Prenatal Exposure to Flavonoids: Implication for Cancer Risk

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Flavonoids are potent antioxidants, freely available as high-dose dietary supplements. However, they can induce DNA double-strand breaks (DSB) and rearrangements in the mixed-lineage leukemia (MLL) gene, which are frequently observed in childhood leukemia. We hypothesize that a deficient DSB repair, as a result of an Atm mutation, may reinforce the clastogenic effect of dietary flavonoids and increase the frequency of MII rearrangements. Therefore, we examined the effects of in vitro and transplacental exposure to high, but biological amounts of flavonoids in mice with different genetic capacities for DSB repair (homozygous/heterozygous knock-in for human Atm mutation [Atm-ΔSRI] vs. wild type [wt]). In vitro exposure to genistein/queretin induced higher numbers of MII rearrangements in bone marrow cells of Atm-ΔSRI mutant mice compared with wt mice. Subsequently, heterozygous Atm-ΔSRI mice were placed on either a flavonoid-poor or a genistein-enriched (270 mg/kg) or quercetin-enriched (302 mg/kg) feed throughout pregnancy. Prenatal exposure to flavonoids associated with higher frequencies of MII rearrangements and a slight increase in the incidence of malignancies in DNA repair-deficient mice. These data suggest that prenatal exposure to both genistein and quercetin supplements could increase the risk on MII rearrangements especially in the presence of compromised DNA repair.

Key Words: genistein; quercetin; MII translocations; infant leukemia; in utero; Atm gene.

Bioflavonoids are a diverse group of polyphenolic compounds found in fruits, vegetables, soy, tea, coffee, and wine. They are most commonly known for their protective effect against cardiovascular diseases, cancer, and inflammation, as a result of their antioxidant capacity (Formica and Regelson, 1995; Ross and Kasum, 2002). Because of the presumed health benefits, flavonoid supplements are worldwide over-the-counter available in pharmacies and drugstores. Unfortunately, there is a general belief that these herbal supplements are safe because they are labeled as “natural.” Reasons for concern are that besides their antioxidant properties, flavonoids are also potent topoisomerase II (topoII) inhibitors. TopoII is a nuclear enzyme that plays an essential role in controlling the topology of DNA during replication and recombination. TopoII binds to specific sites on both DNA strands and generates a transient double-strand break (DSB). These DSBs are normally reeledaged. However, certain topoII-inhibiting drugs, such as etoposide or flavonoids, can stabilize DSBs and increase the risk of chromosomal abnormalities (Skibola and Smith, 2000; Spector et al., 2005; Strick et al., 2000).

One of the most common loci involved in chromosomal translocations because of double-stranded DNA lesions caused by topoII inhibitors is the break point cluster region (BCR) of the mixed-lineage leukemia (MLL) gene. This gene is involved in normal hematopoiesis and is located at chromosome 11q23 (Felix and Lange, 1999; Felix et al., 1995; Libura et al., 2005). Approximately 80% of infants that suffer from acute myelogenous leukemia or acute lymphoblastic leukemia (ALL) have chromosomal translocations involving the MLL gene (Skibola and Smith, 2000; Spector et al., 2005; Strick et al., 2000). These translocations are also identified in patients with secondary leukemia caused by chemotherapy with topoII inhibitors (e.g., etoposide) (Blanco et al., 2004; Felix, 1998; Libura et al., 2008; Skibola and Smith, 2000). It has previously been suggested that dietary flavonoids are also capable of introducing these translocations and cause leukemia (Ross, 2000). Although there are some indications that flavonoids might be involved in leukemogenesis (Spector et al., 2005), the complex nature of human diet made it impossible to elucidate the contribution of flavonoids. Our previous data (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007) demonstrate that exposure of human CD34+ hematopoietic progenitor cells to biologically relevant levels of the flavonoids quercetin, genistein, and kaempferol can induce MLL translocations in vitro. Because the concentrations used in this in vitro study can also be obtained in vivo and knowing that flavonoids can penetrate the placenta and accumulate in the fetus (Adlercreutz et al., 1999; Ross and Kasum, 2002; Schroder-van der Elst
et al., 1998), it is crucial to further examine the safety of flavonoids, especially during pregnancy. Fetal cells are rapidly proliferating cells and thus have high topII activity (Zandvliet et al., 1996), which can theoretically make them more sensitive to the topII-inhibiting effect of flavonoids.

