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Pomegranate intake protects against genomic instability induced by medical x-rays *in vivo* in mice

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Abstract

Ionizing radiation (IR) is a well-documented human carcinogen. The increased use of IR in medical procedures has doubled the annual radiation dose and may increase cancer risk. Genomic instability is an intermediate lesion in IR-induced cancer. We examined whether pomegranate extract (PE) suppresses genomic instability induced by x-rays. Mice were treated orally with PE and exposed to an x-ray dose of 2 Gy. PE intake suppressed x-ray-induced DNA double strand breaks (DSBs) in peripheral blood and chromosomal damage in bone marrow. We hypothesized that PE-mediated protection against x-ray-induced damage may be due to upregulation of DSB repair and antioxidant enzymes and/or increase in glutathione (GSH) levels. We found that expression of DSB repair genes was not altered (Nbs1 and Rad50) or was reduced (Mre11, DNA-PKcs, Ku80, Rad51, Rad52 and Brca2) in the liver of PE treated mice. Likewise, mRNA levels of antioxidant enzymes were reduced (Gpx1, Cat and Sod2) or were not altered (HO-1 and Sod1) as a function of PE treatment. In contrast, PE treated mice with and without IR exposure displayed higher hepatic GSH concentrations than controls. Thus, ingestion of pomegranate polyphenols is associated with inhibition of x-ray-induced genomic instability and elevated GSH, which may reduce cancer risk.

Introduction

Humans are exposed to ionizing radiation (IR) from a variety of natural and anthropogenic sources including environmental, occupational and medical exposures (1). The increased use of IR in diagnostic and therapeutic medical applications has doubled the annual radiation dose over the last two decades (1). Presently, half of the annual radiation dose to the American population is from x-ray and nuclear medicine procedures. IR is one of a few environmental agents that is a well-documented carcinogen in humans (2). The risk of cancer induction by IR depends on multiple factors including dose, dose-rate and quality of radiation, age and organ site (3). The increased risk of fatal IR-induced cancer for the

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general population following chronic exposure is about 5% per Sievert (Sv) of radiation dose (3). Radiation therapy increases the risks of a variety of second cancers such as leukemia, breast, prostate, lung and others. Children and young adults treated for cancer and long-term cancer survivors are at particularly high risk (4–7). In addition, IR exposure from diagnostic computed tomography scans in childhood or adolescence is associated with increased cancer risk (8–10).

Generation of reactive oxygen species (ROS) from radiolysis of water and the induction of oxidative damage is thought to be responsible for the adverse effects of IR, especially cell death, genomic instability and cancer (11, 12). Non-enzymatic antioxidants such as glutathione (GSH) and antioxidant enzymes such as glutathione peroxidase (GPX), cooper-zinc superoxide dismutase (SOD1), manganese superoxide dismutase (SOD2) and catalase (CAT) provide protection against IR-mediated damage (13, 14). The balance of antioxidant enzymes required for optimal protection is however unclear. In particular, an increase in SOD2 activity may have radiosensitizing rather than radioprotective effect, perhaps due to overproduction of hydrogen peroxide (15).

Dietary polyphenols including curcumin, genistein, naringin, grape seed and tea polyphenols are protective against IR in various model systems (12, 16, 17). However, radioprotection by pomegranate polyphenols has been investigated in only one study that was designed to examine IR-induced enteritis (18).

