J Mater Sci: Mater Med (2010) 21:1435–1443
DOI 10.1007/s10856-010-4019-3

The inhibition of collagenase induced degradation of collagen by the galloyl-containing polyphenols tannic acid, epigallocatechin gallate and epicatechin gallate

John K. Jackson · Jinying Zhao · Wesley Wong · Helen M. Burt

Received: 21 August 2009 / Accepted: 30 January 2010 / Published online: 17 February 2010

Abstract Collagen based cosmetic fillers require repeat treatments due to collagenase derived degradation of the filler in the intradermal injection site. The objective of this study was to investigate the inhibition of this degradation by the galloyl-containing compounds tannic acid, epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and gallic acid (GA). A gel permeation chromatography assay was developed to quantitate the collagenase induced reductions in collagen molecular weight. The binding of the compounds to collagen was measured using HPLC. The stabilization of collagen was measured using Differential Scanning Calorimetry (DSC). Tannic acid, EGCG and ECG (but not GA) were found to strongly inhibit collagen degradation at concentrations in the low micromolar range. The compounds bound strongly to collagen and stabilized collagen. It is concluded that tannic acid, EGCG and ECG bind to collagen via extensive hydrogen bonding augmented by some hydrophobic interactions and prevent the free access of collagenase to active sites on the collagen chains.

1 Introduction

Since the injectable collagen implant Zyderm® received FDA approval for soft tissue augmentation in 1981, collagen products have been the most widely used skin cosmetic fillers in Europe and North America [1]. Injectable collagen fillers are ideally suited to correcting cosmetic defects by producing a more natural looking improvement of soft tissue contours than botox-induced muscle relaxation or skin stretching plastic surgery techniques. However, products such as Zyderm® are degraded by endogenous collagenase within 6 months requiring repeated treatments [1]. Mild glutaraldehyde cross linking of collagen provides some protection against this degradation but cross linked collagen products like Zyplast® still lose their cosmetic effect within 1 year [2–4].

Since the mid 1990s, crosslinked hyaluronic acid based skin fillers such as Hyalform® or Restylane® have become more popular than collagen based products [2, 5]. Although these products are non-immunogenic, inject easily and are reported to be more durable than crosslinked collagen, they still require repeat treatments within 1 year [2, 3, 5].

More recently, microparticulate fillers, often suspended in injectable collagen or hyaluronic acid have been developed to provide longer lasting results [2, 3, 6]. Accordingly, non resorbable cosmetic fillers such as Matridex® (cross linked dextran particles suspended in hyaluronic acid) or Artecoll® (poly methyl methacrylate particles suspended in collagen) may offer almost permanent filling effects via induction of collagen formation and fibroblast infiltration into the area. However, serious delayed complications may be associated with the use of these products such as granuloma, requiring surgical treatment [1–3, 6].

Ideally, cosmetic fillers should be biocompatible, easy to inject and provide long term volume retention for 2–5 years. Clearly, crosslinking strategies that maintain the initially designed sculptured contours of the implant are preferable to the unpredictable effects of microparticulate-induced neocollagenesis. Collagen has a 20-year record of success as a short term cosmetic filler but attempts to extend the lifetime of the collagen using glutaraldehyde cross linking have failed due to toxicity related restrictions

J. K. Jackson (✉) · J. Zhao · W. Wong · H. M. Burt
Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC V6T 1Z3, Canada
e-mail: burt@interchange.ubc.ca

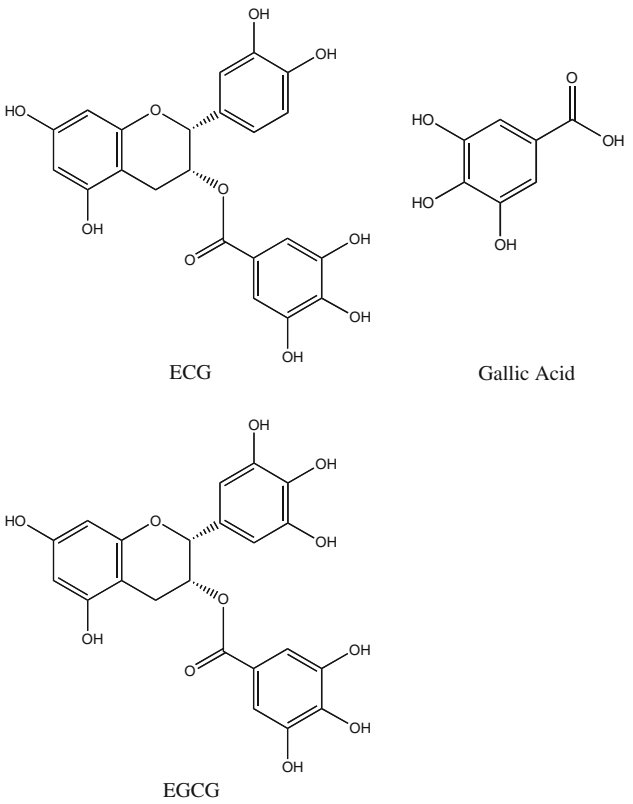

Fig. 1 Schematic of chemical structures of Gallic acid, ECG and EGCG

on permissible glutaraldehyde concentrations. [1, 3, 7]. Recently, in comparison studies with Zyplast®, covalent crosslinking of collagen using ribose has been reported to provide an extended lifetime for collagen implants [7].

