Radiation-induced apoptosis in mouse lymphocytes is modified by a complex dietary supplement: the effect of genotype and gender

Jennifer A. Lemon*, C. David Rollo1, Nicole M. McFarlane and Douglas R. Boreham

Department of Medical Physics and Applied Radiation Sciences and 1Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

This study examined whether radiation sensitivity measured by lymphocyte apoptosis could be ameliorated by a complex anti-oxidant/anti-ageing dietary supplement. We also examined lymphocytes from both genders of normal (Nr) mice as well as transgenic growth hormone (Tg) mice that express strongly elevated reactive oxygen species processes and a progeroid syndrome of accelerated ageing. We introduce Tg mice as a potentially valuable new model to study radiation sensitivity. Isolated lymphocytes from all experimental groups were exposed to gamma radiation and the time course of apoptosis was measured in vitro. Kinetics of radiation-induced apoptosis was similar among groups, which peaked at 8 h, but maximal levels differed significantly between groups. Nr male mice had 60% lower levels of radiation-induced apoptosis than Tg males, supporting our hypothesis that Tg mice would be radiation sensitive. The dietary supplement protected lymphocytes in male mice of both strains, with proportionally greater reductions in Tg mice. Lymphocytes from female mice (both Nr and Tg) were highly radiation resistant compared to males and the supplement provided no additional benefit at the doses used in this study. These results highlight that radiation-induced apoptosis is complex and is modified by genotype, dietary supplements and gender.

Introduction

Oxidative stress, predominantly from increased reactive oxygen species (ROS), is a potent inducer of apoptosis in a wide variety of cells. Increased production of ROS (i.e. through exposure to ionizing radiation) contributes to the initiation of apoptosis in several ways. Key processes include altering the redox status of cells (1) and contributing significant damage to cellular macromolecules (lipids, DNA and proteins) (2) and subcellular organelles of which mitochondria are particularly susceptible (3,4). These perturbations can result in decreased cellular efficiency and functionality, as well as a further increase in ROS production (1), creating an escalating cycle of oxidative stress-induced apoptosis.

Cells have extensive protective mechanisms against oxidative damage. Enzymatic and non-enzymatic antioxidants remove free radicals before they cause damage; while recycling and repair mechanisms remove or repair ROS-damaged macromolecules and organelles. However, if the damage is too extensive, cells are removed through apoptotic or necrotic processes. Several strains of mice with compromised antioxidant systems have illustrated that increases in oxidative stress exacerbates apoptotic cell loss, reduces lifespan and increases the prevalence of age-associated diseases such as cancer and neuropathologies (5,6).

The use of antioxidants and other compounds to ameliorate the physiological signs of oxidative stress have been investigated for decades. However, with few exceptions (7,8), most studies, which have tested materials alone or a few in combination, yielded poor or inconsistent results when looking at higher-order end points such as cognitive ability and longevity (9–11). We developed a complex dietary supplement comprised of 31 ingredients with well-documented effects known to reduce oxidative stress and inflammation, promote membrane and mitochondrial integrity and/or increase insulin sensitivity (12,13).

Transgenic growth hormone (Tg) mice have highly elevated free radical processes in all tissues examined and express a progeroid syndrome resembling accelerated ageing. Characteristically, Tg mice have a shortened lifespan (50% that of Nr mice) and early onset of symptoms associated with senescence in mice including arthritis, reduced activity, cognitive decline, cataracts, sarcopenia and poor fur quality (12,14–20). Younger Tg mice possess remarkable learning and memory abilities, learning an eight-choice radial maze roughly twice as quickly as normal controls (12,21). Within ~11 months, however, 95% of Tg mice were unable to learn, whereas Nr mice showed no cognitive changes at that age (12). The complex dietary supplement completely abolished the age-related cognitive decline of Tg mice (12).

