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Selenium Deficiency Abrogates Inflammation-Dependent Plasma Cell Tumors in Mice

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ABSTRACT

The role of the micronutrient, selenium, in human cancers associated with chronic inflammations and persistent infections is poorly understood. Peritoneal plasmacytomas (PCTs) in strain BALB/c (C), the premier experimental model of inflammation-dependent plasma cell transformation in mice, may afford an opportunity to gain additional insights into the significance of selenium in neoplastic development. Here, we report that selenium-depleted C mice ($n = 32$) maintained on a torula-based low-selenium diet (5–8 μg of selenium/kg) were totally refractory to pristane induction of PCT. In contrast, 11 of 26 (42.3%) control mice maintained on a selenium adequate torula diet (300 μg of selenium/kg) and 15 of 40 (37.5%) control mice fed standard Purina chow (440 μg of selenium/kg) developed PCT by 275 days postpristane. Abrogation of PCT was caused in part by the striking inhibition of the formation of the inflammatory tissue in which PCT develop (pristane granuloma). This was associated with the reduced responsiveness of selenium-deficient inflammatory cells (monocytes and neutrophils) to chemoattractants, such as thioredoxin and chemokines. Selenium-deficient C mice exhibited little evidence of disturbed redox homeostasis and increased mutant frequency of a transgenic *lacZ* reporter gene *in vivo*. These findings implicate selenium, via the selenoproteins, in the promotion of inflammation-induced PCT and suggest that small drug inhibitors of selenoproteins might be useful for preventing human cancers linked with chronic inflammations and persistent infections.

INTRODUCTION

Elucidating the mechanisms by which chronic inflammation facilitates tumor development may be of great significance for human cancer prevention. Human malignancies associated with chronic inflammation include colorectal cancer consequent to Crohn's disease or ulcerative colitis, esophageal carcinoma (reflux oesophagitis and Barrett's esophagus), bronchial carcinoma (smoking and silica), mesothelioma (asbestos), and mucosa-associated lymphoid tissue (MALT) lymphoma (Sjögren syndrome and Hashimoto's thyroiditis). Human cancer can also be initiated by persistent infections that lead to chronic inflammatory processes with the potential to promote neoplastic development. Infectious agents whose causal relationship with chronic inflammation and cancer is well established encompass viruses (hepatitis B and C viruses, hepatocellular carcinoma; papilloma viruses, cervical carcinoma), bacteria (*Helicobacter pylori*, gastric adenocarcinoma and MALT), and parasites (schistosomiasis, bladder cancer; liver flukes, cholangiosarcoma). It has been estimated that approximately 15% of malignancies worldwide can be attributed to chronic infections (1).

Although the relationship between chronic inflammation and carcinogenesis is poorly understood, some general principles have emerged. Inflammatory cells generate trophic factors that can be exploited by the incipient tumor cells. One example is proinflammatory cytokines of the tumor necrosis factor-α family, which play an important role in early tumor development by regulating a cascade of cytokines, adhesion molecules, metalloproteinases, and proangiogenic factors (2). Chronic inflammation also activates the arachidonic acid metabolism, which generates tumor-promoting compounds through the cyclooxygenase (prostaglandins) and lipoxygenase (leukotriens; Ref. 3) pathways. The importance of cyclooxygenase activity for neoplastic progression is illustrated by the reduced risk of colon cancer in long-term users of nonsteroidal anti-inflammatory drugs (4). Another link between inflammation and tumor development is chemokines, which not only recruit leukocytes to inflammatory sites *in situ* but also exert powerful stimulatory effects on tumor growth, angiogenesis, and metastasis. Inflammatory cells can further promote neoplasia by inducing mutations in tumor precursors. Activated phagocytes are able to inflict DNA damage in neighboring cells by releasing reactive oxygen and nitrogen species (5), which can result in "oxyradical overload" (6).

One aspect of inflammation-dependent tumors that has received little attention thus far is micronutrients, such as selenium. Selenium is an essential trace element for all mammals, including humans. It is incorporated as selenocysteine, the 21st amino acid (7), into 25 selenoproteins that comprise the human selenoproteome (8). Although selenium's role in tumor development is complex, its beneficial reduction of cancer incidence and mortality (9), particularly prostate cancer (10), is most well established. Mechanisms by which selenium inhibits cancer include: (*a*) the antioxidant activity of selenoproteins, such as glutathione peroxidase and thioredoxin reductase (TR); (*b*) anti-inflammatory effects derived from interactions with the immune system and cyclooxygenase–lipoxygenase pathway; and (*c*) global gene expression changes, which can block cell cycle progression or induce apoptosis in both tumor precursors and stromal (inflammatory) bystander cells (11). Possible additional tumor-inhibiting effects of selenium are unspecific cytotoxicity (interference with sulfur metabolism), alteration of DNA methylation and polyamine synthesis, and inhibition of DNA replication (12). Generally less well known is that selenium, via the selenoproteins, can also promote tumor development. The clearest example is TR, a family of selenoproteins that maintains thioredoxin in the reduced state (TRX). TRX acts as an autocrine growth factor in tumor cells (13, 14), which often contain elevated levels of TRX (15). In fact, the TR/TRX system may have a dual function in oncogenesis because its tumor-promoting effects (stimulation of cell growth) may be counterbalanced by its tumor-suppressing effects (protection of cells from oxidative damage). The net effect of selenium's positive, negative, and sometimes opposing influences on inflammation-induced tumors may vary according to the specific circumstances.