Tannins have a long history of use in stabilizing collagen in the leather industry [7, 8] and are known to strongly bind to proteins [9, 10]. The gallotannin groups in large tannin molecules are known to provide extensive hydrogen bonding capacity with collagen and thus stabilize the protein chains. More recently catechin molecules which also contain galloyl groups have been shown to stabilize collagen not only through H-bonding but also by hydrophobic interactions of the polyphenols with collagen chains [11–13]. In particular, epigallocatechingallate (EGCG) (Fig. 1) has been shown to stabilize collagen and protect the chains from collagenase degradation more effectively than chemical cross linking agents such as glutaraldehyde or carbodiimides [14]. It is hypothesized that collagen used in dermal filler applications may be stabilized by EGCG so that access of collagenase to cleavage sites may be restricted. This process may therefore preserve the implanted collagen from enzyme induced degradation to extend the lifetime of the implant.

The objective of this study was to investigate the effect of gallic acid, epicatechingallate (ECG) and EGCG on collagenase-induced degradation of collagen. These studies demonstrate strong binding and stabilization of collagen by ECG and EGCG. Tannic acid, ECG and EGCG all provided effective inhibition of collagen degradation by collagenase at concentrations in the low micromolar range.

2 Materials and methods

2.1 Materials

The collagen solution was Vitrogen® supplied by Cohesion (Palo Alto, CA) at a concentration of 2.9 mg/ml, pH 2.5. Collagenase from Clostridium histolyticum type H was obtained from Sigma-Aldrich Canada Ltd., Oakville, ON. Epigallocatechin gallate (EGCG) with a purity >90% was obtained from DSM Nutritional Products Ltd., Wumisweg, Switzerland. Epicatechin gallate (ECG) and gallic acid (GA) (Sigma) were used as supplied. The two commercial collagen fillers used as supplied were Cosmoderm® and Cosmoplast®. Both products are allogeneic human cultured collagen obtained from Inamed Corp, Santa Barbara, CA.

2.2 Collagen degradation assay by gel permeation chromatography

Collagen degradation may be described either by monitoring the amount of collagen decreasing or by the amount of degradation product increasing. The collagen and collagen degradation product concentrations were determined using gel permeation chromatography (GPC). GPC has been previously used to characterize different molecular weight collagens and to measure the degradation products of collagen [15, 16] Samples were analysed using a mobile phase of 0.1 M NaCl (pH 3.5) with a flow rate of 0.8 ml/min and separation was achieved using an Ultrahydrogel 250 column (Waters). Detection was by a Waters model 2410 refractive index detector at 30°C with a Waters model 717 plus auto sampler.

For incubations with collagenase, the acidic collagen solution was neutralized to pH 7.4 using a calculated volume of 0.1 M Tris-HCl (with 140 mg/ml $CaCl_2$, pH 7.4). Fixed amounts of collagen (725 μg/ml) with different collagenase concentrations were incubated at 37°C and sampled at 1, 2, 3, 4, 24 h. Collagenase at a concentration of 1.25 μg/ml was used for the following degradation study.

2.3 Characterization of the inhibition of collagen degradation by polyphenols

The polyphenols EGCG, ECG and GA were used over a concentration range of 2.5 μg/ml to 250 μg/ml in these studies. A collagen solution of 250 μl (pH 2.5, 725 μg/ml)

was neutralized using a calculated volume of 0.1 M Tris-HCl (with 140 mg/ml $CaCl_2$, pH 7.4). Different volumes of each polyphenol in DI water solution were added to achieve the required concentration and then 5 µl of collagenase solution in PBS (pH 7.4) was added to make up to 1 ml in total volume. The contents were mixed and left tumbling in a 37°C oven for appropriate times.

The reactions were stopped by the addition of HCl (20 µl of 1 M). The pH was adjusted to ~2.5 to dissolve the remaining collagen (precipitated at pH 7.4) and ensure zero collagenase activity. In the inhibition studies, some of the collagen that was crosslinked following the addition of the polyphenol could not be dissolved after the pH adjustment. These vials were centrifuged and the supernatant was taken for the GPC analysis.

The original non-degraded collagen gave a single peak at 7 min equivalent to a molecular weight of approximately 80,000 Daltons. Following the addition of collagenase, all the collagen degraded after 24 h so that the peak at 7 min completely disappeared and a new degradation peak appeared at 11 min indicating a drop in molecular weight to approximately 1000 Daltons. The increase in the height of the 11-min degradation peak was used to quantitate degradation. When the inhibitor was present, the height of the degradation peak was reduced. The degradation peak at 24 h with no inhibitor was used as the 100% degradation value.

2.4 Polyphenol analysis by HPLC

Analysis of EGCG, ECG and GA was performed as previously described [17, 18] using a Waters reverse-phase HPLC system with UV detection. The system consisted of a Waters NovaPak C_{18} column with a mobile phase composed of 0.05% phosphoric acid:acetonitrile:ethyl acetate in the volume ratio of 86:12:2 at pH 2.5. The flow rate was 1 ml/min and the detection was at 280 nm.