Pomegranates contain over 100 different phytochemicals, mainly ellagitannins (punicalagin isomers and punicalin) and their hydrolysis products (ellagic and gallic acids), gallotannins, and flavonoids (anthocyanins, flavonols and flavones) (19–21). Pomegranate juice (PJ) exhibits the highest *in vitro* antioxidant capacity compared to other polyphenol-rich beverages including red wine, grape juice, blueberry juice and green tea (22). The relevance of the antioxidant capacity measured *in vitro* should be interpreted with caution for several reasons. First, it is known that antioxidants exhibit pro-oxidant effects under some circumstances (12, 23, 24). Second, ellagitannins are not absorbed through the gastrointestinal tract but are hydrolyzed to ellagic acid and converted to urolithins, which are poor antioxidants (25–27). Ellagic acid mainly accumulates in the intestinal tract with limited absorption into the systemic circulation (28). Moreover, absorbed urolithins or free ellagic acid are rapidly conjugated in the liver and excreted into the urine resulting in low nanomolar range concentrations in plasma (26). Thus, the concentrations of circulating polyphenols and conjugates may be too low to significantly enhance the total plasma antioxidant capacity (29). Yet, intake of PJ reduces lipid and protein oxidation in human blood (30). Pomegranate extract (PE) reduces lipid peroxidation and protein oxidation in chemically-induced mouse models of liver carcinogenesis (31–33), attenuates UV-induced skin damage (34) and lessens IR-induced enteritis (18). These effects may be explained by the capability of polyphenols to upregulate carcinogen detoxifying enzymes, reduce inflammation and/or local effects on the intestinal tract rather than systemic distribution (31–34).

It is presently unknown whether pomegranate-derived phytochemicals protect against systemic genotoxicity induced by IR, and if so, what mechanisms may be involved.

Informed by the facts that oral ingestion of PJ and/or PE leads to limited absorption and rapid biotransformation of pomegranate polyphenols into phase II metabolites that are poor antioxidants, we hypothesized that if PE mitigates IR-induced genotoxicity, this would be due to the induction of endogenous defense mechanisms such as DNA repair and/or antioxidant responses rather than direct scavenging of IR-generated ROS. Thus, we examined whether PE intake leads to upregulation of DNA double strand break (DSB) repair and antioxidant enzymes and/or increase in GSH concentrations. We found that PE intake did not upregulate DSB and/or antioxidant enzymes but increased GSH levels.

Materials and methods

Pomegranate extract

PE is an aqueous polyphenol extract (POMx, POM Wonderful, Los Angeles, CA) prepared from pomegranates grown in California (Wonderful variety, Paramount farms, Lost Hills, CA, USA). PE consists of 95% glycone ellagitannins (mono and oligomeric) standardized to 37–40% punicalagin and 3.4% ellagic acid (26).

Mice and treatments

C57BL/6J p^{mn}/p^{mn} mice (Jackson Laboratory, Bar Harbor, ME) were housed in the virus-free animal facility at the University of Albany's Cancer Research Center and all procedures were approved by the institutional animal use and care committee. Six to 7 week-old mice were orally administered 32 mg/kg of PE (0.8 mg PE per 25 g animal) or vehicle (water) daily for 14 days and were sham or x-ray irradiated 1 h after the last dose. There were four groups of mice consisting of four mice in each group: 1) untreated mice, 2) PE treated mice, 3) untreated mice irradiated with x-rays and 4) PE treated mice irradiated with x-rays. The number of animals was determined from power and sample size calculations and constitute the minimum number of mice required to obtain statistically significant results at power >80% and $\alpha = 0.05$, assuming that a difference between means of PE treated versus untreated irradiated mice is ~50% and a standard deviation is ~25%. The PE dose was based on a previous study in which oral administration of PE inhibited the growth of tumor xenografts (35). Using body surface area normalization method for allometric dose translations (36) and under the assumption that PJ contains up to 2 g/L of ellagitannins such as punicalagin (19), this PE dose to a 70 kg human is equivalent to amount of ellagitannins present in 90 ml (3 oz.) of PJ. PE treated and control mice were exposed to 2 Gy x-rays in a Faxitron irradiator (Faxitron Bioptics LLC, Tucson, AZ) at 130kV and 2mA at a dose rate of 1Gy/min. Peripheral blood was collected into EDTA coated tubes (Braintree Scientific, Braintree, MA) via sub-mandibular vein bleeding using a 5 mm lancet (Braintree Scientific, Braintree, MA) at different time points after irradiation and used for determination of phosphorylated histone H2AX (γ -H2AX) foci and micronuclei, which represent DSBs and permanent chromosomal damage, respectively. DSBs were measured in peripheral blood leukocytes at 20 min and 24 h after irradiation to assess IR-induced DSB induction and repair, respectively. Micronuclei were measured in peripheral blood erythrocytes 24 h after irradiation. Micronuclei represent chromosomal damage (breaks and aneuploidy) that occurs in dividing erythroblasts and can be scored in peripheral blood since damaged erythroblasts complete division and maturation in the bone marrow and subsequently enter the systemic

circulation. The effect of PE alone was determined on independent cohorts of mice from which blood was collected 24 h after administration of the last PE dose. Animals were euthanized 24 h after PE dosing or 24 h after irradiation, and livers were harvested and processed in RNA later for quantitative real-time PCR analysis and flash frozen for GSH measurements, respectively.

