Short Communication

Noninvasive Prediction of Prostatic DNA Damage by Oxidative Stress Challenge of Peripheral Blood Lymphocytes

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Abstract

To move closer to the goal of individualized risk prediction for prostate cancer, we used an *in vivo* canine model to evaluate whether the susceptibility of peripheral blood lymphocytes (PBLs) to oxidative stress-induced DNA damage could identify those individuals with the highest prostatic DNA damage. This hypothesis was tested in a population of 69 elderly male beagle dogs after they had completed a 7-month randomized feeding trial to achieve the broad range of dietary selenium status observed in U.S. men. The alkaline Comet assay was used to directly compare the extent of DNA damage in PBLs with prostatic DNA damage in each dog. Using stepwise logistic regression, the sensitivity of PBLs to oxidative stress challenge with hydrogen peroxide (H_2O_2) predicted dogs in the highest tertile of prostatic DNA damage. Dogs with PBLs highly sensitive to H_2O_2 were 7.6 times [95% confidence interval (95% CI), 1.5-38.3] more likely to have high prostatic DNA damage than those in the H_2O_2-resistant group. This risk stratification was observed in multivariate analysis that considered other factors that might influence DNA damage, such as age, toenail selenium concentration, and serum testosterone concentration. Our data show that the sensitivity of PBLs to oxidative stress challenge, but not endogenous DNA damage in PBLs, provides a noninvasive surrogate marker for prostatic DNA damage. These findings lend support to the concept that oxidative stress contributes to genotoxic damage, and that oxidative stress challenge may stratify men for prostate cancer risk. (Cancer Epidemiol Biomarkers Prev 2007;16(9):1906–10)

Introduction

The lack of validated, noninvasive stratifiers of prostate cancer risk is a major obstacle to finding effective ways to prevent prostate cancer. The inability to segregate men into high- versus low-risk groups mandated the enrollment of more than 32,000 subjects to test a single hypothesis, whether daily supplementation with the antioxidants vitamin E or selenium can substantively reduce prostate cancer incidence (1). Individualized risk prediction would identify those men who have the highest lifetime risk for prostate cancer, so that streamlined, cost-effective prevention trials could be implemented.

Like men, dogs develop spontaneous prostate cancer (2-4), thereby providing an animal model to assess risk. Our previous work showed the dose of selenium that minimized DNA damage in the aging dog prostate was remarkably similar to the level in men that minimized prostate cancer risk (5, 6). The highest prostatic DNA damage was seen in dogs with either low or high selenium status measured noninvasively in toenails, a U-shaped dose response (6). These results provided strong rationale for using the dog model to probe for additional informative biomarkers of prostate cancer risk.

Oxidative stress seems to drive the accumulation of DNA damage within the prostate, thereby contributing to cancer development (7-9). We reasoned that if oxidative stress plays an important role in prostatic carcinogenesis, then evaluating the sensitivity of peripheral blood lymphocytes (PBLs) to oxidative stress challenge might provide a simple test to noninvasively predict cancer risk. In a recent case-control study of 158 men with prostate cancer, risk for prostate cancer was 2-fold greater in men whose PBLs were sensitive to oxidative stress challenge, after adjusting for age, race, smoking, and family history (10). However, no studies have directly compared the extent of DNA damage in PBLs and the prostate of the same individual to confirm whether PBLs provide a reliable predictor of genetic

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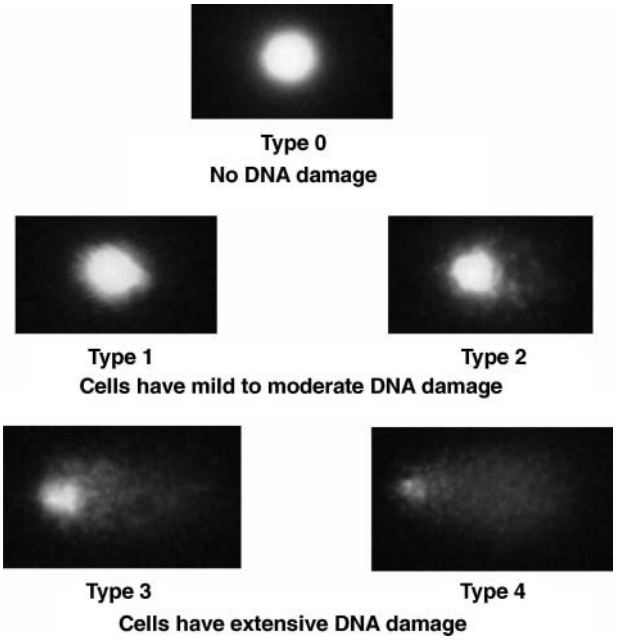

