measurement of mutations in fetuses has been performed yet, fibroblast cells derived from MMR-deficient embryos cultured in vitro revealed high mutation levels [17]. Furthermore, Msh2-deficient ES cells were shown to have mutator phenotypes, but to develop normally when transplanted into blastocysts [15]. These facts suggest that high levels of mutations are compatible with normal embryonic development. This idea, however, is not in accord with other lines of evidence. Many studies on environmental factors which interfere with embryonic development revealed that genotoxic agents disrupt normal development, especially when they are present during the period of organogenesis [18–20]. In addition, many human genetic diseases are known to be associated with a disturbance of developmental processes [21–23]. This suggests a strong correlation between genetic alterations and abnormal development. However, caution is needed in interpreting these results. Since genotoxic agents induce cell death as well as mutations, and since the effects of genetic diseases could be exerted through cell death or an alteration of cellular phenotypes, it is not yet clear if developmental effects are attributable to cell death, to mutations, or to both [18,19].

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In mammals, MMR is a complex process consisting of several steps carried out by more than 10 different proteins [1,2]. The initial step in MMR, the recognition of a DNA mismatch, is performed by the heterodimers Msh2-Msh3 or Msh2-Msh6. The second processing step is performed by Mlh1-Pms2 or Mlh1-Mlh3 [1–3]. In 2006, Hegan et al. compared the effects of different mismatch repair gene deficiencies on spontaneous mutation levels in mice, and found that a knockout of the *Msh2* or *Mlh1* genes produced the most profound effects, and at similar levels. This indicated that the primary importance of these genes is for genome maintenance [24]. Interestingly, these investigators also found that a simultaneous deficiency of the two *Msh2*-homologous genes, *Msh3* and *Msh6*, resulted in an even higher level of spontaneous mutations than the levels obtained by a deficiency of either *Msh2* or *Mlh1*. This suggested that the Msh3 and Msh6 could somehow be involved in MMR, in addition to Msh2-Msh3 and Msh2-Msh6, during the initial step of the mismatch repair process. If this is the case, a Msh2-deficient fetus could perform MMR through a Msh3/Msh6-related pathway, and mutations could be kept at low levels. The high levels of mutation observed in adult tissues of Msh2-deficient mice could be explained if the contribution of Msh3/Msh6 is reduced only after birth. In fact, expression level of Msh6 (also named as Gtbp) is reported to be high during 10.5–15.5 fetal days and reduced at 18.5 fetal day and 2 days after birth [25].

In order to clarify if mutational burden is present in fetal stage of Msh2-deficient mice, mutation levels were analyzed directly in fetuses of *Msh2*-deficient mice. Since mutation levels were found to be elevated during the organogenesis period, the effects on several developmental indices were also examined.

2. Materials and methods

2.1. Mice

Muta™ mice harboring the *lacZ* gene as a marker for mutation assays [26] were provided by Covance Research Products (Denver, PA), and crossed with *Msh2*-deficient mice [14]. The F1 mice $lacZ^{+}/Msh2^{+/-}$ were crossed again to obtain $lacZ^{+}/Msh2^{-/-}$, $lacZ^{+}/Msh2^{+/-}$ and $lacZ^{+}/Msh2^{+/+}$ mice. The genetic background of Muta™ mice was a mixture of BALB/c and DBA/2, and that of *Msh2*-deficient mice was a mixture of 129/Ola and C57BL/6J. Thus the mice examined had a mixed background from the 4 strains.

The mice were sacrificed at different embryonic ages, and also after birth. Intestines of newborn mice were whole tissues, while those of adults were epithelial tissues of about 5 cm in the proximal part of the small intestine [27,28]. The mice used were checked for the absence of any disease with macroscopic examinations. In 6 month-old $lacZ^{+}/Msh2^{-/-}$, however, all of the 13 mice examined showed an abnormality in one or a few tissues. Subsequently, three mice were selected which had small tumors in the small intestine, liver or bladder. To study mutations, normal parts of the tissues were sampled. Whole fetuses or dissected tissues were frozen on dry ice and kept at −70 °C till use. All experimental procedures were conducted according to the guidelines for Animal Welfare and Experimentation of Tohoku University.

2.2. Genotyping and DNA extraction

For genotyping individual fetuses or mice, DNA was extracted from a small portion of the tail, and the presence of the *lacZ* gene, and *Msh2* gene knockout was determined with PCR as previously reported [14,27,28]. For mutation assays, genomic DNA was isolated from whole fetuses or from newborn mice, or from different newborn and adult tissues.