As flavonoids execute their clastogenicity through inhibiting the rejoining of DSBs, one might expect that any defect in DSB repair may predispose cells to clastogenic effects of these compounds. DSBs are normally detected by ataxia telangiectasia–mutated (ATM) protein kinase (Khanna, 2000; Montecucco and Biamonti, 2007; Oguchi et al., 2003). Ye et al. (2004) demonstrated that exposure of human lymphoblastoid cell lines to quercetin or genistein leads to autophosphorylation of ATM on serine 1981 leading to the active form of ATM (Bakkenist and Kastan, 2003). Hence, ATM phosphorylates its downstream targets (e.g., p53, Chk2, and histone H2AX), which based on the severity of the damage will either execute DNA repair or cell cycle arrest and apoptosis (Golding et al., 2004; Guny-Pause et al., 2004; van Gent et al., 2001; Ye et al., 2004). Patients with mutations in the ATM gene are prone to developing chromosomal abnormalities (Khanna, 2000; Oguchi et al., 2003). Heterozygous germ line mutations in the ATM gene are frequent and occur in 0.5–1% of the general population (Barlow et al., 1996; FitzGerald et al., 1997). These mutations are associated with an increased risk for developing different types of cancer, including leukemia (Guny-Pause et al., 2004; Oguchi et al., 2003; Stankovic et al., 2002; Sung et al., 2006). Accordingly, missense mutations in the ATM gene have been detected in 25% of cases with childhood ALL (Guny-Pause et al., 2004).

This study examines the effect of both in vitro and transplacental exposure to high, but biological amounts of flavonoids in mice with different genetic capacities for DSB repair (homozygous/heterozygous knock-in for human ATM mutation [Atm−/−AsRI] vs. wild type [wt]). We examined whether a decreased capacity in DNA DSB repair in Atm−/−AsRI mutant (mut) mice enhances flavonoid-induced clastogenicity.

MATERIALS AND METHODS

Exposure of bone marrow cells to genistein, quercetin, or etoposide. Bone marrow cells collected from one femur of adult wt and homozygote mut Atm−/−AsRI mice were isolated by flushing the marrow with Iscove’s modified Dulbecco’s media (IMDM, Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum. Next, cells were washed, incubated in IMDM, and treated for 1 day with 50 µM of etoposide (Sigma-Aldrich, St Louis, MO), genistein (LC Laboratories, Woburn, MA), quercetin (Sigma), or the vehicle dimethylsulfoxide (0.05% DMSO). After exposure, cells were washed twice and left to recover for another day before DNA isolation. Cell counts and viability were determined by a hemocytometer and trypan blue exclusion.

Mice and sample collection. Female Atm−/−AsRI heterozygous mice (129/Sv1C57BL/6J background) approximately 8 weeks of age received either normal chow (low phytoestrogen content complete feed for mice breeding, in which neither soybean nor alfalfa products were used, resulting in minimized concentrations of the phytoestrogens genistein, daidzein, and coumestrol; ssniff, Soest, Germany, n = 8) or the same chow (ssniff) supplemented with genistein (270 mg/kg feed, n = 9) (LC Laboratories) or quercetin (302 mg/kg feed, n = 8) (Sigma) from 3 days before conception until the end of pregnancy. Male Atm−/−AsRI heterozygous mice were placed in the cage only for the duration of copulation. Heterozygote female and male mice were mated because this gives the opportunity to study the in utero effects of the three expected genotypes in offspring (homozygous/heterozygous Atm−/−AsRI and wt).

After delivery, all mothers and pups received the normal chow. At day 5 after birth, pups were weighted and genotype and gender were determined. At 12 weeks of age, offspring were sacrificed by cardiac puncture and checked for tumor development and blood was collected in EDTA tubes (Terumo, Leuven, Belgium) to determine the blood composition. Bone marrow was isolated by removing the femurs and flushing the marrow with PBS. Offspring that died before sacrificing day were also checked for tumor development.

To study the direct effects of diet on fetal hematopoiesis, wt mice (129/Sv1C57BL/6J background, control n = 5, genistein n = 5, and quercetin n = 3) were mated overnight. The presence of a vaginal plug the next morning was defined as 0.5 day postconception. On day 14.5 of pregnancy (embryonic day 14.5), mice were sacrificed to isolate the fetuses and fetal liver (please note that the liver is the organ for hematopoiesis at this time point).

Blood composition. Blood composition was determined in duplicate using the ADVIA 120 Hematology System (Siemens AG, Erlangen, Germany) following the manufacturer’s protocol. The following blood cell parameters were measured: total number of red blood cells (1012/l), hemoglobin level (mmol/l), hematocrit level (l/l), mean corpuscular volume (fl), mean corpuscular hemoglobin concentration (mmol/l), red blood cell distribution width (%), hemoglobin distribution width (mmol/l), total amount of platelets (109/l), mean platelet volume (fl), total amount (109/l) and percentage of reticulocytes, mean corpuscular hemoglobin concentration of reticulocytes (l/l), total number of white blood cells (109/l), and total amount (109/l) and percentage of neutrophils, lymphocytes, monocytes, eosinophils, and basophils. In case of suspected leukemia by abnormal blood composition, the diagnosis was further confirmed by performing a May–Grünewald staining on blood smear preparations. Briefly, 10 µl of blood was fixed by methanol. Next, smears were stained in May–Grünewald solution (Sigma) for 5 min and washed in tap water. Subsequently, the smears were stained with Giemsa solution (Merck, Darmstadt, Germany) for 20 min and washed again in tap water. All preparations were judged by an experienced animal pathologist.