2.5 Quantification of drug-protein interactions

The binding of polyphenols to collagen was studied by analyzing the free (unbound) polyphenol concentration after incubation with collagen using the HPLC method described above. Collagen solution (pH 2.5) was neutralized using 0.1 M tris-HCl buffer (pH 7.4) and then the polyphenol was added. The vials were mixed and vortexed for 30 s and then centrifuged at 14000×*g* for 90 s. An aliquot of the supernatant was taken to determine the free polyphenol concentration. Polyphenols such as EGCG are known to dimerize slowly in water but not in acidic conditions [19]. Therefore, the pH of the supernatant was adjusted to 2.5 to ensure the stability of polyphenol during HPLC analysis. Depending on the polyphenol concentration, either 10 µl 1 N HCl was added to 500 µl supernatant or 0.05% phosphoric acid was added to dilute the supernatant to pH 2.5 and then injected into the HPLC.

2.6 Thermal analysis of collagen and polyphenol bound collagens

The thermal stability of collagen and polyphenol-treated collagen were measured using differential scanning calorimetry (Q100 DSC with liquid nitrogen cooling, TA instruments, New Castle, DE). The collagen solution was neutralized to pH 7.4 using 0.1 M NaOH and 0.1 M Tris-HCl buffer. The neutralized collagen solution (10 ml) was transferred to a 15 ml centrifuge tube and either EGCG, ECG or GA was added. The contents were vortexed for 30 s and the tubes were centrifuged at 8000×*g* for 10 min. The supernatant was discarded and the gel was removed from the tube and blotted using a tissue. Gel samples were weighed (10 mg) and sealed in a hermetic DSC pans. The contents were heated from 30 to 90°C at a heating rate of 20°C.

2.7 Water absorption studies

The collagen solution (2.9 mg/ml) was neutralized to pH 7.4 using 0.1 M NaOH and 0.1 M Tris-HCl buffer. The neutralized collagen solution (10 ml) was transferred to a 15 ml centrifuge tube and either glutaraldehyde, EGCG or ECG was added. The contents were vortexed for 30 s and the tubes were centrifuged at 8000×*g* for 10 min. The supernatant was discarded and the weight of the gel samples was measured. Since there was 29 mg of collagen present in the sample the water content by weight was determined as a % of collagen.

2.8 Cell cytotoxicity studies

Human umbilical vascular endothelial cells (HUVEC's) (Lorus, Basel, Switzerland) were cultured in endothelial cell media (Lonza) and seeded at 2000 cells per well in 96 well plates. Crosslinked gel samples from water absorption studies were resuspended in culture media at 2.9 mg/ml and 150 µl of this collagen gel was added to each well. The cells were left to proliferate for 24 h. The gel and media were removed, the cells washed in Hanks buffer pH 7.4 and 18 µl of MTS viability solution in 150ul media was added (MTS: Cell titre 96-Non radioactive cell proliferation assay kit, Promega, Madison, WI, USA). The colorimetric conversion of tetrazolium to formazan in live cells was monitored at 480 nm. Cell viability was calculated as % of control cells (control cells were treated with non crosslinked collagen).

3 Results

3.1 Collagenase degradation of collagen

The collagen from Vitrogen was held in solution at low pH (2.5). Therefore, it was necessary to increase the pH to 7.4 to provide physiologically relevant conditions for collagenase enzymatic effects. At pH 7.4 the solution took on a more viscous hydrogel-like state that still allowed homogenous dispersion of both the enzyme and any potential inhibitor. Following incubation of the collagen with the enzyme for a fixed time and restoration of acidic solubilization conditions (pH 3), the sample was analyzed by GPC. Time and collagenase concentration-dependent shifts in molecular weight was observed from approximately 80 Kd to below 10 Kd indicative of collagen degradation. A typical time course for the degradation of collagen and the generation of the degradation product using a collagenase concentration of 1.25 μg/ml is shown in Fig. 2. This concentration of collagenase was used in all further studies.

3.2 Tannic acid inhibition of collagen breakdown

Following the addition of tannic acid to collagen in solution at pH 7.4, there was an observed increase in the viscosity of the collagen solution/suspension. Tannic acid effectively inhibited collagenase breakdown of collagen as shown in Fig. 3. A concentration as low as 12.5 μg/ml reduced collagen degradation by approximately 32% after 3 h incubation. At a tannic acid concentration of 62.5 μg/ml there was over 70% inhibition of collagenase degradation of collagen after 24 h incubation.

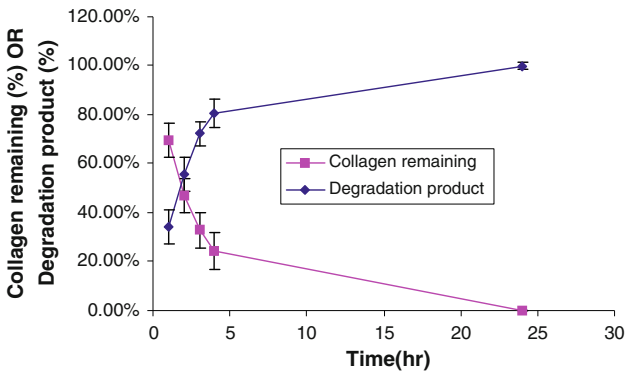

Fig. 2 GPC assay of collagen degradation using collagenase at 1.25 μg/ml. Collagen remaining was quantitated using the 80 KDa peak and collagen degraded was quantitated using the 10 KDa peak. Incubations done at 37°C and pH 7.4. Results show Mean ± SD, $n = 3$

