Cancer Letters 353 (2014) 248–257

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original articles

Systemic DNA damage accumulation under *in vivo* tumor growth can be inhibited by the antioxidant Tempol

Alexandros G. Georgakilas [a,b,1], Christophe E. Redon [c,1], Nicholas F. Ferguson [a], Thomas B. Kryston [a], Palak Parekh [c], Jennifer S. Dickey [c,d], Asako J. Nakamura [c,e], James B. Mitchell [f], William M. Bonner [c], Olga A. Martin [c,g,h,i,*]

[a] *Department of Biology, Thomas Harriot College of Arts and Sciences, East Carolina University, Greenville, NC 27858, USA*
[b] *Department of Physics, National Technical University of Athens, Zografou Campus, Athens GR-15773, Greece*
[c] *Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA*
[d] *Office of In Vitro Diagnostics, Center for Devices and Radiological Health, Food and Drug Administration, Silver Spring, MD 20993, USA*
[e] *Department of Biological Sciences, Faculty of Science, Ibaraki University, Ibaraki 310-8512, Japan*
[f] *Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA*
[g] *Division of Radiation Oncology and Cancer Imaging, Peter MacCallum Cancer Centre, Vic. 3002, Australia*
[h] *Laboratory of Molecular Radiation Biology, Peter MacCallum Cancer Centre, Vic. 3002, Australia*
[i] *Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne, Vic. 3002, Australia*

ARTICLE INFO

Article history:
Received 29 April 2014
Received in revised form 16 July 2014
Accepted 16 July 2014

Keywords:
Tumor-bearing mice
DNA damage
Antioxidants
Tempol
Non-targeted effects

ABSTRACT

Recently we found that mice bearing subcutaneous non-metastatic tumors exhibited elevated levels of two types of complex DNA damage, i.e., double-strand breaks and oxidatively-induced clustered DNA lesions in various tissues throughout the body, both adjacent to and distant from the tumor site. This DNA damage was dependent on CCL2, a cytokine involved in the recruitment and activation of macrophages, suggesting that this systemic DNA damage was mediated via tumor-induced chronic inflammatory responses involving cytokines, activation of macrophages, and consequent free radical production. If free radicals are involved, then a diet containing an antioxidant may decrease the distant DNA damage. Here we repeated our standard protocol in cohorts of two syngeneic tumor-bearing C57BL/6NCr mice that were on a Tempol-supplemented diet. We show that double-strand break and oxidatively-induced clustered DNA lesion levels were considerably decreased, about two- to three fold, in the majority of tissues studied from the tumor-bearing mice fed the antioxidant Tempol compared to the control tumor-bearing mice. Similar results were also observed in nude mice suggesting that the Tempol effects are independent of functioning adaptive immunity. This is the first *in vivo* study demonstrating the effect of a dietary antioxidant on abscopal DNA damage in tissues distant from a localized source of genotoxic stress. These findings may be important for understanding the mechanisms of genomic instability and carcinogenesis caused by chronic stress-induced systemic DNA damage and for developing preventative strategies.

Introduction

Intercellular communication is mediated by substances released by damaged cells which then affect healthy cells. The radiation-induced bystander effect is one example of this phenomenon, where the released factors from irradiated cells may activate pathways in healthy 'bystander' cells leading to the induction of DNA damage [1,2], increased genomic instability and decreased viability [3,4]. The signal transduction from irradiated to bystander cells *in vitro* can occur through both cell media and gap junctions [1] and is reminiscent of the inflammatory response mediated by COX-2 related pathways, involving cytokines, growth factors, and membrane-permeable reactive oxygen and nitrogen species (ROS and RNS) [5,6]. In addition to radiation-damaged cells, recent studies have reported that genetically unstable, senescent, and cancerous cells also can adversely affect their normal neighbors [7–10], suggesting that the radiation-induced bystander effect is a specific instance of a much more general phenomenon of intercellular communication from damaged or abnormal cells to normal cells.

While these bystander-like phenomena have been well-documented *in vitro*, as have *in vivo* counterparts of the

* Corresponding author. Address: Department of Radiation Oncology and Cancer Imaging, Peter MacCallum Cancer Centre and The University of Melbourne, Melbourne, Vic. 3002, Australia. Tel.: +613 96561357; fax: +613 9656 1411.
E-mail address: olga.martin@petermac.org (O.A. Martin).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.canlet.2014.07.030
0304-3835/

radiation-induced bystander (abscopal) effects [11–13], reports of other extensions of the more general phenomenon *in vivo* are not so abundant. An interesting example is that of animal tumors in a chronic inflammatory environment [14], with elevated levels of endogenous stress factors and ROS [15,16], produced either directly by tumors, or indirectly via inflammatory responses, which can induce DNA damage in healthy neighboring cells [17].