Figure 1. Measurement of DNA damage in PBLs and prostate cells from elderly dogs. The extent of DNA damage in PBLs and prostate cells was measured by single cell gel electrophoresis (alkaline Comet assay) as described by Singh (13). Under the assay conditions used in this experiment, comet tails reflect the electrophoretic migration of DNA fragments that result from strand breaks, alkali-labile sites, crosslinks, or base-excision repair sites (13). The extent of DNA damage was visually scored in 100 randomly selected cells from each sample (50 cells from several different fields from each of two replicate slides) by one examiner who was blinded to the treatment group. SYBR Green 1–stained nucleoids were examined at 200× magnification with an epifluorescent microscope. Each cell was visually scored on a 0 to 4 scale using a method described by Collins (42) as follows: no damage (type 0); mild to moderate (types 1 and 2), and extensive DNA damage (types 3 and 4). The extent of DNA damage within PBLs or prostate cells was expressed as the percentage of cells with extensive DNA damage (sum of cells that displayed type 3 or type 4 DNA damage). PBLs isolated from the whole blood of each dog (12) were assayed fresh without cryopreservation. Cytospin preparations confirmed that more than 90% of cells in this enriched cell population were lymphocytes; mean percent viability (trypan blue exclusion) was 90%. The sensitivity of PBLs to oxidative stress was determined by measuring DNA damage before and after *ex vivo* challenge of PBLs with 25 μmol/L hydrogen peroxide (5 min, 4°C). To assess endogenous DNA damage in prostate cells, the prostate was collected from each dog at necropsy, and 50 to 80 mg of prostate tissue was placed in 1 mL of cold HBSS containing 20 mmol/L EDTA and 10% DMSO (43). Tissue was then minced with fine scissors, and 50 μL of the resulting cell suspension was mixed with 1 mL of RPMI 1640 containing 10% fetal bovine serum for subsequent electrophoresis. Cytospin preparations indicated that >90% of cells had epithelial cell morphology; mean percentage cell viability estimated by trypan blue exclusion was 80%. Histopathologic evaluation of formalin-fixed, step-sectioned prostate tissue sections revealed no foci of carcinoma in any of the dogs in this study population.

damage. Herein, using the dog model, we tested the hypothesis that individuals whose PBLs are sensitive to hydrogen peroxide–induced DNA damage are more likely to have high prostatic DNA damage.

Materials and Methods

The hypothesis that oxidative stress challenge of PBLs could provide a useful, noninvasive predictor of prostatic DNA damage was tested in a population of elderly beagle dogs. Sixty-nine elderly (8-10.5 years old; physiologically equivalent to 62- to 69-year-old men) sexually intact male, retired breeder dogs weighing 8 to 21 kg were purchased from a local supplier. Dogs were randomly assigned to either a nutritionally adequate control group (n = 20 dogs) or to receive daily supplementation with selenomethionine (Solgar Vitamin and Herb) or high-selenium yeast (Seleno Excell®, Cypress Systems) at 3 μg/kg/day (n = 29 dogs) or 6 μg/kg/day (n = 20 dogs) for 7 months. This enabled us to study a population with a wide range of steady-state selenium status that mimicked the levels seen in American men (median toenail selenium concentration in lowest, middle, and highest tertiles in dogs were 0.57, 0.76, and 0.99 ppm, compared with 0.66, 0.82, and 1.14 ppm for men in the lowest, middle, and highest quintiles of the Health Professionals Follow-up Study; ref. 11). Dogs were euthanized in accordance with guidelines set forth by the American Veterinary Medical Association Panel on Euthanasia. The protocol was approved by the Purdue University Animal Care and Use Committee.