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Peritoneal plasmacytomagenesis in BALB/c (C) mice, a widely known model of inflammation-dependent plasma cell transformation (16), may further our understanding of the significance of selenium in neoplastic development. We thus generated selenium-deficient (SD) C mice to evaluate the role of selenium on plasmacytoma (PCT) induction by chronic peritoneal inflammation (17). We hypothesized that if the reduction of selenoprotein-dependent antioxidant defense resulted in elevated oxidative mutagenesis in PCT precursors, selenium deficiency may accelerate PCT (18–20). On the other hand, if selenoproteins were essential for promoting the malignant transformation of plasma cells, selenium deficiency may inhibit PCT. We found that SD C mice were totally refractory to PCT. Abrogation of tumor development was caused, in part, by a striking inhibition of the formation of the inflammatory tissue in which PCT develop (pristane granuloma). This was associated with the reduced responsiveness of SD inflammatory cells to chemoattractants, such as TRX and chemokines. These findings implicated selenoproteins in the pathogenesis of inflammation-induced PCT and suggested that small drug inhibitors of selenoproteins might be useful for preventing human cancers associated with chronic inflammation or persistent infection.

MATERIALS AND METHODS

Determination of Selenium Content and Dietary Depletion of Selenium. Selenium levels in tissue and serum specimens of C mice were determined by neutron activation analysis as described previously (21). Among a variety of methods for measuring selenium in biological samples (*e.g.*, enzymatic activity determinations of selenoproteins, radioisotope-induced X-ray fluorescence, fluorometry, and atomic absorption spectroscopy), neutron activation analysis affords the determination of low-selenium concentrations with unsurpassed accuracy. Mice were depleted of selenium by maintenance on a commercially available (ICN Biomedicals, Inc., Aurora, OH) torula yeast diet with a selenium content between 5 and 8 μg/kg (SD diet). The diet contained sucrose, lard and minerals, and was supplemented with zinc and vitamins (22). Mice in the control group were maintained on the same diet, except it was supplemented with 300 μg/kg Na_2SeO_3 [selenium adequate (SA) diet]. Mice in another control group were fed standard Purina chow (Pu diet), which contained 440 μg/kg selenium by neutron activation analysis. The artificial diets were shaped and colored differently from Pu chow to avoid accidental mix-up in the mouse facility. Selenium depletion in mice was propagated by brother-and-sister mating of SD males and females. Fertility was normal in three consecutive generations of low-selenium mice (F1–3 SD mice). At F4, male fertility declined sharply, so that only a few F5 offspring were born. F5 males were infertile, which terminated this study. All mice were maintained in our conventional mouse colony at the National Cancer Institute under Animal Study Protocol LG-020.

Mice. This study used inbred BALB/cAnPt mice (C) and a closely related congenic strain, C.pUR288, which was developed to determine *in vivo* mutant frequencies on the genetic background of C. Strain C.pUR288 was generated by transferring the pUR288 transgene residing on Chr 3 (23) from C57BL/6 (B6) to C, using speed backcrossing. The original B6 mice carrying pUR288 (line 60) were developed by Dr. Jan Vijg, University of Texas, San Antonio. These mice harbor two virtually identical copies of the pUR288 transgene on Chrs 3 and 4. Both copies consist of ~10 individual *pUR288* genes. The chromosomal integration site of the transgene residing on Chr 4 happens to be in a region that contains allelomorphic variants of tumor modifier genes conferring susceptibility to PCT in strain C but resistance to PCT in strain B6 (24). To avoid the risk of replacing the susceptibility alleles of C with resistance alleles from B6, the pUR288 transgene on Chr 4 was not transferred. The intentional loss of one copy of the transgene resulted in the deterioration of the pUR288/lacZ assay by ~50%. Only half the number of pUR288 plasmids was excised from genomic DNA of C.pUR288 mice relative to parental B6.pUR288 mice. The speed backcross protocol for the pUR288 transgene on Chr 3 combined the genotyping for pUR288 (PCR assay) with the monitoring of the transmission of paternal chromosomes by means of simple sequence length polymorphic markers. Simple sequence length polymorphisms were identified by PCR using commercially available primer pairs (Research Genetics, Huntsville, AL). Eighty allelomorphic differences of strains C and B6 covering the centromeric, central, and telomeric portions of all autosomes and Chr X were screened to select the most appropriate breeders for the next generation backcross. At backcross generation N_7, C.pUR288 mice were found to carry B6 alleles only in the 5′ and 3′ flanks of the pUR288 transgene on Chr 3.

Induction of PCTs. PCT were induced in SD 10-week-old C.pUR288 F3 mice by three i.p. injections of 0.5-ml pristane spaced 2 months apart (17). The mice tolerated the treatment with pristane well and showed no adverse health effects. Age-matched C.pUR288 mice maintained on SA or Pu diet were used as controls to evaluate the impact of selenium status on PCT. Inbred C mice on Pu diet served as an additional control to demonstrate that strains C.pUR288 and C exhibit the same susceptibility to PCT. Beginning 1 week after the third injection of pristane, the mice were monitored for tumor development by microscopic examination of cytofuge specimens of ascites cells stained according to Wright's Giemsa. Ascites samples (~50 μl) were obtained by inserting a 16-gauge hypodermic needle into the peritoneal cavity. The presence of $\geq$10, large, hyperchromatic, and atypical plasma cells was indicative of incipient PCT. The mice were scored as tumor positive when the follow-up cytofuge specimen confirmed the presence of PCT cells. In most cases, this specimen contained >100 malignant plasma cells, reflecting the rapid progression of PCT. Mice with advanced PCT were sacrificed to obtain tumor material for histological confirmation of the tumor diagnosis. An attempt to induce PCT in SD mice at F4 had to be terminated for ethical reasons shortly after the first injection of pristane. The mice hyperventilated, had a scrubby coat, and exhibited other symptoms of acute disease. They were sacrificed according to National Cancer Institute guidelines.

GSH Peroxidase, Catalase, and GSH. GSH, GSH peroxidase (GPox, EC 1.11.1.9), and catalase (EC 1.11.1.6) were measured using the Bioxytech colorimetric assays GSH/oxidized GSH (GSSG)-412, GPx-340, and Catalase-520 from Oxis Research (Portland, OR) according to the manufacturer's protocol. The GSH assay included the determination of total GSH, GSSG, and reduced GSH, but it did not include the protein-bound disulfides of GSH (25). Protein samples were prepared by sonicating lymphocytes or peritoneal exudate cell (PEC) on ice (20 s, 60 W) followed by centrifugation (14,000 $\times$ *g*, 10 min, 4°C) to obtain clear supernatant. Protein content was determined with the BCA kit from Pierce (Rockford, IL) using BSA as standard.