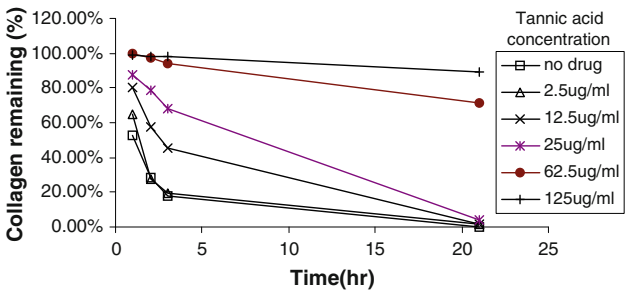

Fig. 3 Effect of tannic acid on collagen degradation using collagenase at 1.25 μg/ml. Incubations done at 37°C and pH 7.4

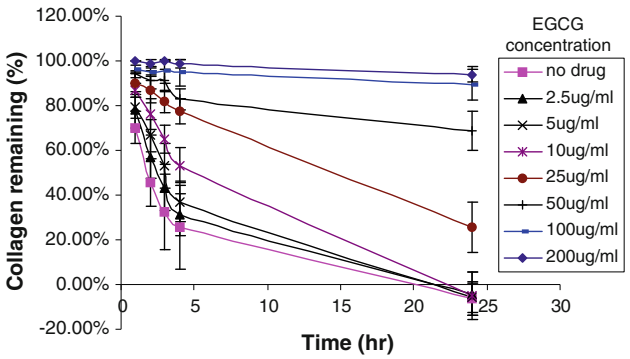

Fig. 4 Effect of EGCG of collagen degradation using collagenase at 1.25 μg/ml. Incubations done at 37°C and pH 7.4. Results show Mean ± SD, $n = 3$

3.3 EGCG inhibition of collagen degradation

Like tannic acid, the addition of EGCG to collagen resulted in a significant increase in viscosity of the solution/suspension indicating a direct interaction between the polyphenol molecules and collagen. EGCG effectively inhibited collagenase degradation of collagen in a concentration dependent manner as shown in Fig. 4. All experiments were completed on four separate occasions and data points represent mean values with standard deviations as error bars. Following 3 h incubation, the inhibition of collagenase activity was statistically significant at 10 μg/ml (Students t-test at $P < 0.05$).

3.4 The effect of ECG and gallic acid on collagenase degradation of collagen

EGC effectively inhibited collagenase degradation of collagen as shown in Fig. 5. However, concentrations in the 25–50 μg/ml range were required to inhibit degradation after 3 h incubation. Although these concentrations were higher than for EGCG, the agent was still effective at almost completely blocking collagenase degradation of collagen at higher concentrations after even 24 h of

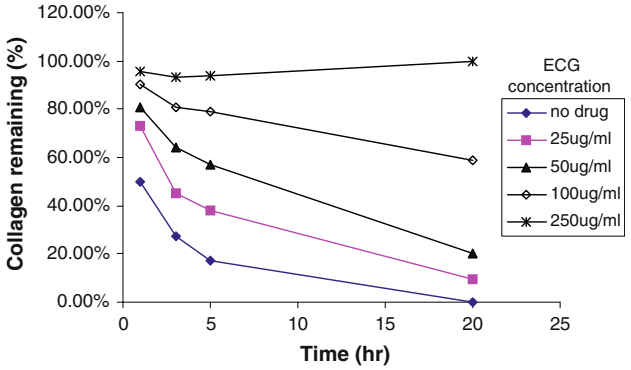

Fig. 5 Effect of ECG on collagen degradation using collagenase at 1.25 μg/ml. Incubations done at 37°C and pH 7.4

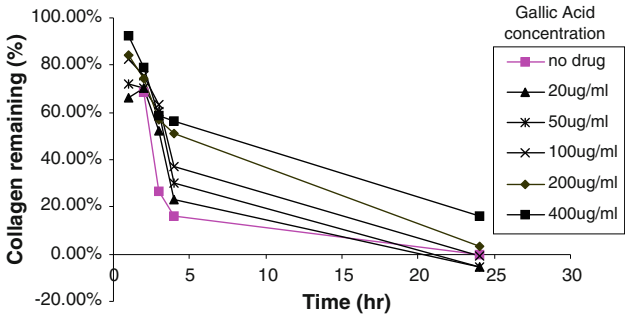

Fig. 6 Effect of gallic acid on collagen degradation using collagenase at 1.25 μg/ml. Incubations done at 37°C and pH 7.4

incubation. Much higher concentrations of gallic acid were required to produce any inhibition of collagenase action on collagen (Fig. 6). Furthermore, although gallic acid inhibited collagnease activity at 3 h using a concentration of between 100 and 400 μg/ml, this inhibition was not sustained as there was about 100% degradation of the collagen at 24 h.

3.5 Binding studies

Both EGCG and ECG bound strongly to collagen in a saturable manner as shown in Fig. 7a and b. Saturation of binding occurred at around 500 μg/ml for ECG and at 1000 μg/ml for EGCG. These binding studies were repeated at different collagen concentrations (150–750 μg/ml) and consistently showed similar saturable binding curves. Scatchard plots of the data gave association constants. This was done using a concentration of 725 μg/ml of collagen for both EGCG and ECG (Fig. 8) and the association constants were 500 M^{-1} and 400 M^{-1}. Gallic acid exhibited only weak binding to collagen using these methods.