Inverse PCR assay and sequencing. Genomic DNA was isolated from bone marrow cells (in vitro culture or in vivo from 12-week-old mice) and from the fetal liver of E14.5 fetuses using DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands). Fetal livers were first homogenized using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). Next, 1 mg of each DNA sample was subjected to SAP digestion (1 unit of shrimp alkaline phosphatase) (Promega, Madison, WI) for 1 h at 37°C followed by an inactivation at 65°C for 20 min. Then, the DNA was digested with 3 units of PstI (Biolabs, Leusden, The Netherlands) for 1 h at 37°C followed by 20 min inactivation at 80°C. The DNA was circularized overnight at 6°C followed by 2 h at 22°C and 20 min of inactivation at 75°C using 3 units of T4 DNA ligase (Promega) in a final volume of 50 µl. Approximately, 80 ng of the circularized DNA was used for the first PCR, in which the circularized DNA was amplified. Both the first PCR and nested PCR were performed using 0.7 µl expand long template polymerase mix (Roche, Mannheim, Germany), system 2 reaction buffer, 20 pmol of each primer (Eurogentec, Liege, Belgium, Table 1), and deoxynucleotide triphosphate (500 µM) in a volume of 50 µl. The reaction was performed using a Biometra TProfessional thermocycler (Biometra, Leusden, The Netherlands) under the following cycling conditions: 94°C for 2 min, 10 cycles: 94°C for 30 s, 56°C for 45 s, and 68°C for 4 min; 25 cycles: 94°C for 30 s, 56°C for 45 s, and 68°C for 4 min (+20 s/cycle); followed by 7 min at 68°C. The amplified fragments from the first PCR were diluted (1/50), and 2 µl was taken for the nested PCR. The nested PCR products were separated by electrophoresis on a 1% agarose gel, aberrant-sized bands were excised, and DNA was isolated.
using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were subsequently sequenced using big dye v3.1 (Applied Biosystems, Foster city, CA) and 5 pmol primer (Table 1) under the following cycling conditions: 1 min at 96°C, 25 cycles: 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min followed by 10 min at 72°C. After purification by sephadex columns, the products were sequenced by ABI 3730 Automatic DNA Sequencer (Applied Biosystems). Nucleotide sequences were analyzed using “Nucleotide blast” tool of the National Center for Biotechnology Information (Basic Local Alignment Search Tool, Bethesda, MD).

**Statistical analysis.** Statistical analysis was performed with Statistical Package for Social Sciences (SPSS version 15 for Windows, SPSS Inc., Chicago, IL). The nonparametric Mann-Whitney test was used to compare the distribution of gender, genotype, pup survival, litter sizes, average weight of pups at postnatal day 5, and tumor development between litters of the different diet groups. Multifactorial univariate ANOVA (2x3x2x2) was performed to identify predictors (diet, genotype, and gender) of persistent hematological changes in mice.

### RESULTS

**Bone Marrow Cells with Atm-∆SRI Mutation Are More Sensitive to Genistein- and Quercetin-Induced Chromosomal Aberrations**

In order to examine whether dietary exposure to topoII inhibitors can induce chromosomal aberrations and whether Atm-∆SRI mutations actually increase this risk, an inverse PCR method was set-up to detect murine Mll translocations. The assay was validated by detecting Mll translocations in murine bone marrow cells after exposure to the known topoII inhibitor etoposide (50 μM). In addition, bone marrow cells from homozygous wt and mut Atm-∆SRI mice were isolated and cultured for approximately 24 h with either genistein (50 μM) or quercetin (50 μM). Cell viability was evaluated by trypan blue exclusion. We observed no significant decrease in cell viability because of exposure to either flavonoids or etoposide or because of genotype of bone marrow cells in comparison with DMSO-treated cells (Fig. 1). Cells were allowed to repair the DSBs for 24 h in a drug-free medium. Subsequently, DNA was extracted and screened for Mll aberrations by inverse PCR. The DNA isolated from DMSO-treated samples resulted in amplification of the wt Mll (5.6 kb) as visualized by agarose gel electrophoresis (Fig. 2), and the identity was confirmed by sequence analysis. In contrast, exposure of bone marrow cells to quercetin, genistein, or etoposide generated multiple bands of different sizes. The variety in the size of PCR products was determined by both the location of the break point junction in Mll and the position of the Pei site in the fusion partner. Smaller sized bands were more likely to be detected by inverse PCR than the wt Mll because wt Mll is more difficult to amplify because of its length. Sequencing of the break point junction of several distinct Mll translocations induced by flavonoids or etoposide confirmed that the bands of aberrant size were indeed translocations (Table 4). Although etoposide and both flavonoids induced Mll rearrangements in bone marrow cells regardless of the genotype of the mice, the Atm-∆SRI mut cells (0, 2.1, 5, or 1.2 translocations/80 ng genomic DNA for the control, quercetin-, genistein-, or etoposide-exposed cells, respectively; Fig. 2) showed more chromosomal translocations than the wt cells (0, 0, 0, 0 or 3 translocations/80 ng genomic DNA for the control, quercetin-, genistein-, or etoposide-exposed cells, respectively). This indicates that the Atm mutation predisposes cells to the clastogenic effects of flavonoids.