Mutant Frequency (MF) in a lacZ Reporter Gene. The pUR288 *in vivo* mutagenesis assay (23, 26) was performed using the commercial kit, RK-16, from Leven, Inc. (Bogart, GA). Genomic DNA (15 μg) was digested with *Hind*III (1 h, 8 37°C) in the presence of magnetic beads (Dynal, Oslo, Norway) precoated with a LacI/LacZ fusion protein. The fusion protein captured the pUR288 plasmids released from genomic DNA. Unbound mouse DNA was removed from the plasmid-enriched bead fraction by three washing steps on a magnetic stand. Linearized plasmid was eluted from beads by the addition of isopropyl β-D-thiogalactoside, circularized with T4 DNA ligase, and electroporated into *Escherichia coli* deficient in β-galactosidase (Δ*lacZ*) and galactose epimerase (*galE*$^-$). The transformed bacteria were plated on agar supplemented with phenyl β-D-galactoside, which selected for β-Gal-deficient (*lacZ*$^-$) colonies. A 2-μl aliquot of the transformed cells (one-1000th of the total volume) was plated on agarose that contained 5-bromo-4-chloro-3-indolyl β-D-galactoside for enumeration of β-Gal-proficient (*lacZ*$^+$), wild-type colonies. After incubation for 15 h at 37°C, the MF was determined as the ratio of mutant colonies to wild-type colonies; *i.e.*, the number of colonies on the phenyl β-D-galactoside selection plate divided by the number of colonies on the 5-bromo-4-chloro-3-indolyl β-D-galactoside titer plate multiplied by 1000 (27, 28).

PEC Differentials. PECs were elicited with pristane, enumerated in a Neubauer hemocytometer, and differentiated into macrophages, neutrophils, and lymphocytes by microscopic examination of cytofuge specimens (Cytofuge 3, Shendon) stained with Diff-Quick (Dade Behring, Deerfield, IL).

Chemotactic Activity of Monocytes, Polymorphnuclear Neutrophils (PMNs), and Lymphocytes. Splenic leukocytes were fractionated into nonadherent (mainly lymphocytes) and adherent (mainly monocytes) cells by short-term culture (~3 h) *in vitro*. PMNs were obtained from pristane-elicited PECs using lymphocyte separation medium (62 grams/liter Ficoll and 94 grams/liter sodium diatrizoate) according to the manufacturer's protocol (ICN Biomedicals). For migration analysis, cells were resuspended in RPMI 1640 supplemented with 1% BSA (A4503; Sigma) and 25 mM HEPES (pH 8.0) and

pipetted into the top wells of a micro-Boyden chamber. Cell density was 1×10^6 cells/ml for monocytes and PMN and 5×10^6 cells/ml for lymphocytes. Chemoattractants were pipetted in the bottom wells of the chamber. Top and bottom wells were separated by a polyvinyl pyrrolidone-free micropore membrane (5 μm) that was treated (only in the case of lymphocytes) with 6.7 μg/ml fibronectin (F1141; Sigma). Chemotaxis chambers were incubated in a humidified CO_2 incubator for 1.5 h (monocytes and PMN) or 3 h (lymphocytes). Cells that migrated through the membrane were stained with H&E and counted under oil immersion using an Olympus microscope. The ratio of cells that traversed the membrane in presence or absence of a chemoattractant defined the chemotactic index, a dimensionless value that ranged from 1 (no response to chemoattractant) to 10 (10-fold increase in migration in response to chemoattractant).

RESULTS

Dietary Deprivation of Selenium in Four Generations of Mice. Neutron activation analysis was used to monitor the depletion of selenium in C mice maintained on SD diet (Fig. 1*A*). Liver was chosen for the analysis because it receives small molecular weight selenium compounds from the intestine, removes selenium from the dietary form (selenomethionine) via the *trans*-sulfuration pathway (29), and also produces the bulk of excretory metabolites of selenium to prevent the accumulation of toxic levels of selenium. The liver thus plays a central role in selenium metabolism. Although the mean selenium content of liver in SA parental (P) mice and three consecutive filial generations (F1-F3) of SA mice was comparable (Fig. 1*A*, *left*), F1 offspring of SD parental mice exhibited a significant drop in liver selenium from 4.8 $\pm$ 0.3 to 0.235 $\pm$ 0.05 μg/grams, a reduction by 95.1%. Similarly low-selenium concentrations were found in SD liver at F2 and F3 (Fig. 1*A*, *right*). These findings demonstrated that maintenance on SD diet caused a severe reduction of liver selenium content in C mice.

Selenium Distribution Pattern in SD Mice. To measure the impact of dietary selenium deprivation on selenium content in different tissues, the selenium in serum, liver, spleen, mesenteric lymph node (MLN), and testis was compared in F3 mice on SD diet and SA diet (Fig. 1*B*). Although the selenium content in SA tissues varied according to the known selenium distribution in rodents (Ref. 30; Fig. 1*B*, *left*), the distribution pattern of selenium was strikingly different in SD tissues (Fig. 1*B*, *right*). Serum and liver from SD mice exhibited the sharpest drop in selenium content, retaining only 11.4 and 8.6%, respectively, of selenium in SA samples, whereas lymphoid tissues, spleen, and MLN retained approximately half the selenium present in SA controls. The striking exception was testis, which in SD mice contained ~90% of the selenium found in SA mice. This is consistent with the hierarchy of selenium distribution in periods of insufficient selenium supply (31) and the ability of testis to retain selenium very efficiently in such periods (31, 32). Spermatogenesis is strictly dependent on selenium (33–35). These findings demonstrated that dietary selenium deprivation resulted in a severe net loss of selenium from liver and serum, two important indicators of selenium depletion. Apparently, selenium depletion triggered the redistribution of selenium not only to central nervous, endocrine, and reproductive tissues (31) but also to lymphoid tissues essential for immune function (36).

