and female *gpt* delta mice treated with DC. Although there were no differences between the male groups, BrdU-LIs in the treated females were elevated with significance ($0.30 \pm 0.17\%$, $p < 0.05$) as compared to the control value ($0.07 \pm 0.04\%$) (Fig. 4c and d).

4. Discussion

It has been reported that 18-month exposure of male and female mice to DC at a concentration of 0.15% in the diet caused hepatocelular adenomas and carcinomas with significantly elevated incidence only in the females, in spite of all negative outcomes in various genotoxicity studies [14]. In the present *in vivo* mutation assay, although there were no changes in Spi^- MFs, suggestive of large size of deletion mutations, among the groups, *gpt* MFs were significantly elevated in the females, but not the males. Their spectrum analyses revealed GC:TA transversion mutations to be predominant in the *gpt* mutations observed in the DC-treated females. To the best of our knowledge, this is the first report showing DC-induced genotoxicity, which was in good concordance with DC carcinogenicity in terms of the sex specificity.

It has been assumed that biotransformation of DC involves oxidative opening the cyclopropyl ring at various positions, followed by further oxidation and cleavage of the cyclopropyl-N-bond [14]. In the males, gene expression analysis using cDNA microarray and RT-PCR from the livers after DC treatment demonstrated upregulation of some metabolism-, reduction- and oxidation-related genes such as *CYP1A*, *Por* and *Txnrd 1*, suggesting a possible generation of reactive oxygen species (ROS) through P450-mediated metabolism of DC [22]. Hepatocyte hypertrophy was apparent in the treated mice of both genders and the present study revealed increases of 8-OHdG levels in liver DNA of female *gpt* delta mice given DC as well as the males, indicating that oxidative DNA damage due to ROS generated during DC metabolism is a phenomenon common to both sexes. By contrast, BrdU-LIs in hepatocytes were only significantly increased in the females, which were in line with the fact that significant increase in liver weights was observed in the treated females, but not the males. In the absence of overt cytotoxicity in the treated females, it seems unlikely that induction of cell proliferation resulted from a regenerative response, so that the underlying mechanisms remain unclear. It is well known that during cell replication, 8-OHdG primarily causes GC:TA transversion by mispairing with A bases [23,24]. Furthermore, the fact that regeneration of hepatocytes after partial hepatectomy does not affect 8-OHdG levels suggests that there is no replication coupled repair of preexisting 8-OHdG [25]. Consequently, high proliferation of cells with accumulated 8-OHdG lead to considerable increase in reporter gene MFs [25–27]. Accordingly, we hypothesized that the dual induction of 8-OHdG and cell proliferation due to DC exposure to female *gpt* delta mice might be responsible for the increment in the MFs. In addition, we have found that 4-week exposure of B6C3F1 mice, a back strain of *gpt* delta mice, to DC at the same dose was sufficient to induce significant elevation of 8-OHdG only in the females, but not the males, albeit without no overexpression of *OGG1*, *MYH* or *MTH* mRNA levels in the livers of both sexes (unpublished data). Therefore, it seems likely that the early onset of DNA oxidation is also responsible for the sex specificity.

As a matter of fact, 8-OHdG levels in nuclear genomic DNA may not always imply high levels of 8-OHdG at *gpt* loci specifically located at chromosome 17 [28]. It has been proposed that the distribution of 8-OHdG following exogenous oxidative stress is not random in the genome [29]. Nevertheless, abundant 8-OHdG modification at the *gpt* loci was reported to be observed in the kidneys of *gpt* delta mice treated with ferric nitrilotriacetate [30]. Partly due to the considerable number of copies of the transgene (approximately 80 copies) per haploid genome in the *gpt* delta mice [31], *gpt* loci indeed appear vulnerable to 8-OHdG modification [30]. Therefore, it is highly probable that DC exposure of the *gpt* delta mice caused accumulation of 8-OHdG at *gpt* loci judging from 8-OHdG levels in the genomic DNA. The present spectrum analysis of *gpt* mutants caused by DC exposure showed GC:AT transition mutations at the second highest incidence, despite this type of mutation being spontaneously observed with a certain incidence. In NIH3T3 cells transfected with the c-Ha-ras gene, which incorporates 8-OHdG at the first position of codon 12 (GGC), show mainly GC:TA transversions, while incorporation at the second position elicits GC:AT transitions to an appreciable extent [32,33]. In addition to our data, the results indicate that types of mutations other than GC:TA transversion mutations are induced by 8-OHdG in DNA [34]. We also found that 85.4% of base substitution mutations occurred at G:C pairs and 14.6% at A:T pairs. Although $1,N^6$-ethenoadenosine formed during lipid peroxidation induces AT:GC transition mutations [35], this type of mutation was not evident among the *gpt* mutants. This might reflect the apparent lack of lipid peroxidation despite oxidative DNA damage due to DC treatment.

In conclusion, DC hitherto categorized as a non-genotoxic carcinogen was here shown to have the

potential to induce gene mutations at target site of DNA, possibly due to 8-OHdG formation. The co-examined data strongly suggest that induction of cell proliferation is required to predispose cells harboring high amounts of 8-OHdG to generation of mutations. Thus, the fact that DC-induced genotoxicity is dependent on cell proliferation in addition to nuclear DNA damage by ROS generated through DC metabolism might provide a reason for why genotoxicity has not been detected previously in various mutation assays. The overall data suggest that examination of several parameters associated with carcinogenesis using reporter gene transgenic rodents is a powerful tool for risk assessment of so-called non-genotoxic carcinogens.

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