### Effect of Prenatal Diet on Litter Characteristics

Heterozygous Atm-∆SRI mice were exposed to genistein (270 mg/kg feed, n = 9) or quercetin (302 mg/kg feed, n = 8)

![FIG. 1. Viability of wt and Atm-∆SRI mut bone marrow cells was determined after 1 day of exposure to 50μM of the flavonoids genistein and quercetin, 50μM etoposide, and the vehicle DMSO (0.05%) by trypan blue exclusion.](image-url)
or control diet ($n = 8$) throughout their pregnancy. There were no differences in gender ratio, average weight of the offspring measured at day 5 after birth, or average litter size between control, genistein-, or quercetin-exposed groups (Table 2).

Interestingly, the incidence of different genotypes was to some extent affected by prenatal diet. Theoretically, the homozygous wt and mut offspring should each count for approximately 25% of the offspring. Accordingly, wt and mut offspring showed equal prevalence (28 and 29%, respectively) in the control group (Table 2). The genistein and quercetin diet groups, however, had different prevalences of the genotypes. The number of mut mice born in the quercetin group was lower than in the control or genistein group ($p = 0.06$ and $p = 0.01$, respectively). In the genistein group, the percentage of heterozygote animals seemed to be low; however, the distribution of distinct genotypes was not significantly different from the control group.

**Changes in Blood Composition and Incidence of Malignancies in Mice Prenatally Exposed to Flavonoids**

A total number of 45 control, 45 genistein-, and 40 quercetin-exposed mice reached the age of 12 weeks. The percentage of animals that died before this time point was not significantly different for all three diet groups (Table 2). We were unable to determine the cause of death, but at autopsy, no tumors were noted. The mice that reached 12 weeks of age were sacrificed, and their internal organs were examined for visual signs of abnormalities and presence of gross tumors (Table 2). In a previous study (Spring *et al.*, 2002), malignancies were detected in heterozygous $Atm$-$∆SRI$ mice on average after 18.6 months. In our study, several mice had already developed malignancies at 12 weeks of age. One mouse in the control group (1/45) demonstrated splenomegaly

**TABLE 2** Characteristics of the Litters and Malignancies Detected in Offspring within Different Diet Groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Genistein</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average litter size</td>
<td>6.38 ± 2.07</td>
<td>5.22 ± 2.28</td>
<td>5.13 ± 2.30</td>
</tr>
<tr>
<td>Genotype (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total numbers of litters</td>
<td>8</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Wt</td>
<td>28</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>Hetero</td>
<td>43</td>
<td>41</td>
<td>53</td>
</tr>
<tr>
<td>Mut</td>
<td>29</td>
<td>28**</td>
<td>8*</td>
</tr>
<tr>
<td>Average pup weight $^b$</td>
<td>3.18 ± 0.71</td>
<td>3.32 ± 0.58</td>
<td>3.13 ± 0.26</td>
</tr>
<tr>
<td>Percentage of males</td>
<td>47</td>
<td>57</td>
<td>51</td>
</tr>
<tr>
<td>% Deceased pups $^c$</td>
<td>10</td>
<td>7</td>
<td>2.5</td>
</tr>
<tr>
<td>Malignancies detected in offspring at 12 weeks of age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals with malignancies/total analyzed (%)</td>
<td>1/45 (2%)</td>
<td>4/45 (9%)</td>
<td>1/40 (2%)</td>
</tr>
<tr>
<td>Tumor type</td>
<td>Erythroleukemia</td>
<td>ALL, thymoma, fallopian tube cancer</td>
<td>Thymoma</td>
</tr>
<tr>
<td>Tissue involved</td>
<td>Bone marrow, thymus, spleen</td>
<td>Bone marrow, thymus, ovarian ducts</td>
<td>Thymus</td>
</tr>
<tr>
<td>Genotype</td>
<td>Mut</td>
<td>Mut, hetero</td>
<td>Mut</td>
</tr>
</tbody>
</table>

*Significant difference between quercetin and genistein group.

