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Galloyl moieties enhance the dentin biomodification potential of plant-derived catechins

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ABSTRACT

Proanthocyanidin-rich plant-derived agents have been shown to enhance dentin biomechanical properties and resistance to collagenase degradation. This study systematically investigated the interaction of chemically well-defined monomeric catechins with dentin extracellular matrix components by evaluating dentin mechanical properties as well as activities of matrix metalloproteinases (MMPs) and cysteine-cathepsins (CTs). Demineralized dentin beams (n = 15) were incubated for 1 h with 0.65% (+)-catechin (C), (−)-catechin gallate (CG), (−)-gallocatechin gallate (GCG), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin-3-gallate (EGCG). The modulus of elasticity (E) and the fold increase in E were determined by comparing specimens at baseline and after treatment. Biodegradation rates were assessed by differences in percentage of dry mass before and after incubation with bacterial collagenase. The inhibition of MMP-9 and CT-B by 0.65, 0.065 and 0.0065% of each catechin was determined using fluorimetric proteolytic assay kits. All monomeric catechins led to a significant increase in E. EGCG showed the highest fold increase in E, followed by ECG, CG and GCG. EGCG, ECG, GCG and CG significantly lowered biodegradation rates and inhibited both MMP-9 and CT-B at a concentration of 0.65%. Overall, the 3-*O*-galloylated monomeric catechins are clearly more potent than their non-galloylated analogues in improving dentin mechanical properties, stabilizing collagen against proteolytic degradation, and inhibiting the activity of MMPs and CTs. The results indicate that galloylation is a key pharmacophore in the monomeric and likely also in the oligomeric proanthocyanidins that exhibit high cross-linking potential for dentin extracellular matrix.

1. Introduction

Plant extracts rich in polyphenols have shown pronounced bioactivity with cellular and extracellular matrix components, and are promising candidates for applications in many disease processes. In particular, sources rich in proanthocyanidin (PAC) have demonstrated applicability in the dental field for enhancement of dentin biomechanical properties and biostability [1,2]. While a strong correlation between the degree of polymerization of oligomeric PACs (OPACs) and an increased modulus of elasticity of the dentin matrix has been demonstrated, the protective effect against bacterial collagenase greatly increases regardless of the fingerprint composition of the PAC plant source [3]. Due to the highly diverse composition of monomeric and oligomeric PACs in the different plant taxa and the associated analytical complexity, their use as standardized intervention material is still limited. While lower-molecular-weight compounds (up to trimers) are available commercially, albeit in very limited quantities for non-monomers, the isolation and standardization of OPACs still represent a challenge and require the development of separation and other analytical techniques, especially for the higher-molecular-weight bioactive compounds.

The OPACs produced by certain plants represent renewable and sustainable resources and are composed of flavan-3-ol building blocks, which are characterized by a saturated C-ring and a hydroxylation in position C-3. The monomeric PAC moieties, named catechins, are abundant in green and white tea, while their dimeric, trimeric and higher oligomeric forms (OPACs) are more abundant in grape seed, pine barks, cocoa and other plant products [3–5]. Catechins can be classified according to their stereochemistry, the different substituents in the B ring, and the presence or absence of

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3-*O*-galloylation. Notably, OPACs can contain two different "gallo" motifs: in the catechin gallates, a gallic acid moiety esterifies the C-3-OH group of the catechin moiety; in gallocatechins, the B-ring of the catechin moiety bears the same 3,4,5-trihydroxy substitution as gallic acid. Variations in the chemical structure of catechins affect their binding affinity with proteins [6]. Galloylated catechins have shown better potential benefits (antiproliferative, apoptotic and antioxidant) as anticancer agents when compared to other monomeric catechins [7,8]. Although it has been proposed that galloylated catechins can stabilize collagen by hydrogen and hydrophobic bonds [9,10], the dentin model explored herein specifically addresses the interactions of monomeric compounds to an already innately highly cross-linked collagen structure. In addition, the dentin matrix contains non-collagenous proteins and endogenous proteases, providing a dynamic system for the study of polyphenols on extracellular matrix.

The aim of the present study was to determine the interactions of the monomeric catechins with dentin organic matrix components (type I collagen, matrix metalloproteinases (MMPs) and cysteine-cathepsins (CTs)) and their effects on mechanical properties and biostability. The hypotheses tested were the following: 1) the modulus of elasticity and the collagen resistance to degradation of dentin varies when treated with the different monomeric catechins; and 2) the activities of recombinant MMPs and CTs are inhibited to a different extent by the various monomeric catechins.

2. Materials and methods

The monomeric catechins (+)-catechin (C), (−)-catechin gallate (CG), (−)-gallocatechin gallate (GCG), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin-3-gallate (EGCG) were obtained commercially (C from Indofine Chemical Company, Inc., Hillsborough, NJ, USA; the others from Sigma–Aldrich, St. Louis, MO, USA), and were comprehensively characterized by nuclear magnetic resonance, mass spectrometry and Liquid chromatography analysis [11]. Force field calculations (MM2) were performed using ChemBioDraw® (PerkinElmer, Waltham, MA, USA). The chemical structure of all monomeric catechins used is depicted in Fig. 1.