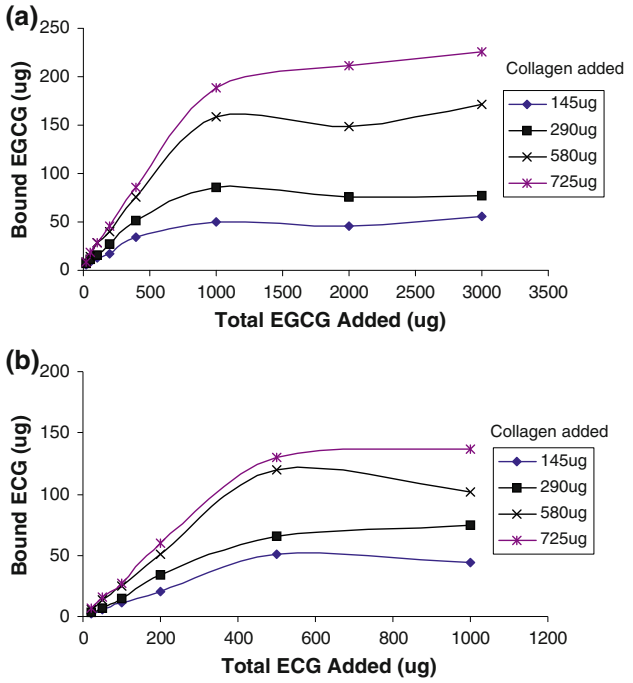

Fig. 7 **a** Binding of EGCG to collagen. **b** Binding of ECG to collagen. The unbound fraction of EGCG or ECG was quantitated. Incubations done at 37°C and pH 7.4

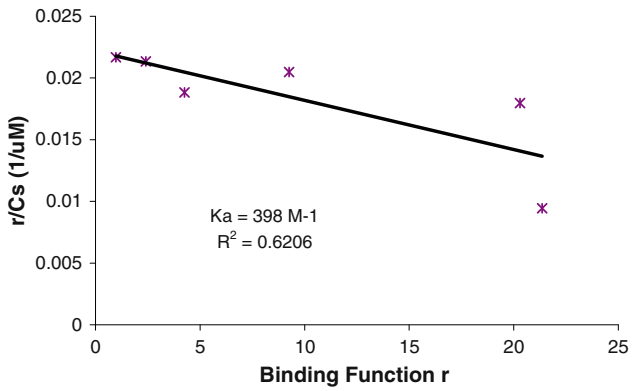

Fig. 8 Scatchard plot of binding of ECG to collagen. Incubations done at 37°C and pH 7.4

3.6 Effect of EGCG and ECG on the thermal stability of collagen

Native collagen undergoes a denaturation effect as evidenced by a shift in baseline at a temperature around 48°C (mid point) using DSC (Fig. 9). Following incubation with either EGCG or ECG, there was a significant increase in the denaturation temperature to around 66°C and 70°C, respectively and evidence of an endothermic peak.

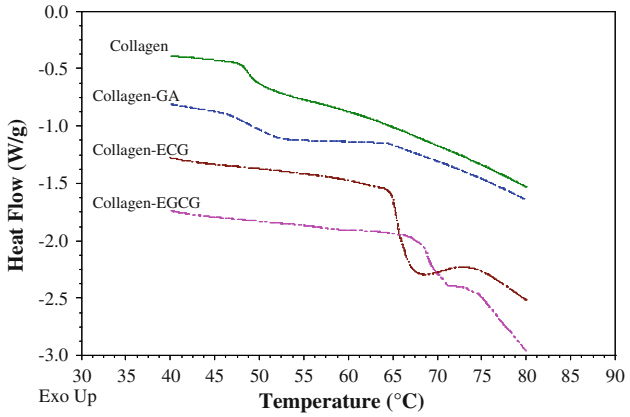

Fig. 9 Thermal analysis (DSC) of collagen treated with EGCG, ECG or gallic acid (GA) (All at a concentration of 400 μg/ml)

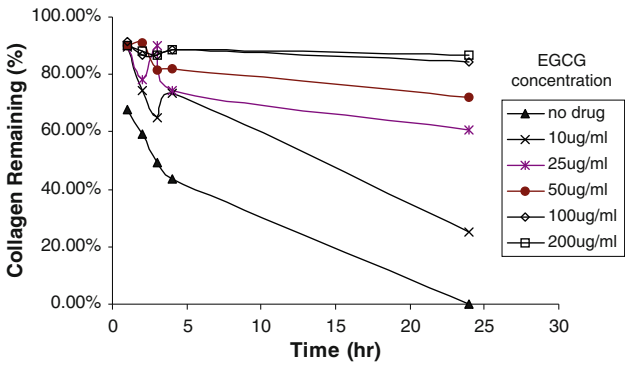

Fig. 10 Effect of EGCG on the degradation of Cosmoderm® by collagenase at 1.25 μg/ml. Incubations done at 37°C and pH 7.4

3.7 The interaction of EGCG with commercial brands of cosmetic collagen implants

Cosmoderm® and Cosmoplast® are allogenic collagen cosmetic fillers manufactured using human fibroblasts. These products were subjected to the same EGCG binding and collagenase inhibition studies as bovine collagen.

Cosmoplast® was found to be essentially unchanged by collagenase. However Cosmoderm® was degraded into lower molecular weight fractions by collagenase in a time and concentration dependent manner, similar to bovine collagen. EGCG inhibited collagenase breakdown of Cosmoderm® at similar concentrations to those used in bovine collagen studies (Fig. 10). This inhibition was evident at a concentration of 10 μg/ml after 3 h incubation. However, there was more effective and durable (24 h) inhibition of collagenase degradation of Cosmoderm® at an EGCG concentration of 25 μg/ml.