The dietary supplement significantly extended lifespan in both Nr and Tg mice. Mean longevity of Nr mice is extended 11 ± 2% [mean ± standard error (SE)], whereas there was a much larger and significant increase of 28 ± 1% for Tg mice (13). Comparison of the physical condition of 12-month-old Tg with age-matched Tg mice on the supplement showed amelioration of most symptoms of ageing including cataracts, sarcopenia and arthritis, coat quality and locomotor activity (13). The superior physical condition of supplemented Tg mice continued for several months after most unsupplemented Tg mice had died (13). Given the powerful effects of the diet supplement on cognition and longevity and the fact that most of the five targets of the supplement directly or indirectly relate to free radical processes, we postulated that the dietary supplement was altering the effects of elevated ROS and associated processes, as such there may be a systemic effect of the supplement which could be radioprotective.

We also considered gender in radiation-induced apoptosis of lymphocytes. There have been numerous reports showing that there is a significant gender difference in apoptosis in several species including mice and humans (22–25). Oestrogen and progesterone have anti-apoptotic actions on several tissues

*To whom correspondence should be addressed. Tel: +1 905 525 9140; Fax: +1 905 522 5982; Email: lemonja@mcmaster.ca

© The Author 2008. Published by Oxford University Press on behalf of the UK Environmental Mutagen Society. All rights reserved. For permissions, please e-mail: journals.permissions@oxfordjournals.org.
including brain (25,26), thymus (27), heart (28,29) and lymphocytes (22,24,30). Alternatively, testosterone is generally pro-apoptotic (22,27,31,32). These actions are mediated through several receptor-independent and -dependent effectors, including regulation of the Bcl-2 proto-oncogene family (22,25) and other proto-oncogenes and oncosuppressor genes (33), tumour necrosis factor-α (24,27), modification of signal transduction pathways (34–38) and modifying oxidative stress and inflammation processes (39–42). The specific actions of sex hormones and their signalling are tissue specific and remain largely unexplored with respect to radiation.

Lymphocyte apoptosis is an established biomarker of oxidative stress. Numerous methods are available to measure this end point and advances in technology have made it a fast and reliable test used in a broad range of applications from markers of age-related diseases to biodosimetry and indicators of radiation risk (43–46). In this study we have compared radiation-induced lymphocyte apoptosis in Nr mice and oxidatively stressed Tg mice. We have tested the postulate that a complex dietary supplement can alter radiation-induced apoptosis in lymphocytes and that gender affects the frequency of apoptotic death.

Materials and methods

Animals

Experimental animals were Nr and Tg male and female C57Bl-6/SJL mice. Tg mice have metallothionein promoters fused to rat growth hormone (GH) structural genes (47). The rat GH genes are incorporated into the mouse genome thereby chronically elevating plasma GH levels >100-fold. Tg mice can be identified morphologically by their significantly larger size by 28 days of age; this technique has been shown to be extremely reliable, negating the necessity for continuous molecular testing (47,48). The kinetics of radiation-induced apoptosis in lymphocytes used 48 mice aged 11–12 months, divided into four experimental groups (three per group). A maximum of four mice were maintained per cage (27). Response experiments consisted of 12 mice in four experimental groups (three per group). A maximum of four mice were maintained per cage (27 × 12 × 15.5 cm) containing woodchip bedding (Harlan Sani-Chips, 7090). A stainless steel hopper provided food ad libitum (Harlan Teklad 8640 22% rodent chow) and supported a water bottle. The housing room maintained a 12:12-h photoperiod. The bagel pieces were always immediately and completely washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 1.5 mM Na2HPO4, 4.3 mM NaHCO3, 14.7 mM KH2PO4, 1.0 mM ethylenediaminetetraacetic acid (EDTA); at room temperature for 15 min). The white blood cells were centrifuged at 200 × g for 10 min at room temperature, washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 1.5 mM Na2HPO4, 14.7 mM KH2PO4, 1.0 mM ethylenediaminetetraacetic acid (EDTA); at room temperature for 15 min), re-suspended in 2.5 ml complete RPMI 1640 and incubated at 37°C (5% CO2, 98% humidity) for designated time periods (see below)..