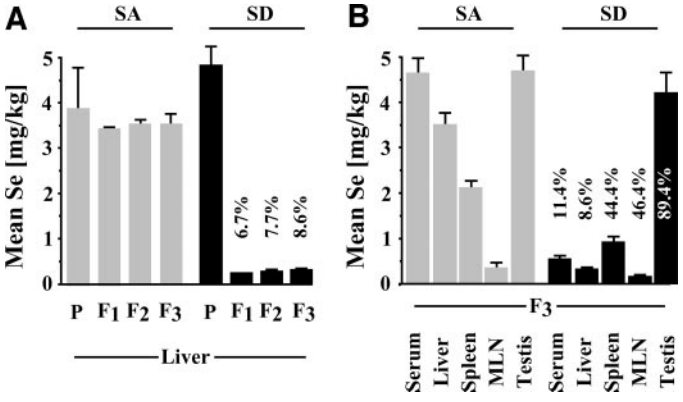

Fig. 1. Selenium deprivation of C.pUR288 mice. *A*, liver selenium in mice maintained on selenium adequate (*SA*; *gray bars*) or selenium-deficient (*SD*; *black bars*) diet. Mean values and SDs of the mean (indicated by *vertical lines above* the *columns*) are plotted. The P, F1, and F2 groups consisted of 2 SA mice and SD mice each. The F3 group contained 8 SA and SD mice. The percentage values *above* the *black bars* denote the residual selenium level in SD F1–3 mice relative to the selenium level in SA F3 mice (100%). *B*, mean selenium content in four tissues and serum in F3 males on SA (*gray bars*) or SD (*black bars*) diet. Each *bar*, the average of 8 mice. The percentage values *above* or within the *black bars* denote the residual selenium levels in SD samples compared with the corresponding SA samples.

Tolerance of C Mice to Selenium Deficiency. Chronic selenium deficiency in mammals leads to characteristic gross pathological and histopathological changes. To evaluate whether such changes occurred in SD C mice, SD F3 mice were necropsied, followed by histological examination of a representative tissue panel. The average body weight of 3-month-old SD mice (27.6 $\pm$ 3.13 grams) was not different from age-matched SA mice (25.8 $\pm$ 1.74 grams). Average organ weights, including spleen (105 $\pm$ 20.7 mg in SD mice *versus* 93.5 $\pm$ 21.8 mg in SA mice), testis (193 $\pm$ 4.5 mg in SD mice *versus* 202 $\pm$ 11.3 mg in SA mice), kidney, brain, and liver (data not shown) were unchanged by Student's *t* test. The ratio of brain:testis weight, a useful parameter for identifying small SD-induced decreases in testis size, was also comparable (2.27 $\pm$ 0.0814 in SD mice *versus* 2.24 $\pm$ 0.0587 in SA mice). In agreement with the unchanged testis size, young SD males at F3 were as fertile as their SA counterparts (pedigree records not shown). This defined a difference to male rats and ICR mice, which lose fertility because of sperm immobility and malformations at F1 or F2 (33, 37). Male Swiss Webster mice may be even more sensitive to selenium deprivation, because a brief 5-week maintenance on a low-selenium diet caused significant alterations of sperm morphology (37).

In contrast to younger SD F3 mice, which exhibited moderate histopathological changes consistent with selenium deficiency (Supplemental Table 1) but few gross pathological changes, SD F3 mice at $\geq$12 months of age showed clear signs of chronic selenium deprivation. Gross pathological changes included scrubby coat, skin lesions, joint swelling, and heart dilation. Corresponding histopathological alterations, which seemed to affect liver and other low-selenium retention tissues more severely, are listed in Supplementary Table 1. These findings indicated that aging (duration of selenium withdrawal) augments selenium deficiency disease. Consistent with the essential role of selenium in male fertility (35, 38) and efficiency with which C males retained testicular selenium (Fig. 1*B*), C males maintained fertility in the face of long-term selenium deprivation remarkably well. The genetic basis for this phenotype is not known.

Abrogation of Inflammation-Induced PCTs in SD Mice. PCT development in genetically susceptible C mice is strictly dependent on chronic peritoneal inflammation. The prominent role of selenoproteins in sustaining chronic inflammatory processes suggested that SD mice might exhibit a reduced incidence and/or delayed onset of pristane-induced PCT relative to selenium-proficient mice. To evaluate this, 32 SD C.pUR288 mice at F3 were treated with three intraperitonel injections of pristane spaced 2 months apart. No PCTs were observed by day 275 after the first injection of pristane (Fig. 2). In contrast to SD mice, 11 of 26 (42.3%) C.pUR288 F3 mice maintained on the SA diet developed PCT with a mean tumor latency of 197 $\pm$ 26.7 days. Tumor incidence and onset in these mice were comparable with that in pristane-treated C.pUR288 mice fed Pu chow (8 of 20, 40%; 180 $\pm$ 43.3 days) and inbred C mice fed Pu chow (7 of 20, 35%;

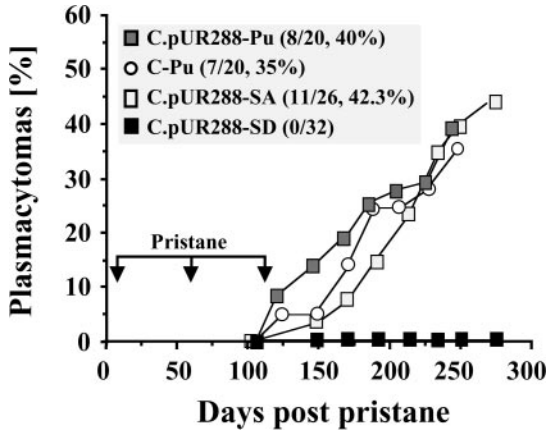

Fig. 2. Abrogation of inflammation-induced plasmacytomas in selenium-depleted mice. Plotted is the cumulative incidence of plasmacytoma in C.pUR288 and C mice maintained on torula-based selenium-deficient (*SD*) or selenium adequate (*SA*) diets or standard Purina chow (*Pu*) diet. Tumors were induced by three i.p. injections of pristane given on days 1, 60, and 120 (indicated by *arrows* pointing down). Plasmacytoma incidence on day 275 after the first injection of pristane is denoted for each group of mice in the *inset* (*top left*). The C.pUR288 mice on the SA or SD diets were from the F3 litter, whose tissue selenium levels are depicted in Fig. 1*B*.