2.1. Sample preparation

Twenty-five intact human molars were selected following a protocol approved by the Institutional Review Board of the University of Illinois at Chicago (#2011-0312). Specimens were obtained from mid-coronal dentin and cut with a slow-speed diamond saw under water lubrication (Isomet, Buehler, Lake Bluff, IL, USA) as previously described [1]. The dentin beams were demineralized in 10% phosphoric acid (Ricca Chemical Company, Arlington, TX, USA) for 5 h [12]. Demineralized dentin beams were randomly divided into eight groups ($n = 15$) and treated with 0.65% (w/v) C, CG, GCG, EC, ECG, EGC and EGCG dissolved in 0.02 M HEPES buffer (pH 7.2) for 1 h at room temperature. A control group was incubated with HEPES buffer under the same experimental conditions. The solutions of GCG and EGC presented as a pale pink color. However, even after 1 h of incubation, no major alterations in the color of the dentin beams were observed. The solutions of all other compounds were colorless.

2.2. Biomechanical analysis – apparent modulus of elasticity

Dentin beams were tested in a three-point bending assay as previously described [13], using a 1 N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at a crosshead speed of 0.5 mm min^{-1}. The apparent modulus of elasticity (E) was determined at baseline and after 1 h exposure to catechins using the following formula: $E = PL^3/4DbT^3$, where P is the maximum load, L is the support span, D is the displacement, b is the width of the specimen and T is the thickness of the specimen. The fold increase in E before and after treatment was also calculated. Data were analyzed by two-way and one-way analysis of variance (ANOVA) and Games–Howell post hoc tests ($\alpha = 0.05$).

2.3. Dentin biodegradation – bacterial collagenase

Dentin biodegradation was assessed by its resistance to bacterial collagenase digestion. After the exposure to catechins and biomechanical analysis, the same specimens were dried in a vacuum desiccator containing anhydrous calcium sulfate for 24 h at room temperature and the mass (M_1) was obtained on an analytical balance (XS105DU, Mettler Toledo Inc., Columbus, OH). Then, beams were rehydrated in distilled water for 1 h and incubated with type I bacterial collagenase (100 μg ml^{-1} *Clostridium histolyticum*; Sigma–Aldrich) in 0.2 M ammonium bicarbonate pH 7.9 at 37 °C for 24 h under constant agitation (0.55 RCF). After digestion, beams were washed, dried as described before and the final mass (M_2) was obtained. Dentin biodegradation rates (R) were determined by the following formula: $R\ (\%) = 100 - (M_2 \times 100)/M_1$, where M_1 is the biomodified dentin matrix dry mass and M_2 is the dry mass after bacterial collagenase digestion. Data were statistically analyzed by one-way ANOVA and Games–Howell post hoc tests ($\alpha = 0.05$).

2.4. Inhibition of proteases

A recombinant human enzyme MMP-9 (Anaspec Inc., Freemont, CA, USA), a generic MMP assay kit (SensoLyte® 520 generic MMP assay kit fluorimetric, Anaspec Inc.) and a CT-B assay kit (SensoLyte® 440 cathepsin B assay kit fluorimetric, Anaspec Inc.) were used to evaluate the inhibition of MMPs and CTs by the catechins. Assays were performed in 96-well microplates. The recombinant enzymes were preincubated with 0.65, 0.065 and 0.0065% C, CG, GCG, EC, ECG, EGC and EGCG, individually, for 15 min at room temperature before adding the substrates. The positive control groups were MMP-9 and CT-B only, without catechins, while the negative controls were MMP-9 incubated with EDTA (2 mM), a broad-spectrum MMP inhibitor and CT-B incubated with 1 μM Ac-LVK-CHO (the inhibitor supplied in the kit). The fluorescence was read at baseline and after 4 and 24 h of incubation in triplicate with microplate readers using Ex/Em = 490 nm/520 nm (Synergy 2 BioTek, Winooski, VT, USA) for MMP-9 and Ex/Em = 354 nm/442 nm for CT-B (Victor™ X5, PerkinElmer Inc., Downers Grove, IL, USA). Fluorescence at the different time points was determined by subtracting the background fluorescence and was expressed in arbitrary fluorescence units. Activity was expressed as a percentage of inhibition according to the positive control group's activity after 4 h of incubation. Statistical analysis was performed by two-way and one-way ANOVA and Games–Howell post hoc tests ($\alpha = 0.05$).

3. Results

The two-way ANOVA test depicted statistically significant differences in E between groups before and after treatment with catechins ($p < 0.001$) and among the different catechins ($p < 0.001$). Dentin treated with CG, GCG, ECG and EGCG presented significantly higher values of E than that treated with C, EC and EGC. These last three groups showed no statistical difference when compared to the control group (Table 1). Significant differences among catechins were observed for the fold increase in E ($p < 0.001$). The highest results were observed for EGCG, followed by ECG, CG and

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