Immunofluorescence of γ -H2AX

Fifty μ l of peripheral blood was incubated with erythrocyte lysis buffer (Qiagen, Germantown, MD) to eliminate red blood cells. Cells were suspended in 50 μ l of PBS and applied onto poly-D-lysine coated cover slips. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100 and washed with PBS as described previously (37). After blocking, cells were incubated with mouse anti-phospho-Histone2AX (Ser139) antibody clone JBW301 (EMD Millipore, Billerica, MA, Catalog #05-636) at a 1:400 dilution followed by incubation with FITC-conjugated anti-mouse IgG secondary antibody (Jackson Immunochemicals, West Grove, PA, Catalog #115-095-008) at a 1:200 dilution. Coverslips were mounted on microscopic slides with 5 μ l of DAPI/Vectashield solution (Vector Laboratories, Burlingame, CA). Slides were visualized under a 100X objective on a Nikon Eclipse TS100 microscope. A minimum of 100 cells were analyzed and cells with more than four distinct foci in the nucleus were considered positive for γ -H2AX foci formation.

In vivo micronucleus assay

Three microliters of peripheral blood was smeared on a microscopic glass slide. The cells were stained with Giemsa (Sigma-Aldrich, St. Louis, MO). Micronuclei were examined under a 100X objective on a Nikon Eclipse TS100 microscope. At least 2000 erythrocytes were scored and the frequency of micronuclei was calculated as the number of micronucleated erythrocytes per 2000 erythrocytes.

Quantitative real time PCR

Total RNA from livers was extracted with TRIzol® reagent (Thermo Fisher Scientific, Franklin, MA). Reverse transcription PCR reactions were performed with 1.5 μ g total RNA using MultiScribe™ Reverse Transcriptase (Thermo Fisher Scientific, Franklin, MA, Catalog #4311235) to synthesize cDNA. Quantitative PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, Catalog #172-5850) on the ABI7900 HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Relative expression levels of each gene were analyzed using the 2^{-CT} method and presented relative to the expression of the housekeeping gene GAPDH. Each sample was replicated twice from independent cDNA preparations. Primer information is provided below:

Gapdh (5' TCGTCCCGTAGACAAAATGG, 3' TTGAGGTCAATGAAGGGGTC)

Mre11 (5' CTACCAGGATGGCAATCTCAAC, 3' CTAATGTCAACCTTCTCCACCG)

Nbs1 (5' GAATACGAGCCCTTGTTGT, 3' TGGTGACTTTAACTGCCGAC)

Rad50 (5' ATTAAGCCTTGGAGAGCCG, 3' GCCGTGCTTCTTTATTCAGC)
 DNA-PK- α (5' AGACTGTTGGAGAGCAGGA, 3' TCCTGCTCTCCAACAGTCT)
 Ku80 (5' TTTGAGGAAGCGAGTCTCCA, 3' TTTCTCTCGAAGGGCTTCCA)
 Rad51 (5' TTCACGGTTAGAGCAGTGTG, 3' ACTCAGTTGCCGTGGTGAAA)
 Rad52 (5' AGTCTCCATTCTTCTGCGA, 3' AGGGAGGATGGTTGTCACAC)
 Brca2 (5' TTACCGAGCATCGGAGAAAT, 3' CAATTGAGGCTTATCGGTCC)
 Gpx1 (5' AAGGCTCACCCGCTCTTTA, 3' CCCACCAGGAAGTTCTCAAAG)
 Cat (5' CTTTGACAGAGAGCGGATTCC, 3' CTTTGACAGAGAGCGGATTCC)
 Sod1 (5' TGTGAGGAGACCTGGGCAAT, 3' ACTGCGCAATCCCAATCAC)
 Sod2 (5' TCAAGCGTGACTTTGGGTCT, 3' TGTTCTTGCAATGGGTCCT)