Dietary supplement

The anti-aging supplement was designed to simultaneously ameliorate five major processes implicated in ageing (oxidative stress, inflammatory processes, sex hormones and their signalling are tissue specific and remain largely unexplored with respect to radiation). Criteria for selecting materials for the supplement were as follows: (i) scientifically documented evidence as effective for one or more of the targeted features, (ii) can be taken orally, (iii) ‘over the counter’ products and (iv) safe for mouse (and human) consumption.

Dosages for the mice were reformulated based on amounts commonly prescribed to humans. Values were adjusted for the difference in body size and nutritional needs for mouse (and human) consumption.

Sample collection and preparation

Mice were anesthetized with Isoflurane™ and blood obtained via cardiac puncture. Samples were kept on ice during preparation unless otherwise stated.

The whole blood was diluted 1:1 in complete RPMI 1640 growth media (10% foetal bovine serum, 1% l-glutamine and 1% penicillin–streptomycin; all components from Invitrogen, Mississauga, Ontario, Canada). Half of the blood from each mouse held at 0°C was irradiated with gamma radiation from a 137Cs source (0.25 Gy/min dose rate). The remaining volume of blood under the same conditions was used as the sham-irradiated control. After irradiation, the red blood cells were removed by lysis using ammonium chloride solution (154 mM ammonium chloride + 1.5 mM potassium bicarbonate 0.1 mM ethylenediaminetetraacetic acid (EDTA); at room temperature for 15 min). The white blood cells were centrifuged at 200 × g for 10 min at room temperature, washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 1.5 mM Na2HPO4, 14.7 mM KH2PO4, 1.0 mM ethylenediaminetetraacetic acid (EDTA); at room temperature for 15 min), re-suspended in 2.5 ml complete RPMI 1640 and incubated at 37°C (5% CO2, 98% humidity) for designated time periods (see below).

Sample staining and flow cytometry

Annexin V was used as the indicator of apoptosis for this assay with 7-amino actinomycin D (7-AAD) as the counterstain. The reagents were purchased as a commercial kit (Annexin V–FITC/7-AAD; IM3614, Beckman Coulter, Miami, FL). Annexin V is a molecule with a high affinity for phosphatidylserine residues that become exposed on the external plasma membrane of apoptotic lymphocytes (50). In later stages of apoptosis, plasma membrane integrity is lost, allowing 7-AAD to enter the cell and bind to DNA. The combination of Annexin V and 7-AAD allowed the enumeration of the various stages of apoptosis. In all groups of mice, apoptotic cells were identified as Annexin V-positive and Annexin V–7-AAD-positive lymphocytes. The relative levels of Annexin V-positive to 7-AAD-positive cells did not differ based on genotype or gender and were consistent between all groups of mice. Loss of Annexin V positivity requires degradation and loss of the cell membrane, as such, events identified as Annexin V negative and 7-AAD positive were not included in the analysis.

Since apoptosis is the predominant mode of death (>99%) for lymphocytes, it was not necessary to further differentiate between apoptotic and necrotic cells.

Cell suspensions were incubated for 0, 1, 2, 4, 6 and 8 h and analysed for apoptosis as described above. A cell suspension of 4 × 103 cells was washed with 4 ml PBS + 0.1 mM EDTA and re-suspended in 100 μl of 1× Binding Buffer (supplied in kit). To each 5-ml polystyrene assay tube, 10 μl Annexin V–FITC and 20 μl 7-AAD were added and incubated for 15 min on ice as per the manufacturer’s instructions. An additional 400 μl of 1× Binding Buffer was then added to each assay tube and analysed within 30 min on a Beckman-Coulter Epics XL flow cytometer. A minimum of 1.5 × 105 lymphocytes were analysed from each sample.

Statistics

All values were represented as the mean and SE of the mean. Student’s t-tests were performed to determine if significant differences existed between groups. Analysis of variance (ANOVA) was carried out to clarify which (if any) of the independent variables (i.e. dietary supplement, gender and genotype) associated with the groups of mice contributed significantly to the differences in mean apoptosis and whether those factors had any interactive effects.

Fig. 1. Experimental groups of mice used in this study consisted of two strains, normal mice (isogenic controls) and transgenic growth hormone mice (TGM).