182 ± 37.3 days). These observations demonstrated that neither genetic differences between strains C.pUR288 and C, nor differences in the basic composition of SA diets (torula-based SA diet *versus* Pu chow diet), influenced tumorigenesis. Instead, the crucial parameter responsible for abrogation of peritoneal plasmacytomagenesis was selenium deficiency.

Biochemical Indications of Disturbed Redox Balance. Selenoproteins, such as Gpox, are important regulators of redox homeostasis and inflammation, both of which have been implicated in the pathogenesis of mouse PCT (20, 39). To evaluate whether selenium deprivation resulted in diminished redox control, Gpox activity was determined. Gpox in splenic lymphocytes in SD mice was significantly lower (71.7 ± 11.7 mU/mg protein) than in SA mice (220 ± 32 mU/mg) or mice fed Pu chow (226 ± 28.9 mU/mg). An even more dramatic reduction in Gpox activity (by ~90%) was observed in the PEC sample: 16.2 ± 9.27 mU/mg in SD mice as opposed to 177 ± 37 mU/mg in SA mice and 163 ± 44.2 mU/mg in Pu chow mice (Fig. 3*A*). These results showed that selenium depletion led to a significant drop in Gpox activity in lymphocytes and myeloid cells. To determine whether the reduced Gpox activity may have been caused by distorted GSH metabolism, the amount of GSSG, a widely used parameter of increased oxidative stress, was determined (Fig. 3*B*). GSSG in splenic lymphocytes was higher in SD mice (0.362 ± 0.0095 nmol/mg protein) than in SA mice (0.24 ± 0.032 nmol/mg) or mice maintained on Pu chow (0.262 ± 0.029 nmol/mg). These results indicated the selenium depletion challenges the maintenance of intracellular redox homeostasis.

Biochemical Indications of Restored Redox Balance. Studies on oxidative stress control in SD rodents have shown that loss of selenium-dependent activities can be compensated for by an increase in selenium-independent oxidant defense mechanisms, including catalase (40), heme oxygenase-1 (41), and γ-glytamyl cysteine synthetase (42), the rate-limiting enzyme of *de novo* GSH synthesis. To assess whether adaptive changes of this sort occurred in SD mice, catalase and total GSH (*i.e.*, the sum of reduced GSH and GSSG) were determined in splenic lymphocytes. SD lymphocytes had a ~3.5 times higher activity of catalase (373 ± 22.3 units/mg protein) than their SA counterparts (103 ± 6.4 units/mg) or Pu chow counterparts (114 ± 8.5 units/mg; Fig. 3*C*). Similarly, splenic lymphocytes from SD mice exhibited an increase in the amount of total GSH (34.7 ± 0.07 nmol/mg protein) relative to lymphocytes from SA mice (22.6 ± 0.14 nmol/mg) or Pu mice (22.5 ± 2.5 nmol/mg; Fig. 3*D*). The total GSH increase in the SD sample counterbalanced the elevation of GSSG in the same sample with regard to the GSH:GSSG ratio, a global parameter of GSH metabolism. The GSH:GSSG ratio in the SD sample (94.4) was not different from that in the SA (92) and Pu (100) samples, indicating that cellular redox buffer capacity was comparable in all three situations (Fig. 3*E*). These results illustrated that long-term depletion of selenium triggered adaptive changes in antioxidant defense. However, it remained unclear whether these changes were sufficient to spare SD mice from the sequelae of chronic oxidative tissue damage, such as oxidative mutations.

Low Mutation Frequency. The transgenic shuttle vector pUR288, which contains a bacterial *lacZ* reporter gene for the determination of mutations *in vivo*, affords a mutagenesis assay that has been successfully used in previous studies to assess somatic mutations associated with chronic oxidative stress caused by aging (43–45), a genetic defect in intracellular redox control (25, 46), and deregulated *MYC* expression (47). To evaluate whether selenium depletion in mice results in elevated mutations, the MF in *lacZ* was determined in spleen, MLN, testis, and liver (Fig. 4). In SD F3 mice, the mean MF in these tissues was $5.82 \pm 3.1 \times 10^{-5}$, $14.3 \pm 6.7 \times 10^{-5}$, $4.2 \pm 1.8 \times 10^{-5}$ and $8.62 \pm 1.96 \times 10^{-5}$, respectively. The corresponding mutant frequencies in SA tissues were $6.33 \pm 1.62 \times 10^{-5}$ (spleen), $9.15 \pm 5.7 \times 10^{-5}$ (MLN), $4.03 \pm 0.8 \times 10^{-5}$ (testis) and $6.8 \pm 1.24 \times 10^{-5}$ (liver). Statistical comparison of SD and SA samples showed that selenium deficiency did not lead to increased *lacZ* mutant levels. The only exception was MLN, which exhibited a borderline difference between selenium depletion and repletion. Furthermore, MF did not correlate with the selenium content. Even the tissue with the lowest selenium retention (liver: 8.6%;