EGCG was also found to bind effectively to both Cosmoderm® and Cosmoplast® as shown in Fig. 11. The saturation of this binding occurred at an EGCG concentration

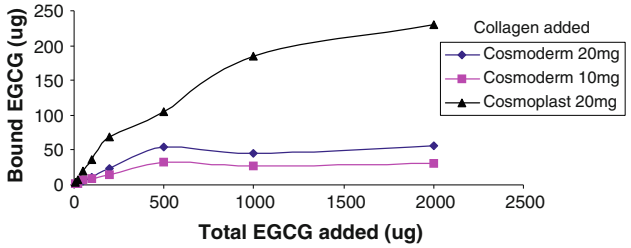

Fig. 11 Binding of EGCG to Cosmoderm® or Cosmoplast®. The unbound fraction of EGCG was quantitated. Incubations done at 37°C and pH 7.4

in the 500–1000 μg/ml range. Scatchard plots for EGCG binding to Cosmoderm® and Cosmoplast® gave similar binding association constants as found for EGCG binding to bovine collagen. These values were 600 M^{-1} and 700 M^{-1}.

3.8 Water absorption studies

All gel samples contained large amounts of water. Control gels (non crosslinked) held 6670% water by weight. Collagen crosslinked with glutaraldehyde (0.0075%), glutaraldehyde (1%), EGC (400 μg/ml) or EGCG (400 μg/ml) absorbed 9850%, 10,637%, 8590% and 9990% water, respectively.

3.9 Cell cytotoxicity studies

Only glutaraldehyde crosslinked gels displayed any significant toxicity to HUVEC's as seen in Fig. 11. Even a concentration as low as 0.0075% (used in commercial collagen injectables) reduced cell viability from 100% in control cells to 69%. A gel crosslinked with a higher concentration of glutaraldehyde (1%) reduced cell viability to just 14%. Cells treated with gels crosslinked with ECG or EGCG at high concentrations (400 μg/ml) had no toxic effect on HUVEC's (Fig. 12).

4 Discussion

Injectable cosmetic fillers have demonstrated biocompatibility of the collagen and hyaluronic acid materials and ease of injection but suffer the problems of short lived cosmetic fillers with the need to repeat injections within 1 year. Clearly, a more durable collagen or hyaluronic acid based filler might allow for a prolonged sculptured cosmetic effect and an acceptable retreatment schedule.

The degradation of collagen or hyaluronic acid implants is largely due to enzymatic degradation by collagenase or hyaluronidase. Techniques to slow this breakdown might include the use of enzyme inhibitors or inhibition of the

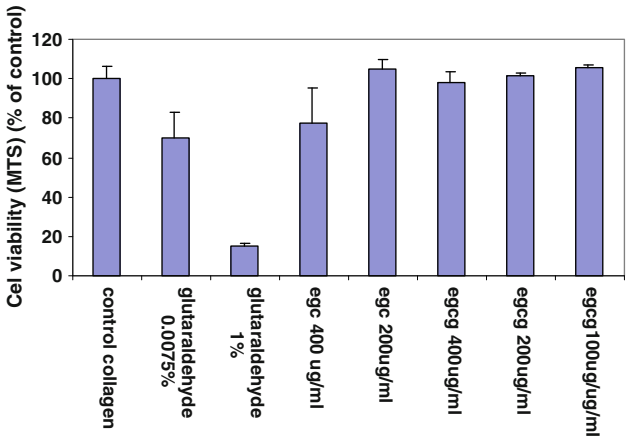

Fig. 12 Effect of glutaraldehyde, ECG or EGCG on HUVEC viability. Cells treated for 24 h, washed and viability measured using an MTS assay

production and secretion of the enzymes by cells surrounding the implants. However, these methods probably require an extended presence of inhibitors and the agents may result in unwanted effects on normal tissue homeostasis [20].

A better strategy might be to stabilize the collagen or hyaluronic acid injectable using chemical crosslinking. This might inhibit access of the enzyme to active sites on the collagen or HA or slow proteolytic breakdown. However, glutararaldehyde or carbodiimide covalent chemical crosslinking is restricted to the use of very low concentrations due to the toxicity of these agents so that only minor improvements in durability of injectables is observed with this strategy.

In this study we have investigated the inhibition of collagen degradation by collagenase using compounds containing galloyl moieties that might stabilize collagen though hydrogen bonding. In order to measure collagen degradation, a method that measured collagenase-induced molecular weight decreases of collagen by GPC was developed, similar to previously described methods [15, 16]. Using this method, the time course of collagenase degradation of collagen and any inhibitory effect of galloyl containing polyphenolic compounds was monitored (Fig. 2).

The binding of tannic acid to collagen has been shown to arise from extensive H-bonding to carbonyl groups in collagen as well as minor roles of both ionic and covalent bonding effects [21, 22]. There was visual evidence of such binding in these studies whereby the collagen suspension became more viscous on the addition of tannic acid prior to the addition of collagenase. It is likely that the inhibition of collagen degradation observed in these studies arose from the stabilization of collagen by tannic acid and the reduction of access of collagenase to potential cleavage sites on the collagen chains. It is also possible that any galloyl moieties on tannic acid (either free or bound to collagen) might also bind the protein collagenase, rendering it inactive.