Half of each strain was fed a complex dietary supplement. Each of these groups consisted of males and females to determine if gender altered radiation-induced lymphocyte apoptosis and the effect of the supplement. Finally, lymphocytes harvested from each mouse were divided and a portion was irradiated (the remainder was the sham-irradiated control).
This is meant to be a complete list of target and action for each of the supplement components, the target and functions of each ingredient are presented only in the context of the supplement and excluding synergistic and recycling interactions among supplements.

**Table I. Components of the dietary supplement and associated cellular targets**

<table>
<thead>
<tr>
<th>Component</th>
<th>Target</th>
<th>Component</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B1</td>
<td>Insulin sensitivity, anti-inflammatory</td>
<td>Flax seed oil</td>
<td>Omega fatty acids for membrane support</td>
</tr>
<tr>
<td>Vitamin B3</td>
<td>Insulin sensitivity, anti-inflammatory</td>
<td>Folic acid</td>
<td>Antioxidant, maintains glutathione levels, endothelial support</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>Insulin sensitivity, anti-inflammatory, scavenges O2^-</td>
<td>Garlic</td>
<td>Antioxidant in lipid membrane, scavenges O2^- , H2O2</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>Insulin sensitivity, anti-inflammatory</td>
<td>Ginger</td>
<td>Antioxidant in cytosol, scavenges OH^-, O2^-, ONOO^-</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Antioxidant in cytosol, scavenges O2^- , H2O2</td>
<td>Ginseng</td>
<td>Antioxidant in cytosol, scavenges OH^-, O2^-, ONOO^-</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Antioxidant in lipid membrane</td>
<td>Green tea extract</td>
<td>Antioxidant in cytosol, scavenges H2O2, OH^-</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Antioxidant in lipid membrane, scavenges O2^- , H2O2</td>
<td>t-glutathione</td>
<td>Enzymatic antioxidant support, antioxidant in cytosol</td>
</tr>
<tr>
<td>Acetyl l-carnitine</td>
<td>Mitochondrial support, antioxidant in mitochondria, insulin sensitivity</td>
<td>Magnesium</td>
<td>Insulin sensitivity, cellular support</td>
</tr>
<tr>
<td>Alpha lipoic acid</td>
<td>Mitochondrial support, antioxidant in mitochondria, insulin sensitivity</td>
<td>Melatonin</td>
<td>Antioxidant in cytosol and nucleus, scavenges OH^-, H2O2, O^-, NO, ONOO^-</td>
</tr>
<tr>
<td>ASA</td>
<td>Anti-inflammatory, scavenges NO^-</td>
<td>N-acetyl cysteine</td>
<td>Mitochondrial support, antioxidant in mitochondria</td>
</tr>
<tr>
<td>Beta carotene</td>
<td>Antioxidant in lipid membrane, scavenges O2^- , H2O2</td>
<td>Potassium</td>
<td>Insulin sensitivity, cellular support</td>
</tr>
<tr>
<td>Bioflavonoids</td>
<td>Antioxidant in cytosol and nucleus, scavenges OH^-, O2^- , metal chelator</td>
<td>Chromium</td>
<td>Antioxidant in lipid membrane, scavenges OH^-, O2^- , metal chelator</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>Omega fatty acids for membrane support</td>
<td>Selenium</td>
<td>Scavenges H2O2, enzymatic antioxidant support, insulin sensitivity</td>
</tr>
<tr>
<td>CoEnzyme Q10</td>
<td>Mitochondrial support, antioxidant in mitochondria</td>
<td>Zinc (chelated)</td>
<td>Neural support (zinc + antioxidants), insulin sensitivity</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>Antioxidant, endocrine support</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This is not meant to be a complete list of target and action for each of the supplement components, the target and functions of each ingredient are presented only in the context of the supplement and excluding synergistic and recycling interactions among supplements.

