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Original Contribution

Tousled kinase activator, gallic acid, promotes homologous recombinational repair and suppresses radiation cytotoxicity in salivary gland cells

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ABSTRACT

Accidental or medical radiation exposure of the salivary glands can gravely impact oral health. Previous studies have shown the importance of Tousled-like kinase 1 (TLK1) and its alternate start variant TLK1B in cell survival against genotoxic stresses. Through a high-throughput library screening of natural compounds, the phenolic phytochemical, gallic acid (GA), was identified as a modulator of TLK1/1B. This small molecule possesses anti-oxidant and free radical scavenging properties, but in this study, we report that *in vitro* it promotes survival of human salivary acinar cells, NS–SV–AC, through repair of ionizing radiation damage. Irradiated cells treated with GA show improved clonogenic survival compared to untreated controls. And, analyses of DNA repair kinetics by alkaline single-cell gel electrophoresis and γ-H2AX foci immunofluorescence indicate rapid resolution of DNA breaks in drug-treated cells. Study of DR-GFP transgene repair indicates GA facilitates homologous recombinational repair to establish a functional GFP gene. In contrast, inactivation of TLK1 or its shRNA knockdown suppressed resolution of radiation-induced DNA tails in NS–SV–AC, and homology directed repair in DR-GFP cells. Consistent with our results in culture, animals treated with GA after exposure to fractionated radiation showed better preservation of salivary function compared to saline-treated animals. Our results suggest that GA-mediated transient modulation of TLK1 activity promotes DNA repair and suppresses radiation cytoxicity in salivary gland cells.

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1. Introduction

Head and neck cancer accounts for nearly 0.5 million new cases each year, worldwide. Radiotherapy alone or in conjunction with surgery and, or, chemotherapy is the standard of care for most patients treated with curative intent. A debilitating side–effect of regional radiation is salivary hypofunction. Within the first weeks from onset of radiotherapy, patients experience oral dryness with a decline in salivary function that continues well beyond the cessation of therapy. Poor salivary function is attributed to dys-function and death of the fluid-producing acinar cells. Unrepaired DNA damage threatens genomic integrity and cell survival, and timely repair can avert the loss of salivary gland function. Since temporary relief of symptoms with sialogogues or pro-secretory drugs is the standard of care for dry mouth, cancer treatment planning centers around its prevention whilst new treatments are explored.

A human homolog of *A. thaliana* Tousled is Tousled-like kinase 1 (TLK1) [1]. The TLK1 protein variant, TLK1B, utilizes an alternate translation start site, and encodes a shorter protein that shares an identical kinase domain. When expressed in normal epithelial cells, TLK1B increases cell survival against genotoxic stresses, and the identification of its substrates, histone H3, anti-silencing factor (Asf1), and Rad9 provided clues to its association with chromatin dynamics [2–4]. Subsequent studies confirmed the role of TLK1B in DNA damage response and in chromatin remodeling at sites of DNA breaks [5,6]. Cells expressing the kinase-defective mutant are sensitive to radiation, and it suggests an important role of the kinase in cell survival after genotoxic stress [7]. Radiation-induced activation of checkpoint kinase, Chk1, was essential for transient downregulation of TLK1 and the establishment of replication checkpoint. TLK1 activity dropped soon after radiation, and

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recovered quickly. An inverse correlation was demonstrated between Chk1 and TLK1 activities [8]. In the current study, we identified a naturally occurring small molecule, GA, as an activator of TLK1B. Since the full-length and the shorter variant of the protein have identical C-terminal kinase domains, they exhibit same substrate specificities. It is, therefore, anticipated that the pharmacological agent will activate TLK1 as well.

Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring phytochemical that has a pyrogallol moiety similar to epigallocatechin – 3-gallate (ECGC), a constituent of green tea. It is abundant in a number of plant foods such as strawberries, blueberries, grape seeds, gallnuts, witch hazel, and processed beverages such as tea and red wine. GA is a part of the ECGC ester, and the pyrogallol moiety of these compounds is attributed to formation of topoisomerase-DNA complexes [9]. Similar to ECGC, GA possesses anti-oxidant and anti-carcinogenic activities. The antiproliferative and apoptotic effects of the compound on cancer cells have been demonstrated [10–13]. And, advantageously utilizing its anti-oxidant property, reports showed that pretreatment protects cellular DNA against radiation-induced oxidative damage. In animal studies, high doses of the drug protected against chromosomal breaks and aberrations in bone marrow cells after total body radiation [14,15]. The radioprotective effect of GA was confirmed in a clinical trial where its supplementation in diet reduced the number of oxidized bases in lymphocytes and increased their resistance to reactive oxygen species damage [16]. To circumvent the influence of the compound's antioxidant effect in our study, GA treatment commenced after radiation to study its effect on TLK1-influenced DNA repair and cell survival.