Just before euthanasia, PBLs were isolated from whole blood (12). DNA damage in PBLs was measured before and after *ex vivo* challenge with hydrogen peroxide (H_2O_2) by single cell gel electrophoresis (alkaline Comet assay; ref. 13; Fig. 1). The prostate was collected from each dog within 15 min after euthanasia. Endogenous DNA damage in the prostate was determined using alkaline Comet assay, and the entire study population of dogs was subdivided into tertiles based on the extent of prostatic DNA damage. The study end point, high prostatic DNA damage, was defined as the highest tertile of prostatic DNA damage found in the study population. Toenail clippings were analyzed for selenium by neutron activation analysis (6, 14, 15). Total serum testosterone was measured by radioimmunoassay.

To determine if the study of PBLs could stratify individuals according to risk for high prostatic DNA damage, two measures of DNA damage in PBLs were evaluated: endogenous DNA damage (DNA damage without *ex vivo* H_2O_2 exposure) and H_2O_2-inducible DNA damage index [(damage after H_2O_2 challenge − endogenous damage)/(100 − endogenous damage)], which reflects the sensitivity of cells to oxidative stress-induced DNA damage. Odds ratios (OR) for high prostatic DNA damage were calculated by subdividing dogs into tertiles based on the response of their PBLs to oxidative stress challenge: resistant, moderately sensitive, and highly sensitive. Unconditional logistic regression was used to evaluate the influence of additional variables, including age, body weight change over the 7-month feeding trial, toenail selenium concentration,

and total serum testosterone. Receiver operating characteristic (ROC) curves were constructed to assess predictive accuracy of the models (16).

Results

In elderly dogs, the level of endogenous DNA damage in PBLs failed to predict high prostatic DNA damage. In contrast, the sensitivity of PBLs to *ex vivo* challenge with H_2O_2 was a strong predictor (Table 1). In univariate analysis, dogs with sensitive PBLs were 5.6 times [95% confidence interval (95% CI), 1.3-24.3] more likely to have high prostatic DNA damage. High prostatic DNA damage was found in 10 of 22 (45.5%) dogs with sensitive PBLs, but in only 3 of 23 (13.0%) dogs with resistant PBLs ($P = 0.037$). In stepwise multivariate logistic regression, two predictors of high prostatic DNA damage were identified: sensitivity of PBLs to oxidative stress challenge and toenail selenium concentration. The relationship between sensitivity of PBLs to oxidative stress and prostatic damage strengthened in multivariate analysis; dogs with PBLs sensitive to H_2O_2 were 7.6 times (95% CI, 1.5-38.3) more likely to have high prostatic DNA damage than dogs in the resistant group. Dogs with middle-range toenail selenium concentration had the lowest risk for high prostatic DNA damage. Compared with dogs with the lowest selenium status, dogs with middle-range toenail selenium concentration (0.67-0.92 ppm) had an 84% reduction in the risk for high prostatic DNA damage (OR, 0.16; 95% CI, 0.04-0.63). Risk in dogs with the highest selenium status did not differ significantly from dogs with the lowest selenium (OR, 0.55; 95% CI, 0.12-2.6). There was no apparent interaction between the sensitivity of PBLs to oxidative stress challenge and toenail selenium concentration; interindividual differences in the sensitivity of PBLs to H_2O_2 could not be explained by differences in selenium status.

Receiver operating characteristic (ROC) curves were constructed to assess the ability of noninvasive predictors to discriminate between a dog in the highest tertile of prostatic DNA damage and a dog with less extensive prostatic DNA damage (Fig. 2). As the area under the ROC curve approaches 1.0, predictive accuracy increases. For the model using sensitivity of PBLs to oxidative stress challenge alone, the area under the ROC curve was 0.66 (95% CI, 0.53-0.80). For the model using sensitivity of PBLs to oxidative stress challenge and toenail selenium concentration, the area under the ROC curve was increased to 0.77 (95% CI, 0.65-0.89). These 95% CIs do not include 0.5, indicating that both models are better than a coin toss. Predictive accuracy was not increased by adding total serum testosterone to either model.