**Results**

**Kinetics of apoptosis**

Spontaneous and radiation-induced apoptosis was monitored in all groups of mice (Figure 1, Groups A–P.) over 12-h post-irradiation to determine if differences in kinetics existed between experimental groups (Figure 2A and B). In all groups, both spontaneous and radiation-induced apoptotic levels from *in vitro* samples peaked at 8 h. Levels plateaued out to 12 h, after which a continuous decline in levels of apoptotic cells was observed out to 16 h (data not shown). Since maximal differences between groups were observed after an incubation period of 8 h, this incubation time was used for all remaining analyses. Although the number of apoptotic lymphocytes varied between groups, all groups demonstrated similar kinetic patterns during the 8-h incubation period (Figure 2A and B). There was no difference between spontaneous and radiation-induced apoptosis for any group of mice between 0 and 2 h incubation and overall levels of apoptosis did not increase during this time period. The proportion of apoptotic lymphocytes then began to increase substantially at 4 h and continued to increase up to 8 h (Figure 2A and B). At 8 h, male Tg mice (Figure 1, group L) had the highest level of apoptosis after a 2 Gy dose (Figures 2A and 4). Nr male mice were the next most sensitive (Figure 1, Group D). The female mouse lymphocytes were much more resistant than males and did not differ significantly from each other (Nr versus Tg) (Figure 1, Groups B and J).

**Dose response**

The dose of radiation was proportional to the apoptotic response for all unsupplemented mice (Groups B, D, J and L) (Figure 3). Lymphocytes from male Tg mice had significantly higher levels of apoptosis compared to Nr male mice and both groups of female mice at doses of ≥2 Gy and above (2 Gy: \( P < 0.005 \), 4 Gy: \( P < 0.01 \) and 6 Gy: \( P < 0.04 \)). Female Nr and Tg mice had apoptosis levels that were not significantly different from each other at any dose (\( P > 0.05 \)),

**Fig. 2.** Cell kinetics of mouse lymphocyte apoptosis. The graph shows a comparison of radiation-induced lymphocyte apoptosis with time (hours) in male mice (squares) and female mice (circles) after a 2 Gy dose of gamma radiation. (A) Kinetics of radiation-induced apoptosis in mice not on a diet supplement. (B) Kinetics of radiation-induced apoptosis in mice given a diet supplement. Graph values represent mean ± SE.
although female Tg mice demonstrate moderately greater radiosensitivity >4 Gy. Female mice had significantly lower levels of lymphocyte apoptosis than Nr and Tg males at doses of ≥2 Gy (P < 0.01; Figure 3).

Spontaneous apoptosis

Unirradiated lymphocytes in (Figure 1, Groups A, C, E, G, I, K, M and O) exhibited spontaneous induction of apoptosis in vitro. The frequency of spontaneous apoptosis was subtracted from the respective irradiated group to distinguish radiation-induced levels. After 8-h incubation at 37°C, there was no significant difference in spontaneous levels (0 Gy dose groups) of lymphocyte apoptosis between unsupplemented Nr and Tg males (26.87 ± 0.96 and 27.67 ± 4.08%, respectively, P < 0.85; Figure 3). Spontaneous apoptosis was also similar in unsupplemented Nr females (23.70 ± 2.16%) and Tg females (17.73 ± 3.30%; P < 0.16). Spontaneous lymphocyte apoptosis in diet-supplemented male Nr mice was 34.98 ± 3.17% which was not statistically significantly different (P > 0.01) from diet-supplemented male Tg mice at 22.47 ± 6.46%.

Spontaneous lymphocyte apoptosis in diet-supplemented female Nr mice (25.23 ± 4.49%) was similar to supplemented female Tg mice (19.87 ± 2.50%; P < 0.32). When spontaneous lymphocyte apoptosis was compared within genotypes, unsupplemented male Nr mice had a similar level of apoptotic lymphocytes compared to diet-supplemented male Nr mice (P < 0.73). Unsupplemented and diet-supplemented male Tg mice did not differ significantly (P > 0.11). There was also no significant difference between unsupplemented and diet-supplemented female Nr mice (P < 0.50) or unsupplemented and diet-supplemented female Tg mice (P < 0.62). There was no significant difference in spontaneous apoptosis between male and female mice in any experimental group (P > 0.09 in all cases).