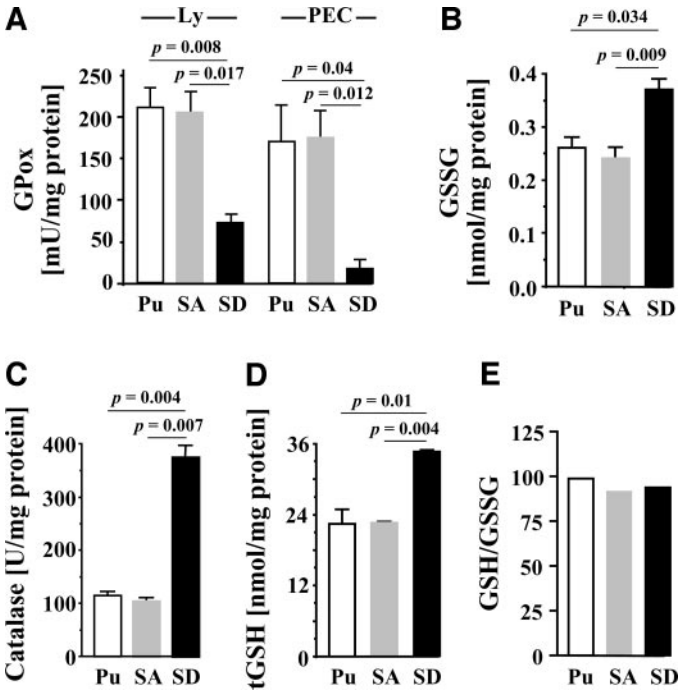

Fig. 3. Biochemical indications of disturbed redox balance (*A* and *B*) and adaptive changes restoring redox balance (*C–E*) in selenium-deprived mice. Plotted are mean values and SDs of the mean (*vertical lines above* the columns) of glutathione peroxidase activity (*A*), oxidized glutathione (*B*), catalase activity (*C*), total glutathione (*D*), and the ratio of total:oxidized glutathione (*E*). Mice maintained on Purina chow diet (*Pu*; *white columns*) were compared with mice on selenium adequate (*SA*; *gray columns*) or selenium-deficient (*SD*; *black columns*) diet. Each experimental group consisted of 3 mice. Student's *t* test was used to evaluate differences between mean values.

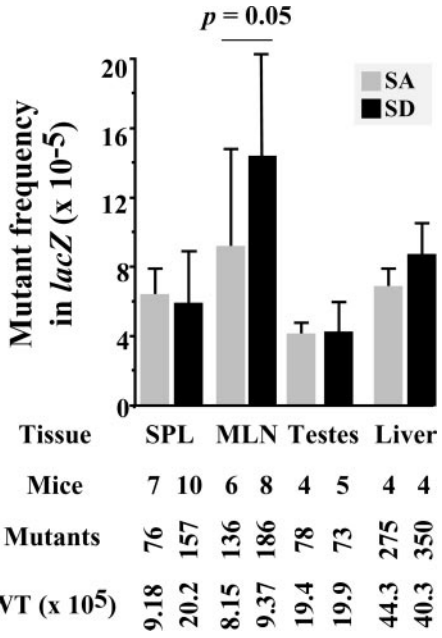

Fig. 4. Normal mutant frequency in a transgenic *lacZ* reporter gene in selenium-depleted tissues. Shown are the mean mutant frequency in four different tissues (*SPL*, spleen; *MLN*, mesenteric lymph node) from C.pUR288 mice maintained on selenium adequate (*SA*; *gray columns*) or selenium-deficient (*SD*; *black columns*) diet. The mutant frequency was calculated as the ratio of pUR288 plasmids with mutations in *lacZ* (*Mutants*) to wild-type pUR288 plasmids without mutations in *lacZ* (*WT*). The number of mice in each experimental group is denoted *below* the abscissa. Mean mutant frequency between SD and SA tissues was different for MLN but not the other tissues (Student's *t* test).

Fig. 1*B*) had comparable MF values under SD and SA conditions. The finding that selenium depletion in mice did not cause increased oxidative mutagenesis lent support to the above-stated hypothesis that redox balance in SD mice was largely restored by increased *de novo* synthesis of GSH and elevated catalase activity.

Distorted Inflammatory Response. i.p. administration of pristane provokes a massive influx of phagocytes (mainly monocytes and neutrophils) into the peritoneal cavity of C mice. This attempt to remove the foreign material provokes the formation of a chronic inflammatory tissue, the pristane granuloma, which is crucial for PCT development. To investigate whether the influx of inflammatory cells in selenium-deprived mice was altered, PECs were obtained from pristane-treated SD, SA, and Pu mice. Total cell number and composition of PEC varied dramatically according to selenium status. Mean PEC number in SD mice (5.9×10^6) was only one-third of that in SA mice (18.3×10^6) or Pu mice (17.7×10^6; Fig. 5*A*). Furthermore, although PMN predominated in PEC of SA (60%) and Pu (58%) mice, macrophages comprised the major cell type in PEC of SD mice (63%). These findings indicated that selenium deficiency might interfere with the formation of the pristane granuloma. Histological examination of mesentery from pristane-treated SD and SA mice confirmed this prediction, because SD mice contained only very small amounts of granuloma (estimated to be $<5\%$ compared with SD mice; results not shown). Consistent with the low number of PMN in the SD PEC, SD granulomas were virtually devoid of PMN (results not shown). PMN, which usually infiltrate SA granulomas in great numbers, have been implicated in the pathogenesis of PCT (18, 19, 48). These findings strongly suggested that reduced granuloma formation and/or altered granuloma composition contributed to PCT abrogation in pristane-treated SD mice.

Impaired Chemotactic Response. Changes in PEC cellularity and composition caused by selenium deficiency might be caused by diminished responsiveness of monocytes and neutrophils to chemoattractants that direct inflammatory cells to incipient pristane granulomas *in situ*. To evaluate whether inflammatory cells from SD mice demonstrated an altered migratory response to chemoattractants, splenic monocytes and PMN were tested in transwell experiments (micro-Boyden chamber) using TRX and chemokines. TRX, a redox enzyme released in inflammation, is a unique chemoattractant for PMN and monocytes (49). Monocytes from SD mice responded poorly to mouse TRX when compared with monocytes from SA mice (Fig. 5*B*). At 10 ng/ml TRX, nearly five times as many SA monocytes migrated through the filter membrane of the micro-Boyden chamber than unstimulated SA monocytes (chemotactic index: 4.83). In contrast, the migration of SD monocytes remained unchanged by TRX. Similar