Discussion

Our results show that detectable interindividual differences in the response of PBLs to oxidative stress can be exploited to predict the extent of DNA damage within the prostate. Importantly, these differences in genetic instability were not found by measuring endogenous DNA damage in PBLs. However, when dogs were segregated into tertiles according to sensitivity to oxidative stress challenge, there was a greater than 7-fold difference in risk for high prostatic damage. Moreover, in our dog model, PBLs provide a valuable window to the prostate across the broad range of dietary selenium status observed in U.S. men.

Our study is the first to document the relationship between DNA damage in PBLs and prostatic cells in the same individual. Two previous studies have evaluated DNA damage in PBLs as a surrogate for target tissues. In one study, there was a weak correlation ($r^2 = 0.11$) between endogenous DNA damage in PBLs and upper airway mucosal cells harvested from 60 surgical patients; response to oxidative stress challenge was not evaluated (17). In another study, a weak correlation ($r^2 = 0.20$) between endogenous DNA damage in PBLs and rectal mucosal cells from 19 human subjects was strengthened ($r^2 = 0.37$) when both PBLs and rectal mucosal cells were challenged with hydrogen peroxide, supporting the

Table 1. Sensitivity of PBLs to oxidative stress challenge, but not endogenous DNA damage in PBLs, is associated with high prostatic DNA damage in elderly beagle dogs

Endogenous DNA damage in PBLs	Range of extensive DNA damage (%)	Univariate OR (95% CI)	Multivariate* OR (95% CI)
First tertile ($n = 28$)	5-12	1.0 (reference)	1.0 (reference)
Second tertile ($n = 20$)	13-18	0.6 (0.16 to 2.1)	No selection †
Third tertile ($n = 17$)	19-28	1.1 (0.33 to 3.8)	No selection
Oxidative stress-induced DNA damage in PBLs	**Range of extensive DNA damage (%)**	**Univariate OR (95% CI)**	**Multivariate* OR (95% CI)**
First tertile ($n = 23$)	15-30	1.0 (reference)	1.0 (reference)
Second tertile ($n = 20$)	31-39	4.9 (1.1 to 22.1)	6.6 (1.3 to 32.2)
Third tertile ($n = 22$)	40-97	5.6 (1.3 to 24.3)	7.6 (1.5 to 38.2)

NOTE: Endogenous DNA damage measured by alkaline Comet assay was quantified by the percentage of cells with extensive DNA damage (visually scored as type 3 or type 4 damage). Oxidative stress-induced PBL damage is expressed as the H_2O_2-inducible DNA damage index, which is calculated as follows: (damage after H_2O_2 challenge − endogenous damage)/(100 − endogenous damage). The end point of the study, high prostatic DNA damage, was defined as the highest tertile of prostatic DNA damage found in the prostates of the 65 dogs in the study population for which complete data were available. Mean prostatic DNA damage in elderly beagle dogs was 67%, with a range of 40% to 97%. Ranges for tertiles of prostatic DNA damage were as follows: lowest (40-54%), middle (55-77%), and highest (78-97%) tertiles of prostatic DNA damage.

*Multivariate OR adjusted for toenail selenium concentration, age, change in body weight, and serum testosterone.

† Not selected as a significant predictor of high prostatic DNA damage in stepwise multivariate logistic regression.