The catechins, EGCG and ECG, which also contain many galloyl groups, behaved similarly to tannic acid, inhibiting collagenase activity at concentrations as low as 10 μg/ml (Figs. 4, 5). EGCG has been previously reported to inhibit collagenase degradation of collagen using higher concentrations of EGCG [11, 23]. Binding studies confirmed a strong binding interaction between both EGCG and ECG with collagen (Figs. 6, 7). It is likely that the mechanism of inhibition of collagen degradation is similar to that described for tannic acid, namely catechin induced cross linking of collagen chains and protection of potential cleavage sites from collagenase attack. There are some reports of EGCG directly inhibiting collagenase activity [14, 24] but others report that EGCG has no effect on collagenase [11]. The strong binding interaction of galloyl containing catechins (but not catechins alone) arises from both H-bonding and hydrophobic interactions with the collagen chains [11–13]. The particularly strong binding observed for EGCG and EGC arises from the high levels of galloyl group flexibility on the catechin molecule allowing for optimal molecular conformation to potential binding sites [11, 13]. For EGCG, this binding is more similar to chemical cross linking of collagen with aldehydes or carbodiimdes [11, 23]. It was interesting to note that all samples absorbed large amounts of water but collagen treated with glutaraldehyde, EGCG or ECG all absorbed approximately 50% more water than control collagen gels. It is likely that the collagen chains in the control sample are more closely associated than the crosslinked chains may be held apart to some degree by the crosslinking molecules. Therefore the crosslinked chains may have more room for water retention within the matrix than the more tightly packed, non-crosslinked samples.

Tannin or aldehyde stabilization of collagen has traditionally been studied using thermal analysis. Untreated collagen undergoes a thermal destabilization process (triple helix unraveling to random coil) at approximately 65°C but cross linking causes this to occur at higher temperatures (approximately 70–80°C) [13, 25–29]. In these studies, EGCG and EGC were found to cause an increase of 15°C in the destabilization temperature indicating crosslinking of the collagen chains (Fig. 9). Although strong chemical crosslinking of collagen may negatively affect the triple helix structure of collagen, EGCG has been reported to leave the structure intact [11]. Therefore, this non-covalent crosslinking effect of collagen by EGCG may leave the material in a more natural and physiologically preferable form. All collagen gels (control or chemically crosslinked) held over 6000% water (as reported in these absorption

studies) so it is unlikely that any difference in water association with polymer chains could result in the change in denaturation temperature described here.

These studies were extended to include collagen based commercial cosmetic fillers. Cosmoplast® is a glutaraldehyde crosslinked version of Cosmoderm® and was found to be largely unaffected by collagenase over the normal course of a 24 h incubation. However, EGCG was found to effectively inhibit collagenase induced degradation of the collagen in Cosmoderm® (Fig. 10) and to bind strongly to both Cosmoderm® and Cosmoplast® (Fig. 11).

HUVEC's are primary endothelial cells that make up capillaries in angiogenic beds of growing or repairing tissues. In this lab we routinely measure the biocompatibility of implantable polymeric material using HUVEC cells as these cells are relevant to most implant settings since vascularization (capillary growth around implant area) is generally required for long term biocompatibility. HUVEC growth is particularly sensitive to culture media components and therefore the growth of these cells is a sensitive barometer of local toxicity. Furthermore, a preferred outcome of subdermal implantation and filling methods is tissue remodeling with new collagen and subsequent vascularization [5, 30, 31]. These factors all support the use of HUVEC cells in the cytotoxicity assay. The ECG or EGCG crosslinked gels had no detrimental effect on the viability of HUVEC's even when crosslinked at concentrations as high as 400 μg/ml (Fig. 12). On the other hand, glutaraldehyde crosslinked gels demonstrated significant toxicity to these cells which was apparent at a concentration as low as 0.0075% (used in commercial formulations). Although a 20% reduction in cell viability might be acceptable in vivo with the 0.0075% glutaraldehyde treatment, the lack of toxicity associated with the catechin treated collagen illustrates the excellent biocompatibility of these treated gels for subdermal applications. It should be noted that EGCG is a powerful antioxidant molecule and is currently included in many soft drinks worldwide and regarded as a healthy food additive, further establishing the suitability of this compound for use in cosmetic formulations.

Overall, these studies using both acid soluble collagen as well as commercial cosmetic collagen fillers strongly support the use of EGCG as a adjunct treatment for collagen based injectables. Although EGCG increased the viscosity of both Cosmoderm® and Cosmoplast® products the products were still readily injectable. it did not render the material non-injectable. Therefore EGCG might be premixed with the collagen filler prior to injection, coadministered using a dual syringe system or, injected any time after the initial collagen injection. This novel use of EGCG as an inhibitor of collagen degradation is the first report of the potential application of this compound as a stabilizing agent in collagen cosmetic fillers.

Acknowledgement This work was supported using funds from Angiotech Pharmaceuticals, Station Street, Vancouver, BC, Canada.