While there are several methods for detecting ROS *in vitro*, they are difficult to monitor *in vivo*. All ROS detection methodologies have to overcome various limitations such as time, dye specificity, species specificity, and others [18,19]. In our study with tumor-bearing mice, we employed two endpoints to monitor the effects of oxidative stress, the presence of two potentially lethal DNA lesions, bistranded oxidatively-induced clustered DNA lesions (OCDLs) [20,21] and foci of phosphorylated histone H2AX (γ-H2AX), a surrogate marker of DNA double strand breaks (DSBs) [22–24]. Both biomarkers have been used to detect and monitor radiation- and cancer-related DNA damage in mouse and human tissues [25–28]. While induction of γ-H2AX foci has been reported at non-DSB sites, such as dysfunctional telomeres [29] or in the absence of DNA damage [30], numerous studies related to the bystander effect, have shown a direct link between DSBs and γ-H2AX foci [1,2,9,31,32]. In our recent study with mice implanted with localized tumors, we showed that the levels of these two types of complex DNA lesions were elevated in several distant tissues [26]. We also showed that the elevated levels of these lesions in distant tissues were mediated by inflammatory macrophages in a CCL2-dependent manner. The elevation of OCDLs and the participation of macrophages both point to ROS involvement in this distant DNA damage.

While ROS homeostasis can be maintained in unstressed healthy cells by a balance of the pathways that produce and destroy ROS, excessive ROS levels may be beyond the capacity of these endogenous systems to regulate. However, they can often be lowered by exogenous antioxidants such as Tempol, a cell-permeable superoxide dismutase mimetic and a free radical scavenger [33]. Belonging to nitroxide stable free radical family, Tempol is a promising agent for clinical use as an antioxidant and radioprotector [34]. It significantly reduces superoxide anion and peroxynitrite-associated inflammation, lowers blood pressure in a variety of animal models and also displays neuroprotective effects [35–39]. It has been found to be efficient in restoring mitochondrial and cardiac functions in tumour necrosis factor (TNF)α-induced oxidative stress and reducing cardiac hypertrophy in chronically hypoxic rats [40]. It reduces the incidence of hematopoietic neoplasms, increases the survival of irradiated mice [41] and topically protects mice against radiation-induced mucositis [42]. Preclinical studies in guinea pigs, and a Phase I clinical trial in patients receiving whole-brain radiotherapy, suggest that Tempol is effective in suppressing radiation-induced alopecia [43–45].

To test the hypothesis for an oxidative mechanism fueling these non-targeted effects in the organism, we examined whether an exogenous antioxidant treatment could lower systemic or abscopal oxidative DNA damage levels in tumor-bearing mice. For this reason we incorporated a well-known antioxidant Tempol, into the diets of several tumor-bearing mouse cohorts. Here we report that the local tumor-induced DSB and OCDL accumulation in normal tissues of tumor-bearing mice can be suppressed by feeding the mice a Tempol-supplemented diet. These findings show that oxidative stress pathways leading to elevated DSB and OCDL levels can be interrupted with exogenous antioxidants. Since these two lesions are often precursors to genomic instability and carcinogenesis, and it is estimated that as many as 20% of cancers may be due to chronic inflammatory conditions [14], these findings may have important implications for development of clinical strategies to mitigate chronic stress-induced systemic DNA damage.

Materials and methods

Mice and tumors

All necessary permits were obtained for the described study. The protocols were approved by the National Cancer Institute Animal Care and Use Committee. Six-week-old C57BL/6NCr (B6) and nude female mice were obtained from the Animal Production Area, National Cancer Institute (NCI) – Frederick. Cryopreserved murine B16 melanoma (MEL, host strain: B6) and Lewis lung carcinoma (LLC, host strain: B6) were obtained from the Division of Cancer Treatment and Diagnosis tumor repository, NCI – Frederick. One vial of cryopreserved tumor tissue was thawed according to the provided protocols. The tissue was minced into fragments of ~8 mm^3 ($2 \times 2 \times 2$ mm). These tumor fragments were placed into an 11- to 13-gauge trocar. One fragment of each tumor was implanted s.c. into each of three B6 mice. These "donor" mice were subsequently used as the source of tumors grown in test animals, implanted using a similar procedure. B6 mice were implanted with both MEL and LLC tumors, and nude mice were implanted with the LLC tumor.