Diet and radiation-induced apoptosis: dependence on gender and genotype

After an exposure of 2 Gy, there was no significant difference in the levels of apoptosis in lymphocytes from female mice in any group (Groups B, F, J and N) (Figure 4). Diet, genotype and gender did not appear to affect the radiation response of lymphocytes from females (P < 0.17). However, there were large differences in radiation sensitivity in lymphocytes from male mice (Groups D, H, L and P) depending on the genotype and if the diet supplement was given (Figure 4). In Nr male mice on the standard diet, lymphocyte sensitivity was statistically significantly higher than any female group (24.11 ± 0.54%; P < 0.010), but Tg males were nearly twice as sensitive as the Nr males (40.39 ± 4.19%; P < 0.00023). Lymphocytes from diet-supplemented Nr mice had lower levels of apoptosis, but the decrease was not statistically significant (17.97 ± 3.91%; P < 0.15). However, diet-supplemented Tg mice had a 68% reduction in radiation-induced apoptosis levels (13.14 ± 3.49%; P < 0.00054) compared to unsupplemented Tg mice (Figure 4).

ANOVA

ANOVA (Table II) determined that individually, the variables ‘gender’ and ‘supplementation’, but not ‘genotype’ significantly influenced the level of radiation-induced apoptosis. The interactive effect of variables genotype and ‘diet supplement’ also significantly influenced radiation-induced apoptosis. Significant interactions were also found for genotype, gender and diet supplement (Table II).

Table II. ANOVA was performed for radiation-induced apoptosis to determine which of the variables from the experimental groups influenced lymphocyte apoptosis in the mice

<table>
<thead>
<tr>
<th>Factor</th>
<th>Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>2.2442</td>
<td>0.14</td>
</tr>
<tr>
<td>Gender</td>
<td>30.0966</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Diet supplement</td>
<td>18.4215</td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Genotype × gender</td>
<td>0.6004</td>
<td>0.44</td>
</tr>
<tr>
<td>Genotype × supplement</td>
<td>3.1435</td>
<td>0.084</td>
</tr>
<tr>
<td>Gender × supplement</td>
<td>5.4542</td>
<td>0.025*</td>
</tr>
<tr>
<td>Genotype × gender × supplement</td>
<td>5.8634</td>
<td>0.020*</td>
</tr>
</tbody>
</table>

The symbol ‘*’ denotes variables that had a significant impact on apoptosis.
Discussion

Lympocytes are frequently used for studying the physiological impacts of radiation since immunocytes are relatively radiosensitive, readily accessible and relatively long lived. Lympocyte apoptosis is a well-established and relatively specific biomarker for radiation-induced damage and free radical impacts. There is great interest in developing interventions to prevent radiation damage to biological tissues (particularly ingestible substrates) but to date, there are few materials with exceptional efficacy (8–11).

Spontaneous apoptosis

Apoptosis was examined in Nr mice, which are assumed to have normal metabolism and free radical production representative of a typical mouse population, and Tg mice, which have substantially elevated levels of endogenous free radicals (19). We hypothesized that the apoptosis levels in Tg mice would be higher under normal growth conditions because of increased oxidative stress. However, although Tg mice had significantly higher endogenous ROS production, spontaneous levels of lymphocyte apoptosis were not altered and were essentially identical for all groups of mice; these results did not support our postulate that spontaneous apoptosis levels might be different in these two mouse strains. There may be several reasons for this, including the possibility that Tg cells were able to adapt to the effects of the chronically elevated ROS through up-regulation in cellular recycling and repair mechanisms and/or antioxidant systems (51) and cope with tissue culture stress like normal cells. The increased radiosensitivity of Tg mice indicates that these systems may be saturated and are unable to cope with the increased radiation-induced ROS. Alternatively, the elevated endogenous ROS production could suppress the ability of the Tg lymphocytes to undergo apoptosis since chronic low doses of ionizing radiation have been shown to suppress leukocyte apoptosis (52). Although the treatment used by Joksic and Pretrovic (52) involves radiation-induced generation of ROS, the outcome is likely to be similar to that in Tg cells with endogenous ROS. Either postulate could also be exacerbated by the known anti-apoptotic actions of GH and its downstream effector, insulin-like growth factor-I (IGF-I), both strongly up-regulated in Tg mice, which could increase the apoptotic threshold of Tg cells (53,54). Interestingly, all groups of female mice had similar levels of spontaneous apoptosis, which were lower than that of the male mice (data not shown), indicating that, regardless of the level of cellular oxidative damage, the anti-apoptotic actions of oestrogen and progesterone may play an important role in reducing lymphocyte apoptosis in these mice.