$^b$Average pup weight (grams) at day 5.

$^c$Average number of deceased pups after birth and before sacrifice day (week 12).

$^{*}p = 0.06$.

$^{**}p \leq 0.01$.  

**FIG. 2.** Inverse PCR products representing $Mll$ rearrangements found in bone marrow of wt and $Atm$-$∆SRI$ mut cells after 24 h in vitro exposure to quercetin (50 μM), genistein (50 μM), or etoposide (50 μM). Equal aliquots of DNA were used for each inverse PCR reaction. The samples were visualized by gel electrophoresis. Each band with a size different from the wt 5.6-kb band represents a translocation that results in an aberrant size of the PCR product. Wt $Mll$ is indicated with “*.”
with concomitant erythroleukemia and thymoma. On the other hand, thymomas were detected in three mice (3/45, 7%) of the genistein-exposed group and in 1 of the 40 mice (2.5%) that were prenatally exposed to quercetin. Blood count and blood smear examination showed concomitant ALL in two mice that were exposed to genistein. In general, leukemia and thymomas were only detected in mice that were homozygous for the Atm mutation. The only heterozygous mouse that was diagnosed with malignancy (fallopian tube tumor) belonged to the genistein group. Altogether, mut mice prenatally exposed to genistein showed a slightly higher risk for developing tumors and leukemia compared with wt control mice.

The complete blood count at 12 weeks of age was successfully carried out in duplicate for 41 control, 41 genistein-, and 39 quercetin-exposed mice. The three mice that suffered from leukemia (as assessed by ADVIA 1200 Hematology System and subsequent May-Grünewald staining of blood smears) were excluded from the analysis because of the extreme outlying measurements. As shown in Table 3, 2 × 2 × 2 multifactorial univariate analysis was used to adjust for confounding variables (gender and genotype). Analysis of the red blood cell fraction demonstrated an increase in the mean cell volume (MCV) for both genistein and quercetin group in comparison to the control (mean ± SE, respectively, 46.31 ± 0.34 fl, p = 0.01; 46.33 ± 0.50 fl, p = 0.4; and 44.96 ± 0.45 fl for control). The red blood cell distribution width (RDW) was only significantly increased in the genistein group (13.92 ± 0.19% vs. 13.35 ± 0.12% for control, p = 0.03). A combined high MCV and RDW could be an indication of a higher reticulocyte count. Indeed, the total amount of reticulocytes was elevated in the genistein group (157.57 ± 17.51 × 10⁹/l vs. 106.79 ± 15.88 × 10⁹/l in control, p = 0.4). Although prenatal quercetin exposure was not associated with an increased reticulocyte count, the mean hemoglobin content of reticulocytes (MCHR) was higher (1.03 ± 0.01 fmol) compared with the control group (0.96 ± 0.01 fmol, p = 0.07). Prenatal exposure to genistein on the contrary significantly decreased the MCHR (0.94 ± 0.03 fmol, p = 0.02).

In the genistein group, the total number of red blood cells (7.44 ± 0.12 × 10¹²/l vs. 7.03 ± 0.13 × 10¹²/l for control, p = 0.07) and consequently the hemoglobin (7.25 ± 0.09 mmol vs. 6.78 ± 0.11 mmol for control, p = 0.009) and hematocrit levels (0.35 ± 0.01 l/l vs. 0.32 ± 0.01 l/l for control, p = 0.02) were significantly elevated.

Detection of Mll Translocations in Mice Prenatally Exposed to Flavonoids

In order to investigate the clastogenic effects of prenatal genistein and quercetin exposure, we studied the occurrence of Mll translocations in the fetal liver of E14.5 wt fetuses by inverse PCR. Genomic DNA obtained from the fetal liver was screened for chromosomal aberrations (Fig. 3). Interestingly, regardless of the maternal diet, Mll rearrangements could be detected in all the fetuses. The rate of translocations between the control group and both exposure groups showed no differences (control: 4.3 translocations/80 ng genomic DNA vs. 2.5 translocations/80 ng genomic DNA and 6.5 translocations/80 ng genomic DNA for the genistein- and quercetin-exposed