GSH determination and protein estimation

Liver homogenates (5% w/v) were prepared in PBS containing 1mM EDTA. The samples were de-proteinated using 5% meta-phosphoric acid (Sigma-Aldrich, St. Louis, MO). Fifty μ l of the sample was used for GSH determination using Bioxytech® GSH/GSSG-412 assay (Oxis International, Foster City, CA, Catalog #21040) according to the manufacturer's instructions. Oxidized GSSG was undetectable. Thus, only reduced GSH was measured. Protein levels in tissue homogenates were determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, Catalog #23227) according to manufacturer's instructions. GSH levels were normalized to protein levels.

Statistics

Data were analyzed by one-way ANOVA followed by the *post hoc* Tukey's Multiple Comparison test or Student's *t* test, as appropriate, using Graphpad Prism (Graphpad Software, San Diego, CA) and presented as means $\pm$ SEM. Differences between groups were considered statistically significant if *p* value was less than 0.05.

Results

PE reduces IR-induced DSBs

To determine whether PE intake protects against systemic genotoxicity induced by IR we determined DSBs in peripheral blood leukocytes by measuring γ -H2AX foci formation. γ -H2AX foci rapidly form after DSB induction and resolve after repair (38, 39). γ -H2AX foci were measured at 20 min and 24 h after irradiation to assess DSB formation and repair, respectively. PE treated mice displayed 2-fold fewer γ -H2AX foci than untreated controls at 20 min post-IR, indicating prevention of DSB formation following IR exposure (Fig. 1A). Both PE treated and untreated mice displayed similar levels of residual γ -H2AX foci at 24 h post-IR. The basal levels of γ -H2AX foci were slightly but not significantly higher in PE treated than untreated mice. Overall, these data demonstrate that PE consumption protects against IR-induced DSBs in the systemic circulation.

PE abolishes IR-induced chromosomal damage

To determine whether PE intake protects against permanent genome alterations we utilized the micronucleus assay, which detects chromosomal breaks and chromosome loss in dividing cells. Presence of micronuclei in peripheral blood erythrocytes reflect chromosomal damage that occurred during erythroblast development in bone marrow (40). We examined micronuclei in peripheral blood erythrocytes as a function of PE treatment, irradiation or a combination of PE treatment and irradiation. To allow for damaged erythroblasts to complete cell division and maturation and enter the systemic circulation, micronuclei were scored at 24 h post-IR. We found that PE treatment abrogated IR-induced micronucleus formation (Fig. 1B), indicating that PE consumption protects against genomic instability induced by IR.

PE modulates DNA repair genes

To examine whether PE prevented IR-induced genomic instability via upregulation of DNA repair we examined the effect of PE on expression of DSB repair genes. In mammalian cells, most DSBs are repaired by the nonhomologous end-joining (NHEJ) pathway, which is active during all cell cycle phases. DSBs that occur during S and G2 phases can also be repaired by homologous recombination (HR) (41–43). We examined genes that represent important enzymes of both NHEJ and HR pathways. DNA-PK catalytic subunit (DNA-PKcs) and Ku proteins form the DNA-PK enzyme that is involved in the first steps of NHEJ. Mre11, Rad50 and Nbs1 form the MRN complex that has a well-documented role in HR. In addition, the MRN complex, and Mre11 in particular, has a direct role in NHEJ (44). Rad51, Rad52 and Brca2 are major enzymes of the HR pathway. We found that PE intake reduced expression of Mre11, DNA-PKcs, Ku80, Rad51, Rad52 and Brca2 genes (Fig. 2A). Nbs1 and Rad50 were unaltered by PE treatment (data not shown). These data indicate that PE-mediated suppression of IR-induced DSBs and chromosomal breaks is not associated with upregulation of genes that encode DSB repair enzymes.