Radiation-induced apoptosis

Radiation-induced apoptosis differed significantly between groups of male mice. Unsupplemented Nr and Tg males had highly elevated apoptosis following irradiation, with unsupplemented Tg showing the greatest radiation sensitivity >1 Gy compared to any other group of mice (Figure 3). Consequently, our hypothesis of increased radiation sensitivity was confirmed, despite the lack of spontaneous/basal response observed in unirradiated cells. Although chronic low doses of ionizing radiation/free radicals can ameliorate leukocyte apoptosis (52), the mechanism probably involves adaptive up-regulation of stress-response systems against a background of otherwise normal endogenous ROS generation. This mechanism may be otherwise engaged in Tg mice, thus up-regulation of endogenous repair, replacement and defensive systems to offset elevated ROS processes in Tg mice is consistent with increased radiosensitivity if these systems are stressed to limited capacity. In that case, further ROS generated by radiation would overwhelm these protective capabilities. It is also likely that general levels of oxidative damage in Tg mice are higher (see above) before radiation exposure (18–20), so that further damage triggers apoptosis, even though the threshold is set higher by anti-apoptotic regulatory impacts of GH and IGF-I.

As might be expected from their low spontaneous levels of apoptosis in vitro, female mice demonstrated greater radioresistance than male mice at doses >2 Gy (Figure 3). There was also no significant difference in radiation-induced apoptosis between any groups of female mice. These results are supported by other studies which indicate that females of other mammalian species also show greater radioresistance than their male counterparts (53,54). Perhaps in females, the apoptotic threshold is higher such that radiation impacts are ameliorated or absorbed and are not significant enough to elicit the response. This might explain why the diet had no apparent benefit in females. We are currently assessing other biomarkers of ROS damage since it is possible that females do derive benefit from the diet, despite the fact that apoptosis does not respond. It is possible that the anti-apoptotic actions of oestrogen and progesterone (22,24,25,28,36) are reducing lymphocyte apoptosis in Nr females and could be interacting either additively or synergistically with the anti-apoptotic effects of GH/IGF-I (55–58) in female Tg mice, completely masking the effects of increased oxidative damage caused by radiation. This would likely work in combination with the antioxidant activity of oestrogen (40,41) which of itself could reduce oxidative damage in female mice, thereby reducing both spontaneous and radiation-induced apoptosis.

Impact of the dietary supplement

There is likely an imbalance in the two major subsets of antioxidants (endogenous versus nutritional) in cells experiencing chronic oxidative stress in Tg mice. Typically, there is an up-regulation of enzymatic antioxidants in response to the redox status of the cells (as long as sufficient substrates are available) (59), with a concomitant depletion of non-enzymatic antioxidants unless replenished from some external source (i.e., through diet) (60,61). The results for supplemented mice suggest that chronic oxidative stress in Tg cells has depleted non-enzymatic antioxidants, and possibly enzymatic antioxidants, although further study is required for confirmation. Radiation-induced apoptosis is significantly reduced in supplemented male mice and there was a trend (while not significant) for a reduction in lymphocyte apoptosis in supplemented female mice that were already resistant.

We speculate that the reduction in radiation-induced apoptosis could be due to two aspects of the dietary supplement:

(i) The first may be higher inter- and intracellular concentrations of enzymatic and non-enzymatic antioxidants that scavenge ROS before they can damage cellular macromolecules. The baseline level of damage to the cellular components of male Nr and Tg mice on the dietary supplement is likely to be reduced compared to unsupplemented mice since the increase in antioxidants provides a protective
effect from on-going cellular ROS production as well as for acute radiation insult (59). The supplement was designed to provide antioxidant protection to all critical cell components, including nuclear material, membranes, the cytosol and associated proteins and subcellular organelles (particularly the mitochondria), using compounds with documented specific protective effects for each of these subcellular regions (12). Reduction in the quantity of oxidatively modified cellular components allows recycling and repair processes to function more effectively, an issue particularly important in older organisms since these processes appear to be compromised in senescent animals (62–65).