FIG. 3. Inverse PCR products representing Mll rearrangements found in liver cells of fetuses exposed to genistein or quercetin. Equal aliquots of DNA were used for each inverse PCR reaction. The samples were visualized by gel electrophoresis. Each band with a size different from the wt 5.6-kb band represents a translocation that results in an aberrant size of the PCR product. Wt Mll is indicated with ‘*’.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Multifactorial Univariate Analysis of the Long-Term Effects of Genistein and Quercetin on the Blood Composition of 12-Week-Old Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Control Mean ± SE</td>
</tr>
<tr>
<td>Red blood cells (10¹²/l)</td>
<td>7.03 ± 0.13</td>
</tr>
<tr>
<td>Hemoglobin (mmol/l)</td>
<td>6.78 ± 0.11</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>44.96 ± 0.45</td>
</tr>
<tr>
<td>Hematocrit (l/l)</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin reticulocytes (fmol)</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>Absolute amount of reticulocytes (10⁹/l)</td>
<td>106.79 ± 15.88</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>13.35 ± 0.12</td>
</tr>
</tbody>
</table>

*p Value calculated for dietary effects adjusted for genotype and gender. Bold: p < 0.05; italic: p = 0.07
Selection of *Mll* Translocations Detected in Fetal Liver of E14.5 Fetuses or in Bone Marrow Cells *In Vitro* Exposed or of Mice Prenatally Exposed to the topoII Inhibitors

<table>
<thead>
<tr>
<th>Exposurea</th>
<th>Genotypeb</th>
<th><em>Mll</em></th>
<th>Fusion partner</th>
<th>Sequence of the translocation junction</th>
<th># Basepair homology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> exp.</td>
<td>Q Mut</td>
<td>44 648 590</td>
<td><em>Mll</em>-chromosome 4</td>
<td>CGTTAATAATTTCTTCATCTT…</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>G Mut</td>
<td>44 648 585</td>
<td><em>Mll</em>-chromosome 13</td>
<td>AAAAAATACCAGGTTGCGGTTG</td>
<td>6</td>
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<tr>
<td></td>
<td>G Mut</td>
<td>44 648 365</td>
<td><em>Mll</em>-chromosome 12</td>
<td>TATAAAAAATTTTTGTTTTATAA</td>
<td>1</td>
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<tr>
<td></td>
<td>G Mut</td>
<td>44 648 168</td>
<td><em>Mll</em>-chromosome 17</td>
<td>GAACAATCGAGGCTGACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q Mut</td>
<td>44 648 870</td>
<td><em>Mll</em>-chromosome 8</td>
<td>CGCAGTCTAGAGTAAATGCCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q Mut</td>
<td>44 648 162</td>
<td><em>Mll</em>-chromosome 17</td>
<td>CATGTCTCAAAAAATAAAAAT</td>
<td>119</td>
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<tr>
<td></td>
<td>Q Mut</td>
<td>44 648 585</td>
<td><em>Mll</em>-chromosome 17</td>
<td>ATTTAGAGTAAATAAAAATAGAGGACAGAC</td>
<td>143</td>
</tr>
<tr>
<td>Adult mice</td>
<td>C Mut</td>
<td>44 648 573</td>
<td><em>Mll</em>-chromosome 3</td>
<td>GAGGTGGTGGGACACATG…</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C Mut</td>
<td>44 648 853</td>
<td><em>Mll</em>-chromosome 8</td>
<td>CCCACACTCGGTCGTTACAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C Mut</td>
<td>44 648 870</td>
<td><em>Mll</em>-chromosome 8</td>
<td>GACAATCAGAGCTGACAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C Mut</td>
<td>44 648 942</td>
<td><em>Mll</em>-chromosome 2</td>
<td>AAAAAAATTITCAAGAAA…</td>
<td>162</td>
</tr>
</tbody>
</table>

Note. The nucleotide sequence of the *Mll* gene and its fusion partners at the break point junction are, respectively, indicated in normal and italic. The nucleotide sequence homology between *Mll* and the fusion partner is underlined. The nucleotide sequence homology of the translocations, which showed an extensive homology, could not be demonstrated in this table.
aExposure of murine bone marrow cells *in vitro* to quercetin (Q), genistein (G), or etoposide (E) or diet condition during prenatal period: quercetin (Q), genistein (G), or control diet (C).
bGenotype of examined mice: wt or *Atm*-ASRI mut.

The nucleotide numbering at the break point junction is based on the *Mll* genomic sequence National Center for Biotechnology Information database NC_000075.

The average number of translocations is calculated as average number of alternative-sized band seen in one inverse PCR reaction/DNA input in an inverse PCR reaction. However, when both flavonoids-exposed groups were compared, the quercetin-exposed fetuses showed more chromosomal translocations compared with the genistein-exposed fetuses. In order to confirm the mut-sized bands as genuine translocations instead of PCR artifacts, a selection of aberrant products was sequenced to confirm that the *Mll* products were sequenced (Table 4) and all appeared to be real *Mll* translocations with various chromosomes as fusion partners.