PE modulates antioxidant enzymes

To determine whether PE protects against IR-induced genomic instability through the induction of antioxidant enzymes we examined the expression of genes that encode the major ROS metabolizing enzymes Gpx1, Cat, Sod1 and Sod2 and the oxidative-stress responsive enzyme heme oxygenase-1 (HO-1). We found that expression of Gpx1, Cat and Sod2 was reduced in PE treated mice (Fig. 2B), while expression of Sod1 and HO-1 did not change (data not shown). This suggests that mechanisms other than transcriptional upregulation of antioxidant enzymes are responsible for PE-mediated protection against IR-induced genome damage.

PE modulates GSH levels

GSH is the most abundant non-enzymatic intracellular antioxidant (45). We therefore determined whether PE-mediated radioprotection is associated with changes in cellular GSH content. We found that PE treatment increased hepatic GSH levels by 30% (Fig. 3). In addition, PE attenuated GSH depletion following irradiation, resulting in a more than 50% difference in GSH levels between PE treated and untreated irradiated mice (Fig. 3). These

data suggest that inhibition of IR-induced genotoxicity in PE treated mice may be mediated by increased GSH content.

Discussion

This study demonstrated that PE consumption prevents IR-induced DNA and chromosomal damage, providing the first evidence that PE intake may protect against cancer development associated with IR exposure. The PE dose used contains the equivalent ellagitannin content in 90 ml of PJ and thus represents a physiologically relevant dose. Of particular interest is determination of the mechanisms by which PE ingestion provides protection against IR-induced genomic instability. We postulated that activation of DNA repair and antioxidant responses may be responsible for the observed effects of PE.

It has been suggested that dietary polyphenols contribute to cancer prevention through upregulation of DNA repair (29, 46). In support of this notion, intake of ellagic acid, a constituent of PE, upregulated nucleotide excision repair genes in mouse liver (47). However, the same study also reported downregulation of the DSB repair gene Brca2. Overall, there is limited evidence as to whether dietary polyphenols modulate DNA repair genes and further studies are needed to understand their impact, if any, on DNA repair processes. In our study, PE intake had no effect or resulted in a moderate (~2-fold) reduction in the expression of genes associated with the NHEJ and HR pathways of DSB repair. The outcomes of these gene expression changes are currently unclear. It is important to note, however, that downregulation of NHEJ genes by PE may reduce genomic instability, especially, in the context of IR exposure. It has been well-documented that NHEJ enzymes can generate aberrant genome rearrangements. Small deletions are usually introduced during the NHEJ repair process that often involves degradation of a few terminal nucleotides prior to end ligation (41, 42). In addition, chromosomal translocations occur, when NHEJ enzymes incorrectly rejoin two DSBs that are present on different chromosomes (48). In contrast, deficiency in NHEJ genes results in reduced frequency of IR-induced genome rearrangements (49). Thus, downregulation of NHEJ genes by PE may reduce the frequency of genome rearrangements following IR exposure. A complementary DSB repair pathway, HR, is a high fidelity repair pathway that uses an undamaged DNA template on the sister chromatid or homologous chromosome to repair the break (43). However, HR can result in genome rearrangements, if recombination occurs at non-allelic homologous sequences such as repeated sequences (50). Whether downregulation of HR genes affects normal tissues is unclear given that HR is only active during the S and G2 phases, while most cells in an adult organism are quiescent and, in addition, somatic cells utilize NHEJ as the major DSB repair pathway irrespective of the cell cycle phase (43, 51).

Upregulation of antioxidant genes may serve as a cellular defense mechanism against IR-generated ROS and resultant genomic instability. In our study, PE intake did not upregulate enzymes involved in ROS metabolism but instead downregulated some of these genes. These data suggest that PE intake alters the cellular redox status to a more reducing environment, modulating expression of ROS scavenging genes. These findings are compatible with the reported decreased activities of the same enzymes (GPX, CAT and SOD) in mice treated with PJ (52). Similarly, decreased SOD1 and SOD2 activities were reported in PE treated

HepG2 cells (24). In contrast, the same study reported an increased CAT activity, while GPX activity did not change (24). Mixed results on CAT activity were obtained from human studies on PJ (30, 53). Overall, findings from our current study and other studies do not support the notion that pomegranate intake leads to upregulation of ROS scavenging enzymes.