(ii) The second effect may be an amelioration of processes associated with oxidative stress, which can further increase ROS production and cellular oxidative stress. These include disruption of mitochondrial metabolism and reduced electron transport chain substrate availability, resulting in reductions in ATP production (64,66), impaired glucose metabolism (67–69) and inflammatory processes typically associated with oxidative damage (70,71), which are normally exacerbated in senescent animals (64,72). The lower radiation-induced apoptosis in diet-supplemented Nr and Tg mice lend support to the idea that a broad-spectrum dietary supplement can provide a significant protective effect, even to cells near limiting capacity (Tg mice) or exposed to an acute increase in ROS damage due to radiation exposure. While it is unknown if some factors provide greater protective effects than others, it has been established that several of the components of the supplement act additively or synergistically (73–75). It is also likely that the immediate free radical scavenging effects of some of the components and the factors that provide additional support for the processes associated with oxidative stress act synergistically to enhance the supplement’s overall protective effect.

Time course studies showed that the kinetics of lymphocyte apoptosis among all groups was similar despite GH levels, gender or diet supplementation. Only the magnitude of the response varied; we postulate that this indicates that overall initial damage levels must be altered in the various groups due to enhanced free radical scavenging and/or DNA repair capacity.

This study emphasizes the importance of genotype, gender and diet on the modulation of radiation response. The data support the contention that male Tg mice provide an excellent model to study the effects of long-term oxidative stress and its effects on radiation response in male mice only. There is a relative paucity of studies addressing gender differences in radiation biology. The striking sexual dichotomy in apoptotic response indicates female mice may prove to be an interesting model for radiation risk modification associated with apoptotic mechanisms. Although results vary, males and females generally acquire oxidative damage at the same rate (76–78). Unsupplemented Tg mice (both genders) accumulate greater oxidative damage to cellular components than age-matched unsupplemented normals (19), which would imply that Tg mice are less able to adequately respond to additional oxidative stress from external sources (i.e. ionizing radiation) and more importantly, from the escalating ROS production normally associated with ageing (2,4,10,72). However, cells from female mice (Nr and Tg) appear to have the capacity to cope with greater amounts of oxidative damage before apoptotic processes are triggered. The fact that females of both genotypes are similar suggests that the regulation of the threshold in females differs from males, and was not affected by GH transgenesis or background ROS processes implying that oestrogen may have a priority role in determining an apoptotic threshold over other factors.

The greater overall resistance to radiation-induced apoptosis demonstrated by female mice requires further study to determine what processes (i.e. involving sex hormones or other factors) are contributing to this increased resistance. This might be extended to considerations of dwarf (GH deficient) mice that were recently shown to express increased resistance to free radical stress than normal animals (79,80).

Conclusion

Regardless of genetic factors that influence radiation-induced apoptosis (i.e. Nr versus Tg mice), we have shown that diet can change the probability of a cell undergoing radiation-induced apoptosis. Since apoptosis is regarded as an essential mechanism associated with genomic instability, dietary supplements could have important ramifications for human diseases associated with apoptosis, such as ageing and cancer, and health risks from exposure to environmental mutagens and carcinogens. We have also demonstrated that gender plays a significant role in the modulation of apoptosis, indicating that the use of apoptosis for issues such as biodosimetry and risk assessment may be problematic if gender is not taken into account.

Funding

CANDU Owners Group (03.014), the Chemical Biological Radiological Nuclear Research and Technology Initiative (015ssH1021-021608) and the National Science and Engineering Research Council (238495).

Acknowledgements

Conflict of interest statement: None declared.

References

Factors effecting radiation-induced apoptosis


Received on December 4, 2007; revised on June 12, 2008; accepted on June 15, 2008