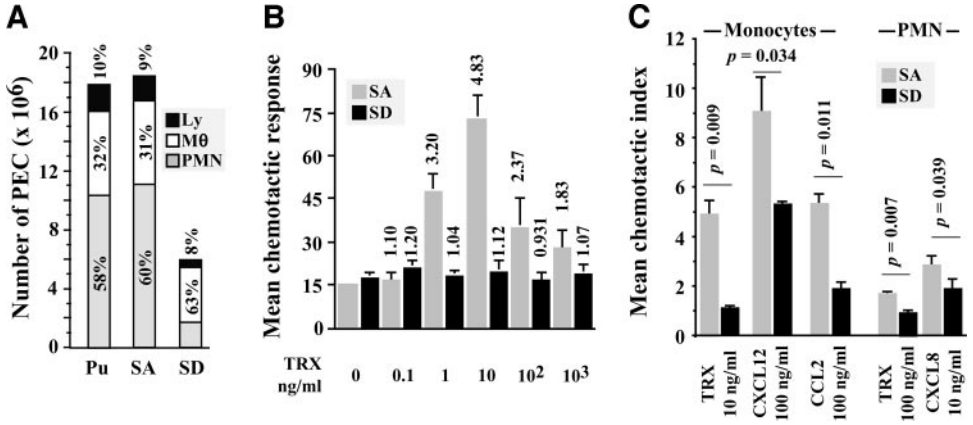

Fig. 5. Impaired inflammatory and chemotactic response in selenium-depleted mice. *A*, number of peritoneal exudate cells (*PEC*) recovered from pristane-primed C.pUR288 mice maintained on Purina chow (*Pu*; 17.7×10^6), selenium adequate (*SA*; 18.3×10^6), or selenium-deficient (*SD*; 5.9×10^6) diet. PECs were harvested 6 weeks postpristane (0.5 ml). PEC composition regarding lymphocytes (*black*), macrophages (*white*), and polymorphnuclear neutrophils (*gray*) is given within or *above* the *columns*. The percentage values add $\leq$100% for each sample. This indicated that other cell types (basophils and mesothelial cells) were rare in PECs. The amount of polymorphnuclear neutrophils (*PMN*) in SD PECs was 29%. Cell differentials were determined three times giving similar results. Average values are shown. *B*, chemotactic effect of mouse recombinant thioredoxin (*TRX*) on splenic monocytes obtained from SA (*gray bars*) or SD (*black bars*) mice. Chemotactic response is expressed as the average number of monocytes that migrated through the filter membrane of a micro-Boyden chamber. Mean values and SDs of the mean (indicated by *horizontal lines above* the *columns*) are based on three independent determinations using 3 mice each. The chemotactic index (*i.e.*, the ratio of cells migrated in the presence of TRX relative to the cells migrated in absence of TRX) is given for each TRX concentration *above* the *columns*. A similar bell-shaped, dose-response curve was obtained with human recombinant TRX (results not shown). *C*, chemotactic effect of mouse recombinant chemokines on monocytes and PMN from SA (*gray bars*) and SD (*black bars*) mice. For each chemokine, dose-response curves and chemotactic indices similar to those shown in *B* were determined. The chemokine concentration resulting in the best discrimination between the SA and SD cells was plotted. Student's *t* test was used to determine whether the differences between corresponding SA and SD samples were significant (indicated by the *P*s *above* the *columns*).

differences between SA and SD monocytes were observed for the chemokines CXCL12 and CCL2 (Fig. 5*C*, *left*). Furthermore, SD PMNs were less responsive to TRX and CXCL8 (interleukin-8) than their SA counterparts (Fig. 5*C*, *right*). These results established that inflammatory cells from SD mice exhibited reduced chemotactic activity. This reduction did, however, not reflect a general motility defect in SD cells, because the responses of splenic lymphocytes to CXCL12 and CCL5 and of PMN to formylmethionylleucylphenylalanine were not affected by selenium status (Supplementary Table 2). The response of PEC to formylmethionylleucylphenylalanine, C5a, CCL3, and CCL5 was also comparable in SA and SD mice (Supplementary Table 2). These findings confirmed earlier observations on impaired neutrophil migration and secretion of chemotactic factors under conditions of selenium depletion (50, 51). Furthermore, the distortions in chemotactic activity were selective.

DISCUSSION

This study has demonstrated that C mice, which are genetically susceptible to inflammation-induced PCT (52, 53), become completely refractory to pristane induction of PCT when deprived of selenium. To the best of our knowledge, this is the first time that dietary selenium deprivation has been used to prevent inflammation-induced cancer. Peritoneal plasmactyomagenesis in mice may thus provide a good experimental model system to elucidate the mechanisms by which lack of selenium inhibits cancers associated with chronic inflammation. Our findings extend previous results on the inhibition of PCT by anti-inflammatory drugs of the steroid family (cortisol; Ref. 54) and the nonsteroid family (indomethacin and sulindac). Cortisol's inhibitory effect on PCT is caused by the broad anti-inflammatory activity of glucocorticoids. Treatment with cortisol also causes a redistribution of selenium in mice (55), but it is not known whether this is important for PCT inhibition by cortisol. Continuous administration of indomethacin in the drinking water of C mice inhibited PCT development without inhibiting the formation of the mesenteric oil granuloma. Sulindac added to the mouse diet had similar effects (56). Indomethacin and sulindac are inhibitors of prostaglandin-generating cyclooxygenases, suggesting that prostaglandins may be important for inflammation-induced PCT in mice (57). In support of this interpretation, pristane-elicited, prostaglandin-stimulated macrophages produce elevated amounts of the PCT growth factor interleukin-6 (58, 59). Unlike selenium deficiency, cortisol and COX inhibitors reduced but did not abrogate PCT. Selenium depletion is thus the most effective chemoprevention of peritoneal PCT currently available.