2.3. Mutation assays

Extracted mouse genomic DNA was mixed with a packaging extract solution (Transpack Packaging Extract, Stratagene, La Jolla, CA). *LacZ*-containing lambda DNA was retrieved as phages. The number of phages retrieved was estimated from the number of plaques formed on an *Escherichia coli* (C strain, $lacZ^{-}$, $galE^{-}$) plate. Phages containing mutated *lacZ* genes were identified as plaques formed in the presence of phenyl beta-D-galactoside. The experimental details have been previously described [27,28].

2.4. DNA sequencing

Mutant clones (12–24 clones) were selected randomly from each DNA sample. Phage DNA was extracted from a single plaque and the *lacZ* gene DNA was amplified with PCR as a set of 4 overlapping fragments. The primers used for PCR were described previously [29]. The amplified DNA was sequenced with a sequencer (ABI Prism 3100) using the Big-Dye Terminating Cycle Sequencing System (Applied Biosystems, Foster City, CA). The sequenced DNA was compared with the wild-type *lacZ* gene to identify mutations. The entire *lacZ* sequence of each mutant clone was analyzed for alterations.

In the analysis of mutation spectra, mutations which could have been produced through DNA replication of mutated *lacZ* in tissues were eliminated and not counted. Identical mutations found in individual mouse DNA samples were counted only once as a non-redundant mutation.

2.5. Analysis of developmental indices

The $lacZ^{-}/Msh2^{+/+}$ and $lacZ^{-}/Msh2^{-/-}$ mice found in the F2 generation were crossed with the same genotype. Pregnancy was judged by a vaginal plug. Some pregnant mice were irradiated with 1.5 Gy of X-rays (0.72 Gy/min, 200 kV, 10 mA, 1 mm Al and 0.5 mm Cu filters) when they were 9.5 dpc fetuses. At 18.5 dpc, the dams were sacrificed and the number of corpora lutea, implants, early and late resorption, dead fetuses, and live fetuses were counted. A remnant without a placenta was judged as an early resorption, and those with a placenta as a late resorption. Live fetuses were weighed and examined for external malformations. The details have been previously described [30].

2.6. Apoptosis

12.5 dpc fetuses of $Msh2^{+/+}$ and $Msh2^{-/-}$ genotypes were frozen and sagittal sections of 5 micrometer thickness were stained with TUNEL kit (Roche, In Situ Cell Death Detection Kit, Penzberg, Germany). Apoptotic cells were examined under microscope.

2.7. Statistical analysis

The average mutation frequencies and developmental indices were analyzed with the *t*-test. The number of abnormalities was examined with the contingency test. In both cases, *p* values of less than 0.05 were judged as statistically significant.

3. Results

Mutation frequencies in the transgenic *lacZ* gene were studied on 9.5 dpc and 12.5 dpc fetuses, as well as on newborn mice. The 9.5 dpc time point was the earliest stage at which DNA samples were sufficient for determining mutation frequencies in single individuals. Mutation levels were compared among $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ genotypes (Fig. 1). The $Msh2^{-/-}$ fetuses revealed higher levels (33.6×10^{-5}) than those of $Msh2^{+/-}$ (6.6×10^{-5}) and $Msh2^{+/+}$ (5.8×10^{-5}) mice at 9.5 dpc ($p = 0.0005$ and 0.0003, respectively), and levels increased further at 12.5 dpc (60.0×10^{-5}) and in newborns (99.7×10^{-5}). During this period, fetuses of $Msh2^{+/+}$ and $Msh2^{+/-}$ showed low levels of mutations ($6–9 \times 10^{-5}$) and no appreciable differences were observed between the two genotypes ($p = 0.69$ at 9.5 dpc, and $p = 0.40$ at newborn). Mutation levels were also examined in the brain, liver, spleen and small intestines of newborn mice (Fig. 2). All of the tissues from $Msh2^{-/-}$ mice showed similar high levels ($80–130 \times 10^{-5}$) of mutations comparable to

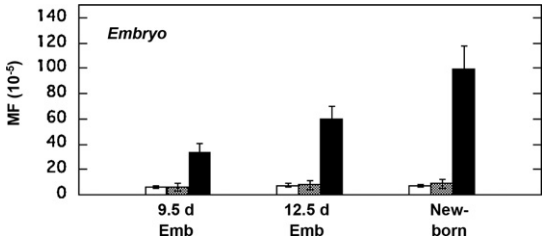

Fig. 1. Spontaneous mutation levels in fetuses of $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ mice. Mutation frequencies (MF) in the *lacZ* gene were examined at the 9.5 dpc and 12.5 dpc embryo stages, and at the newborn stage. Open columns indicate $Msh2^{+/+}$, gray columns $Msh2^{+/-}$ and closed columns $Msh2^{-/-}$ mice. Averages from 3 to 4 individuals and their standard deviations are shown.