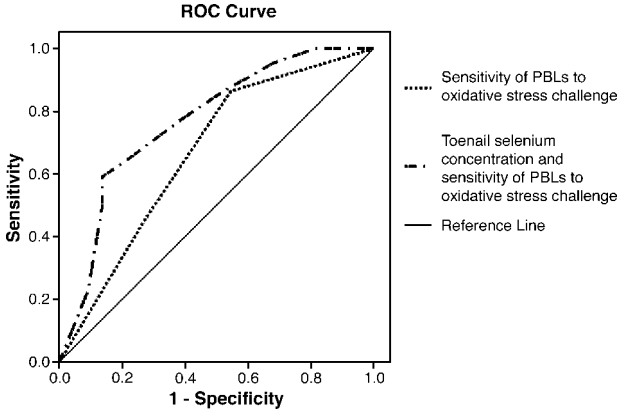

Figure 2. ROC curves for prediction of high prostatic DNA damage in elderly dogs. The area under the curve estimates the probability of correctly discerning a dog in the highest tertile of prostatic DNA damage from a dog with less extensive prostatic DNA damage. Curves are plotted for two models: (*a*) sensitivity of PBLs to oxidative stress challenge alone (area under the curve, 0.66); and (*b*) sensitivity of PBLs to oxidative stress challenge and toenail selenium concentration (area under the curve, 0.77). The reference line is the line of unity at which no discrimination of prostatic DNA damage occurs.

notion that PBLs and epithelial cell targets respond similarly to oxidative stress challenge (18). Higher endogenous DNA damage in PBLs (19-24) or more damage in PBLs was found after mutagen challenge (19, 21-23, 25-29) in cancer cases than in benign controls. However, for the prostate, endogenous DNA damage of PBLs did not stratify individuals according to risk for high DNA damage (dogs in this study) or cancer (10), indicating that *ex vivo* challenge of PBLs with hydrogen peroxide was required to stratify risk. In our dogs, we found a much greater interindividual heterogeneity in the extent of DNA damage induced by oxidative stress challenge (15-97%) compared with a relatively narrow range of endogenous DNA damage in PBLs (5-28%). We propose that challenging PBLs with oxidative stress to predict risk for genotoxic damage in the prostate is analogous to the treadmill challenge that cardiologists rely on to stratify men in terms of their risk for cardiac events.

The rationale for using toenail selenium concentration as a noninvasive predictor of prostate cancer risk is that low selenium status in men has been associated with increased risk for prostate cancer (11, 30-32). In our study, toenail selenium concentration was a significant predictor of high prostatic DNA damage. However, the 7-fold higher risk for high prostatic damage in dogs with PBLs most sensitive to oxidative stress challenge was seen even after differences in selenium status were taken into consideration. The ROC curve analysis shows that the accuracy of predicting high prostatic DNA damage is best when both selenium status and oxidative stress sensitivity are included in the model.

The nature of interindividual differences in sensitivity of PBLs to oxidative stress challenge is not known. The results in dogs likely reflect differences in the intrinsic sensitivity of DNA to mutagenic damage or early DNA repair (25, 33-37). Having sensitive PBLs seems to indicate a sensitive prostate and a greater likelihood of genotoxic damage. The alkaline Comet assay is ideally suited to probe for interindividual differences in mutagen response because DNA damage can be measured on a single cell basis within a population of cells (38, 39). In this study, we maximized our ability to detect subtle interindividual differences in lymphocyte response to oxidative stress by (*a*) using fresh PBLs rather than cryopreserved cells to reduce artifactual damage introduced by cell storage and thawing; (*b*) challenging PBLs with a low dose of H_2O_2 (25 μmol/L instead of 50-500 μmol/L used by others; refs. 40, 41); and (*c*) recognizing the heterogeneity of cell response by reporting each subject's genetic instability as the percentage of cells with extensive DNA damage rather than mean level of damage.

In summary, our findings show that sensitivity of PBLs to hydrogen peroxide challenge, but not endogenous PBL damage, is a noninvasive predictor of prostatic DNA damage. These results lend further support to the concept that oxidative stress contributes to DNA damage and cancer development within the aging prostate. The ability of the alkaline Comet assay to detect heterogeneity in the response of PBLs to oxidative stress challenge enables detection of interindividual differences in latent genetic instability. This creates an opportunity to stratify individuals in terms of low versus high prostatic DNA damage. A prospective cohort study in men is now needed to validate whether this approach is indeed an informative cancer risk stratification tool.

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