We postulated that modulation of intracellular GSH levels is a possible mechanism of action through which PE confers protection against IR-induced genome damage. GSH is the most abundant intracellular thiol that acts as a direct ROS scavenger and cofactor to antioxidant and detoxification enzymes (45). We found that hepatic GSH levels increased following PE intake. This observation is consistent with elevated GSH blood levels in humans and rats following PJ intake, monocyte-derived macrophages from diabetes patients consuming PJ and macrophages from atherosclerotic mice treated with PE (30, 54–56). The molecular mechanism of PE-mediated increases in GSH levels is unknown but at least four complementary mechanisms can be postulated, including increased *de novo* GSH synthesis, enhanced enzymatic reduction of glutathione disulfide (GSSG) to GSH, increased protein deglutathionylation and sparing of GSH by pomegranate polyphenols in antioxidant defenses. In support of the first postulated mechanism, some plant polyphenols were shown to upregulate γ -glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, leading to increased GSH levels (57). Enzymatic reduction of GSSG may produce some free GSH but is unlikely to result in a significant increase because intracellular GSSG levels relative to GSH are typically low. In addition, activity of the enzyme responsible for this reduction, GSH reductase, was shown to be reduced in PJ treated mice (52). GSH can bind to proteins under normal conditions and mild oxidative and nitrosative stress and form mixed disulfides with the protein sulfhydryl groups through a reversible process of S-glutathionylation (14). GSH can be removed from mixed disulfides through enzymatic or non-enzymatic deglutathionylation, which occurs when the environment becomes more reducing. Treatment of cells with thiols causes an increase in the free GSH content by triggering deglutathionylation (14). Diabetes patients who consumed PJ displayed increased serum levels of sulfhydryl groups that correlated with increased GSH in monocyte-derived macrophages but whether the increase in GSH was due to protein deglutathionylation is unknown (55). Lastly, polyphenols from PE may substitute for GSH in antioxidant defenses and thereby increase its concentration (30). This however contradicts with the data from bioavailability studies reporting low plasma concentrations of free ellagic acid following PJ intake (25–27). However, the total amount of all absorbed pomegranate phytochemicals is likely to be underestimated given their diversity, complexity and lack of commercially available standards (58).

In summary, our data demonstrate that PE intake protects against IR-induced genomic instability and thereby may provide a protection against IR-associated cancer. An increase in GSH levels may be responsible for the observed radioprotective effects of PE.

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Fig. 1. PE protects against IR-induced DSBs and chromosomal damage

A) Effect of PE on DSBs. B) Effect of PE on chromosomal damage. DSBs were measured as γ -H2AX foci formation in peripheral blood leukocytes. Chromosomal damage was measured as micronucleus formation in peripheral blood erythrocytes. γ -H2AX foci were measured 20 min post-IR to assess DSB formation and 24 h post-IR to assess DSB repair. Micronuclei reflect permanent chromosomal damage and were measured 24 h post-IR. Filled bars show PE-treated mice and open bars show respective untreated controls, mean $\pm$ SEM is shown, n = 4 mice/group, *, p < 0.05, **, p < 0.01, ***, p < 0.001 compared to untreated mice or as indicated by solid lines.

Fig. 2. PE modulates expression of DSB repair and antioxidant genes

A) Effect of PE on expression of DSB repair genes. B) Effect of PE on expression of antioxidant genes. Gene expression was analyzed by qPCR of liver RNA isolated from untreated and PE treated mice. Data was normalized to GAPDH expression. Relative fold change is shown as mean $\pm$ SEM, n = 4 – 5 mice/group, *, p < 0.05, **, p < 0.01 compared to untreated mice.

Fig. 3. PE modulates GSH levels

Hepatic GSH levels were determined in response to PE treatment, irradiation and a combination of PE treatment plus irradiation. GSH levels were normalized to protein levels. Mean $\pm$ SEM is shown, $n = 4 - 5$ mice/group. Stars above solid line (**, $p < 0.01$) indicate comparison between PE treated irradiated and untreated irradiated mice.