Next, bone marrow of 12-week-old wt or *Atm*-ASRI mut mice prenatally exposed to genistein or quercetin was screened for *Mll* translocations. As shown in Figure 4, prenatal exposure to both genistein and quercetin increased the frequency of *Mll* rearrangements. In wt control mice, we identified 0.9 translocations/80 ng genomic DNA by inverse PCR. Prenatal exposure to genistein increased the mutation rate in wt mice to 2.5 translocations/80 ng genomic DNA, whereas wt mice prenatally exposed to quercetin showed no change in the frequency of *Mll* rearrangements (1 translocation/80 ng genomic DNA). All three diet groups increased the incidence on *Mll* translocations in mice carrying the *Atm* mutation. In the control group, the mutation rate increased up to 2.7 translocations/80 ng genomic DNA. This is comparable with the rate seen in wt mice prenatally exposed to genistein. *Atm*-ASRI mut mice prenatally exposed to genistein had a twofold higher rearrangement rate (5.6 translocations/80 ng genomic DNA) compared with their wt siblings. Although prenatal exposure to quercetin had no effect on the occurrence of *Mll* translocations in wt mice, it increased the mutation rate in *Atm*-ASRI mut mice considerably (up to 16 translocations/80 ng genomic DNA). This suggests that prenatal exposure to genistein leads to a modest increase in the number of *Mll* rearrangements, whereas prenatal quercetin exposure considerably elevates the rearrangement rate in *Atm*-ASRI mut mice. Again, a selection of aberrant *Mll* products was sequenced to confirm that the alternative *Mll* products were comprised of genuine...
translocations (Table 4). No genuine translocations were detected in the wt mice on control diet.

**DISCUSSION**

It has been suggested that high intake of dietary flavonoids contributes to infant leukemia (Ross et al., 1996; Spector et al., 2005). However, no direct experimental evidence has yet been presented. Our previous study (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007), together with the study of Strick et al. (2000), showed that flavonoids could induce MLL translocations in CD34+ cells. However, we are the first to report that prenatal exposure to flavonoids can increase the risk for leukemia onset, as assessed by the frequency of Mll translocations in a mouse model prone to develop cancer. To detect potential translocations, an inverse PCR method was developed. In order to validate this method, murine bone marrow cells of wt and Atm-ASRI mut mice were exposed to 50μM of genistein, quercetin, and the known topoII inhibitor etoposide. DNA isolated from these cells was then subjected to inverse PCR amplification to detect murine Mll translocations. Although all exposures induced Mll translocations, Atm-ASRI mut bone marrow cells that lack adequate DSB repair were more susceptible for flavonoids- and etoposide-induced Mll rearrangements.

To investigate the effect of prenatal exposure to flavonoids and the predisposing role of deficient DSB repair, Atm-ASRI hetero mice were mated to obtain pups with different genetic capacity for DNA repair from a single mother. Bone marrows of 12-week-old wt and Atm-ASRI mut offspring mice that were prenatally exposed to genistein or quercetin were screened for Mll translocations by using the inverse PCR method. Both flavonoids were capable of inducing Mll translocations in wt and Atm-ASRI mut mice, but Atm-ASRI mut mice showed a higher susceptibility to develop these chromosomal aberrations, which was expected from the in vitro results. This suggests that exposure to flavonoid supplements can have more severe consequences in individuals with a malfunctioning DNA repair system. Alk flavonoids, radiation induces DNA DSBs. Accordingly, radiation exposure has been shown to induce significantly more cell death and chromosomal aberrations in Atm-ASRI mut thymocytes than in wt cells (Spring et al., 2001).

In order to study the direct effects of flavonoid exposure on the growing fetus, fetal livers were isolated at day 14.5 of pregnancy and screened for Mll translocations. These translocations were detectable in all fetuses regardless of the maternal diet. This indicates that such translocations might be common events during fetal development, which are probably generated because of high topoII activity of the proliferating cells (Zandvliet et al., 1996). However, the fact that less Mll translocations were detected at 12 weeks of age suggests that the cells carrying these translocations are either eliminated or have restricted expansion capacity.