2. Materials and methods

2.1. Library screen

Compounds from the NIH Clinical Collection, the Prestwick Chemical Library, the ChemDiv Library, and the Enzo Life Sciences Redox Library were screened for modulators of TLK1B. Bacterially isolated recombinant protein TLK1B was reacted with its phosphorylation substrate, Rad9, in Kinase Assay buffer (ADP-Hunter Plus Assay, Discoverx). The ADP generated during the kinase reaction was measured by the formation of florescent Resorufin, at 590 nM using Synergy 4 Hybrid Microplate Reader (Biotek).

2.2. In vitro kinase assay

Bacterially isolated recombinant proteins, 0.5 μg TLK1B and 3 μg histone H3 (New England Biolabs) were incubated in kinase buffer (15 mM HEPES pH 7.5, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Tween 20, and 200 μM ATP) with or without GA (Sigma, 50 μM) for indicated times at room temperature. Reactions were stopped with the addition of Lammeli buffer, heated, and loaded on a SDS-PAG. Immunoblots were reacted with anti-serum to phospho-S – 10 Histone H3 (Millipore) followed by HRP-conjugated anti-rabbit antibody (Vector Laboratories).

2.3. Cell culture

The human submandibular acinar cells, NS-SV-AC, were a kind gift from Dr. Masayuki Azuma (University of Tokushima School of Dentistry, Tokushima, Japan) [17]. The immortalized cell line was cultured in complete keratinocyte growth medium 2 (KGM – 2; Lonza) supplemented with antibiotic/antimycotic (Invitrogen) at 37 °C in a humidified CO_2 incubator. Knockdown of TLK1/1B in NS-SV-AC was achieved by transfecting cells with human 29-mer TLK1 shRNA (Origene; ATTACTTCATCTGCTTGGTAGAGGTGGCT), and selecting a multiclonal cell population under puromycin (1 μg/ml) challenge. GA was dissolved in water, and 10 mM solutions were made fresh for each experiment.

2.4. MTS assay

To quantitatively determine cell viability, a colorimetric assay based on the ability of viable cells to reduce tetrazolium compound, MTS (3-(4,5-dimethythiazol – 2-yl) – 5-(3-carboxymethoxyphenyl) – 2-(4-sulfophenyl) – 2H-tetrazolium), into a soluble formazan dye was used. Exponentially growing NS–SV–AC cells were trypsinized, and cell suspensions (1×10^5 cells/ml) were exposed to radiation (0, 2, 4, 8, or 12 Gy). Cells were seeded in triplicates into 96-well plates at different densities (500–3000 cells/well), and treated with 50 μM GA 30 min later. After 16 h, the drug was washed out and cells were placed in drug-free medium. Cell viability was quantified on day 3 using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) as per the manufacturer's protocol. After 2 h incubation with MTS, absorbance was measured at 490 nm using a multi-well plate spectrometer reader. Absorbance values were normalized to non-irradiated controls, and cell viability was expressed as percentage in relation to controls. The experiment was repeated, and the data were averaged and expressed as mean ± SEM.

2.5. Colony survival assay

Cells from sub-confluent culture plates were trypsinized and resuspended at 1×10^5 cells/ml. they were irradiated at 0, 2, 4, 8, or 12 Gy, and seeded in triplicates at different densities in 12-well plates. Cells were similarly treated with GA and thereafter, placed in fresh medium. On day 10 post-radiation, cell colonies were stained with crystal violet and counted. The results are an average of 3 experiments and are expressed as fraction of surviving colonies in irradiated wells compared to non-irradiated (plating efficiency) controls.

2.6. Single-cell alkaline gel electrophoresis (COMET assay)

DNA single and double strand breaks were analyzed by single-cell electrophoresis in alkaline conditions using the Trevigen Comet Assay kit (Trevigen). Sub-confluent cell cultures in 35 mm plates were irradiated (8 Gy) on ice, and cells were collected immediately or allowed to recover at 37 °C. To determine the effect of GA on DNA repair, cells were treated 30 min after radiation, and at stated recovery times, cells were trypsinized, washed, and resuspended in Ca^{+2} and Mg^{+2} -free ice cold PBS, pH 7.4. An aliquot of resuspended cells was mixed with low melting agarose at 37 °C, and 50 μl of mix was evenly spread in each well of the comet slide. The agarose was allowed to set at 4 °C, 10 min, before immersing the slides in prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X – 100, pH 10) overnight at 4 °C. DNA denaturation was performed in alkaline solution (300 mM NaOH, 1 mM EDTA, pH > 13) in the dark for 20 min at room temperature before electrophoresis (1 V/cm, 30 mA) at 4 °C for 30 min in prechilled alkaline solution. Slides were rinsed in distilled water twice followed by a rinse in 70% ethanol. Slides were dried before staining with 100 μl propidium iodide (Sigma; 10 μg/ml in PBS, pH 7.4) for 20 min. Images were captured using a TRITC filter mounted on an Olympus epi-fluorescence microscope, and the DNA tail length and tail moment were measured using CASP software (http://www.casp.of.pl). DNA tail lengths of > 50 cells/treatment group were measured, and the data were statistically evaluated.