Bacon-flavored Tempol-supplemented chow (10 mg/g of chow) and control bacon-flavored chow were obtained from Bio-Serv, Frenchtown, NJ, USA. Mice were given the food 1 week prior to the experiment to acclimatize animals to the diet and during 16 days of the experiment. Body weights were taken at the feeding start point (~7 days before the experiment), the first day of experiment (Day 0), and at euthanasia (Day 16).

Six cohorts of B6 mice were used: (1) "MEL": five animals (fed control chow) were implanted with minced fragments of B16 tumor harvested from donor mice; (2) "LLC": five animals (fed control chow) were implanted with minced fragments of LLC tumor harvested from donor mice; (3) "PBS": five control animals (fed control chow) were subjected to a single s.c. injection of 0.05 mL sterile PBS (i.e. mock tumor implantation); (4) "MEL + Tempol": five animals implanted with B16 tumor and fed Tempol chow; (5) "LLC + Tempol": five animals implanted with LLC and fed Tempol chow; (6) "PBS + Tempol": five control animals were subjected to PBS injection and fed Tempol chow. Four cohorts of nude mice were used: (1) "LLC"; (2) "PBS"; (3) "LLC + Tempol"; (4) "PBS + Tempol".

Growth of tumors was monitored at least twice during the course of the experiment. Mice of all 10 cohorts were euthanized with CO_2 when grafted tumors in the test cohorts reached a volume of ~200 mg (L = 7 mm, W = 5 mm). Stomach, duodenum, colon, rectum, liver, kidney, lung, ovary, spleen, brain, tumor mass and skin proximal to the tumor were harvested, and for stomach, duodenum, colon and rectum, fresh tissue samples were "touch-printed" to a slide surface for the γ-H2AX assay, as described below. All tissues were then frozen and fixed by formaldehyde exposure, and paraffin-embedded. Pathology reports were prepared for all organs of test and control mice.

γ-H2AX immunostaining and analysis

Touch-print specimens were prepared by gentle pressing stomach, duodenum, colon and rectum tissues against a microscopic slide surface. The samples were air-dried, fixed in 2% para-formaldehyde for 20 min at room temperature, permeabilized with 1% Triton X-100, and processed for γ-H2AX immunostaining, as previously described [26,46]. Primary rabbit polyclonal anti-γ-H2AX antibody (Novus Biologicals) and secondary goat anti-rabbit Alexa-488-conjugated IgG (Invitrogen) were used. Nuclei were stained with propidium iodide. Laser scanning confocal microscopy was performed with a Nikon PCM 2000 (Nikon, Inc.). γ-H2AX foci were counted in three randomly selected microscopic fields per data point (at least 300–500 cells per mouse) using FociCounter software (http://focicounter.sourceforge.net/index.html) [47].

Isolation and processing of mouse DNA and OCDL detection

The High Pure PCR Template Kit (Roche) was used for isolation of DNA from the tissues as previously described [48,49]. To minimize oxidation artifacts during DNA isolation, all buffers were freshly prepared, autoclaved, purged with argon, and supplemented with 50 μM phenyl-tert-butyl nitrone, a free radical scavenger (Sigma-Aldrich) [50].

Detection of OCDLs was performed as analytically described in [25]. Briefly, DNA isolated from the tissues was digested with human BER repair enzymes APE1 and OGG1, and *Escherichia coli* EndoIII (New England Biolabs). The specific enzymatic DNA damage probes detect a variety of DNA lesions covering a wide spectrum of oxidatively-generated DNA damage. Specifically APE1 detects abasic DNA lesions, OGG1 primarily oxidized purines and EndoIII will cleave mainly oxidized pyrimidines [51]. For both glycosylases, OGG1 and EndoIII, the minor detection of some abasic sites cannot be excluded. Forty nanograms of isolated DNA were mixed with 7 μL of the appropriate enzyme reaction buffer and left on ice for 30 min. The enzyme buffers used were: APE1 buffer (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, pH 7.9), OGG1 buffer (50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.9), and *E. coli* EndoIII buffer (20 mM Tris–HCl, 1 mM EDTA, pH 8.0). In each case, repair enzyme was added in order to achieve optimum cleavage i.e. APE1 and OGG1: two enzyme units and EndoIII: two enzyme units [40]. For each enzyme-treated sample, a corresponding non-enzyme containing sample was also run as a control following the same steps. An adaptation of constant field gel