References

1. Goldberg DJ. Fillers in cosmedic dermatology. London, PA: Informa Healthcare; 2006.
2. Eppley BL, Dadvand B. Injectable soft-tissue fillers: clinical overview. Plast Reconstr Surg. 2006;118(4):98e–106e.
3. Werschler WP, Weinkle S. Longevity of effects of injectable products for soft-tissue augmentation. J Drugs Dermatol. 2005; 4(1):20–7.
4. Baumann L, Kaufman J, Saghari S. Collagen fillers. Dermatol Ther. 2006;19:134–40.
5. Owens JM. Soft tissue implants and fillers. Otolaryngol Clin North Am. 2005;38:361–9.
6. Laeschke K. Biocompatibility of microparticles into soft tissue fillers. Semin Cutan Med Surg. 2004;23:214–7.
7. Pitaru S, Noff M, Blok L, et al. Long-term efficacy of novel ribose-cross-linked collagen dermal filler: a histologic and histomorphometric study in an animal model. Dermatol Surg. 2007; 33:1045–54.
8. Covington AD, Lilley TH, Song L, Evans CS. Collagen and polyphenols: new relationships and new outcomes. Part 1. Flavonoid reactions for new tanning processes. Jalca. 2005;100:325–43.
9. Deaville ER, Green RJ, Mueller-Harvey I, Willoughby I, Frazier RA. Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths. J Agric Food Chem. 2007;55:4454–561.
10. Hagerman AE, Butler L. Protein precipitation method for the quantitative determination of tannins. J Agric Food Chem. 1978; 26(4):809–11.
11. Goo HC, Hwang YS, Choi YR, Cho HN, Suh H. Development of collagenase-resistant collagen and its interaction with adult human dermal fibroblasts. Biomaterials. 2003;24:5099–113.
12. Liao XP, Lu ZB, Shi B. Selective adsorption of vegetable tannins onto collagen fibers. Ind Eng Chem Res. 2003;42:3397–402.
13. Tang HR, Covington AD, Hancock RA. Structure-activity relationships in the hydrophobic interactions of polyphenols with cellulose and collagen. Biopolymers. 2003;70(3):403–13.
14. Demule M, Brossard M, Pagé M, Gingras D, Béliceau R. Matrix metalloproteinase inhibition by green tea catechins. Biochim Biophys Acta. 2000;1478:51–60.
15. Deyl Z, Macek K. High-performance gel permeation chromatography of collagens. J Chromatogr. 1982;230:409–14.
16. Meyer M, Morgenstern B. Characterization of Gelatine and acid soluble collagen by size exclusion chromatography coupled with multi angle light scattering (SEC-MALS). Biomacromolecules. 2003;4:1727–32.
17. Saito ST, Froelich PE, Gosaman G, Bergold AM. Full validation of a simple method for determination of catechins and caffeine in Brazilian green tea (Cameillia sinensis assamica) using HPLC. Chrmoatographia. 2007;65:607–10.
18. Xu J, Tan T, Janson JC. One step purification of epigallocatechin gallate from crude green tea extracts by mixed mode adsorption chromatography on highly crosslinked agarose media. J Chromatogr A. 2007;1169:235–8.
19. Hong Jungil, Lu Hong, Meng Xiaofeng, Ryu Jae-Ha, Hara Yukihiko, Yang ChungS. Stability, Cellular uptake, biotransformation, and efflux of tea polyphenol (-)-Epigallocatechin-3-Gallate in HT-29 human colon adenocarcinoma cells. Cancer Res. 2002;62:7241–6.
20. Peterson JT. Matrix metalloproteinase inhibitor development and the remodelling of drug discovery. Heart Fail Rev. 2004;9:63–79.

21. Covington AD. Modern tanning chemistry. Chem Soc Rev. 1997;26:111–26.
22. Calderon P, Van Buren J, Robinson WB. Factors influencing the formation of precipitates and hazes by gelatine and condensed and hydrolysable tannins. J Agric Food Chem. 1968;16(3): 479–82.
23. Cho HH, Matsumura K, Nakajima N, Han DW, Tsutsumi S, Hyon SH. Degradation control of collagen by epigallocatechin-3-o-gallate. Key Eng Mater. 2007;342–343:781–4.
24. Makimura M, Hirasawa M, Kobayashi K, et al. Inhibitory effect of tea catechins on collagenase activity. J Periodontol. 1993;7: 630–6.
25. Celej MS, Montich GG, Fidelio GD. Protein stability induced by ligand binding correlates with changes in protein flexibility. Protein Sci. 2003;12:1496–506.
26. Fathima NN, Madhan B, Rao JR, Nair BU, Ramasami T. Interaction of aldehydes with collagen: effect of thermal, enzymatic and conformational stability. Int J Biol Macromol. 2004;34: 241–7.
27. Tang HR, Covington AD, Hancock RA. Use of DSC to detect the heterogeneity of hydrothermal stability in the polyphenol-treated collagen matrix. J Agric Food Chem. 2003;51:6652–6.
28. McClain P, Wiley E. Differential scanning calorimeter studies of the thermal transitions of collagen. J Biol Chem. 1972;247(8): 692–7.
29. Miles CA, Avery NC, Rodin VV, Bailey AJ. The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibers. J Mol Biol. 2005;346:551–6.
30. Cotran RS, Kumar V, Robbins SL. Pathologic basis of disease. Philadelphia: W.B. Saunders Company; 1989. p. 39–87.
31. Seandel M, Noack-Kunnmann K, Zhu D, Aimes RT, Quigley JP. Growth factor induced angiogenesis in vivo requires specific cleavage of fibrillar type 1 collagen. Blood. 2001;97:2323–32.