Prenatal quercetin exposure in this study led to a minor decrease in average litter size. It seems that quercetin exposure is more lethal to fetuses with homozygous Atm-ASRI mutations. In fact, studying the direct effect of the flavonoids on the fetuses shows that transplacental quercetin exposure induces more chromosomal translocations than genistein. Both prenatal genistein and quercetin exposure had no effect on gender ratio and average birth weight, which suggests that in utero dietary supplementation did not affect normal development. Also the number of pups that died spontaneously after birth was not influenced by the diet. Three mice, all Atm-ASRI mut, developed leukemia (ALL or erythroleukemia) at 12 weeks of age, confirming our hypothesis that a decrease in DNA DSB repair (ATM dysfunction) could enhance the leukemia risk. One mouse that developed erythroleukemia was not prenatally exposed to flavonoid supplements, suggesting that in this case the Atm-ASRI predisposition was responsible for the development of leukemia. Indeed, Atm-ASRI mut mice are known to have a 50% chance on developing malignancies (Spring et al., 2001). The ratio of malignancies between control and genistein-exposed mice suggests that prenatal genistein exposure may increase the risk on developing malignancies. All malignancies, except ALL detected in our mice, were previously described in Atm-ASRI mice (Spring et al., 2001, 2002). Atm-ASRI hetero mice develop different categories of tumors at an average age of 18.6 months. However, 44% of the Atm-ASRI mut mice die of thymic lymphomas up to 10 months of age. In our study, 1 out of 13 Atm-ASRI mut mice in the control group

**FIG. 4.** Inverse PCR products representing Mll rearrangements found in bone marrow cells of wt and Atm-ASRI mut offspring mice at 12 weeks of age, prenatally exposed to genistein or quercetin. Equal aliquots of DNA were used for each inverse PCR reaction. The samples were visualized by gel electrophoresis. Each band with a size different from the wt 5.6-kb band represents a translocation that results in an aberrant size of the PCR product. Wt Mll is indicated with "*."
had developed malignancy at the age of 3 months. The percentage of malignancies in the genistein and quercetin group was, respectively, 23% (3 out of 13 Atm-ΔSRI mut mice) and 25% (one out of four Atm-ΔSRI mut mice). The decrease in the number of Atm-ΔSRI mut mice born in the quercetin group further suggests the hazards of such exposure in a vulnerable population that lacks an effective DNA DSB repair.

A complete blood count analysis showed that prenatal exposure to genistein not only influenced the lymphoblastic cell development but also the myeloblastic cell development and overall erythropoiesis. Prenatal exposure to genistein associates with an upregulation of erythropoiesis in adult mice, as seen in the increase in the number of red blood cells, which results in an increase in hemoglobin and hematocrit levels (ratio cells/plasma). The increase in RDW could be the result of an increase in the number of reticulocytes, which could also explain the increase in mean cell volume. However, it could also indicate that there is a coexistence of young and old red blood cells, suggesting a longer survival of these cells. Prenatal exposure to quercetin on the other hand only increased the size of the red blood cells and the amount of hemoglobin in reticulocytes. The increase in red blood cell size could be the result of an increase in the amount of hemoglobin per cell. An increase in the amount of hemoglobin in reticulocytes would automatically lead to an increase in the amount of hemoglobin in erythrocytes. Knowing that quercetin has iron ion chelating capacities (Ferrali et al., 1997) and the fact that the heme part of hemoglobin contains iron, it can be suggested that the effects seen is because of a disturbed iron metabolism in these mice. Further investigation is needed to unravel these aspects of prenatal genistein/quercetin exposure and the mechanisms behind the long-term effects of diet during pregnancy on blood composition.

Due to the fact that flavonoids are present in a variety of food sources, the incidental daily intake can be as high as 1 g but can increase up to several grams in those supplementing their diet with flavonoids. The average daily dietary intake of quercetin in the range of 4–68 mg. Daily isoflavone intake in Western countries is approximately 1–9 mg. In Asian countries, however, the daily intake of isoflavones is much higher, reaching levels of 20–240 mg, because of the high consumption of soy (Skibola and Smith, 2000). In our study, pregnant mice were exposed to approximately 26.7–36.7 mg/kg body weight genistein and 33.3–46.7 mg/kg body weight quercetin per day. Record et al. (1995) determined that mice fed 20 mg/kg genistein had an average plasma concentration of 10μM. In this study, pregnant mice were exposed to a higher dose of genistein and quercetin, probably resulting in even higher plasma concentrations. These concentrations are also higher compared with plasma concentrations seen in humans on both a Western or Asian diet (Morton et al., 2002). However, both flavonoids are freely available as supplements, and the daily dose recommended by manufacturers can be as high as 1–2 g/day. When supplements are taken, plasma concentrations can be 10–20 times higher (Ferry et al., 1996) than the levels we tested in mice, indicating that the doses used in this study are biologically relevant. It is also noteworthy that the metabolism of phytoestrogens is different in the fetus and adults. In human, fetal cord blood has a higher genistein level than maternal serum (Adlercreutz et al., 1999; Schroder-van der Elst et al., 1998; Todaka et al., 2005). Taken together, it is important to establish clear guidelines for the use of flavonoid supplements during pregnancy.

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