Our observation that selenium deficiency abrogates neoplastic plasma cell development in mice was paradoxical in light of the large body of evidence that links reduced cancer risk with selenium supplementation. Epidemiological studies have established an inverse relationship between selenium status and cancer incidence/mortality (60). Most animal studies have shown that selenium supplementation results in decreased incidence of tumors from cancer-causing chemicals or viruses (61). These studies have primarily attributed selenium's suppressive effect on cancer to the antioxidant function of selenoproteins, such as glutathione peroxidases (five different proteins), TRs (three different proteins), and selenoproteins P and W. Selenium's beneficial effect on viral infections, atherosclerosis, cardiovascular diseases, and other conditions associated with oxidative stress has indirectly strengthened this interpretation. Indeed, on the basis of evidence that virtually any condition associated with increased levels of oxidative stress or inflammation might benefit from selenium supplementation, it has been argued that the selenium intake should be increased to supranutritional levels in the general population (60). The present findings in the mouse PCT model neither contradict these conclusions nor suggest that low-selenium intake should be recommended to prevent inflammation-induced tumors. Instead, they indicate that selenium, via the selenoproteins, can sometimes play a critical role in tumor promotion.

The mechanism by which depletion of selenium abrogates PCT has not been elucidated; however, some potential explanations were suggested by our experimental results. The first hypothesis considers the adverse effects of selenium deficiency on the innate and adaptive immune system. Numerous functions of macrophages and neutrophils, components of the innate immune system, are linked with inflammatory processes that are involved in plasmacytomagenesis. SD macrophages are impaired in their ability to synthesize leukotrien B4, which is essential for neutrophil chemotaxis. SD neutrophils remove ingested pathogens poorly. The free radicals that are produced in the respiratory burst, but inefficiently eliminated because of the loss of cytosolic GSH peroxidase activity, are ultimately fatal to the neutrophil. The reduced number of neutrophils in PECs (Fig. 5*A*) and pristane granulomas of selenium-deprived mice was in agreement with these alterations. PCT development is further dependent on T-cell help (62, 63) and the ability of B cells to mount a normal antibody response (64, 65). SD B cells proliferate less vigorously in response to mitogen and antigen, which can lead to decreased antibody titers (36). Selenium-depleted T cells produce only a weak delayed-type hypersensitivity skin reaction (66). Another indication of the importance of selenoproteins for cellular immunity is that activated T cells exhibit elevated levels of selenophosphate synthase 2 (67), which is essential for synthesis of selenocysteine, the unique building block of selenoproteins. Thus, disturbed immune functions in SD mice might be involved in inhibition of PCT.

Reduced chemotaxis of SD inflammatory cells provides another link between low-selenium, impaired granuloma formation, and abrogated PCT development. Our finding that SD monocytes and neutrophils responded poorly to some chemokines (Fig. 5*C*) but normally to others (Supplementary Table 2) suggested that chemokine receptor genes were selectively down-regulated. Low selenium has been associated with global gene expression changes (68–70). Selenium deficiency-associated gene expression changes interfering with the TR/TRX pathway may also be the underlying reason why SD monocytes and neutrophils failed to respond to TRX. TRX, a redox enzyme released in inflammation (49), appears to be of great importance for neutrophil chemotaxis (71). Impaired redox status of chemokine receptors attributable to diminished activity of selenium-dependent oxidoreductases, such as TR (72), may have further confounded chemokine signaling. Chemokines, chemokine receptors, and TRX contain redox-sensitive cytosines in their functional sites (73). Of particular relevance for plasmacytomagenesis may have been the observation that SD cells reacted poorly to the chemokine CXCL12. Interaction of CXCL12 with its receptor, CXCR4, is critical for plasma cell trafficking and homing to the bone marrow (74). CXCL12 enhances the survival of chronic lymphocytic leukemia B cells (75) and normal peritoneal B1 cells (76), the presumptive precursors of inflammation-induced PCT (77). CXCL12-CXCR4 signaling has been strongly implicated in the pathogenesis of multiple myeloma, a malignant plasma cell tumor in humans (78). Studies in mice in which chemokines, their receptors, and TR/TRX have been selectively inactivated in B or inflammatory cells should help to elucidate the mechanism by which selenium deficiency inhibits chemotaxis, granuloma formation, and PCT.

Diminished function of selenoproteins with putative tumor-promoting activities is another explanation for PCT abrogation in selenium-deprived mice. The TR/TRX system is the strongest candidate along this line (13, 14). TRX catalyzes dithiol-disulfide oxidoreductions and

serves as a hydrogen donor for ribonucleotide reductase, which is essential for DNA synthesis. TRX is a growth factor in tumor cells, and TR-null mice are not viable (79). TRX's ability to augment interleukin-6 production by inflammatory cells (80) may specifically promote PCT because interleukin-6 is a B-cell growth, differentiation, and survival factor that is crucial for plasma cell tumor formation in mice (81, 82) and humans (83). The significance of the TR/TRX system for cell proliferation and tumor progression is further underscored by the emerging understanding that the therapeutic benefit of widely used cancer drugs (*e.g.*, carmustine and cisplatin) must be attributed in part to TR inhibition. What is more, TR's biological activity seems to depend on the selenium status of the host. Although selenium-replete TR promotes cell viability (a potential tumor-promoting function), SD TR induces apoptosis (a potential tumor-inhibiting function; Ref. 84). Thus, partial loss and/or altered properties of TR may be involved in PCT suppression in low-selenium mice.

In conclusion, our observation that long-term dietary selenium depletion abrogates inflammation-dependent PCT has implicated selenoproteins as an essential cofactor in neoplastic plasma cell development in mice. Mouse PCT may provide a valuable model system for the design and testing of specific small drug inhibitors of proinflammatory and/or tumor-promoting selenoproteins, such as organometallic compounds that oxidize or complex the selenium moiety *in vivo* (85). However, the potential benefit of these inhibitors in preventing and treating inflammation-dependent cancers must be carefully balanced against the risk of facilitating other cancers. Considering that the mouse PCT model is available as a set of inbred, congenic, and transgenic strains exhibiting varying degrees of tumor susceptibility/resistance, the PCT model may also be useful to associate the genetics of selenoprotein metabolism with the predisposition to inflammation-induced tumors. Two allelomorphic variants of genes encoding selenoproteins have recently been linked to increased cancer risk in humans (86, 87).

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