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Protease inhibition by oleic acid transfer from chronic wound dressings to albumin

J. Vincent Edwards [a,*], Phyllis Howley [a], Rachel Davis [b], Andrew Mashchak [b], Steven C. Goheen [b]

[a] *United States Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, Cotton Textile Chemistry Research Unit, 1100 Robert E. Lee Blvd., New Orleans, LA 70124, United States*
[b] *Pacific Northwest National Laboratory, National Security Division, 1 Battelle Boulevard, Richland, WA 99352, United States*

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Abstract

High elastase and cathepsin G activities have been observed in chronic wounds to inhibit healing through degradation of growth factors, cytokines, and extracellular matrix proteins. Oleic acid is a non-toxic elastase inhibitor. Cotton wound dressing material was characterized as a transfer carrier for affinity uptake of oleic acid by albumin under conditions mimicking chronic wounds. The mechanism of oleic acid uptake from cotton and binding by albumin was examined with both intact dressings and cotton fiber-designed chromatography. Raman spectra of the albumin–oleic acid complexes under liquid equilibrium conditions revealed fully saturated albumin–oleic acid complexes with a 1:1 weight ratio of albumin:oleic acid. Liquid–solid equilibrium conditions revealed oleic acid transfer from cotton to albumin at 27 mole equivalents of oleic acid per mole albumin. Comparing oleic acid formulated wound dressings for dose dependent ability to lower elastase activity, we found cotton gauze > hydrogel > hydrocolloid. In contrast, the cationic serine protease cathepsin G was inhibited by oleic acid within a narrow range of oleic acid–cotton formulations. 2% albumin was sufficient to transfer quantities of oleic acid necessary to achieve a significant elastase-lowering effect. Oleic acid bound to cotton wound dressings may have promise in the selective lowering of cationic serine protease activity useful in topical application for chronic inflammatory pathogenesis.
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Keywords: Occlusive wound dressings; Oleic acid; Cationic proteases; Cotton; Albumin; Micelles; Chronic wounds

1. Introduction

Human neutrophil elastase (HNE) and cathepsin G (CG) are cationic proteases found in high concentration in the non-healing wound, and are the result of elevated neutrophil levels in the chronic wound environment (Yager and Nwomeh, 1999). An overexuberant neutrophil ingestion is part of the inflammatory pathology characteristic of chronic wounds (Diegelmann, 2003). High neutrophil levels mediate a variety of prolonged chemotactic (Galkowska et al., 2006), proteolytic (Weckroth et al., 1996) and oxidative events (James et al., 2003) that occur in the wound environment and are deleterious to healing. The proteolytic environment of the wound resulting from high neutrophil concentrations is a combination of cationic serine proteases (Grinell and Zhu, 1994; Yager et al., 1997) and matrix metalloproteases (Trengove et al., 1996; Yager et al., 1996). These two families of proteases, which normally augment wound healing (Broughton et al., 2006), at high levels are associated with degradation of important growth factors (Yager et al., 1997) and major proteins of the extracellular matrix like fibronectin (Grinell and Zhu, 1994; Herrick et al., 1997).

Approaches to neutralizing the deleterious effect of high protease levels in chronic wounds include investigation of doxycyline as a topical metalloproteinase inhibitor (Chin et al., 2003) on diabetic foot ulcers, and use of the enzyme inhibitor aprotinin for treatment of corneal ulcers (Salonen et al., 1987). Development of mechanism-based wound dressings designed to sequester proteases directly from the wound environment (Edwards, 2006) and dressings that release protease inhibitors into the wound environment (Lobman et al., 2005) are two approaches to chronic wound treatment, which utilize traditional concepts in enzyme sequestration and inhibition to modulate

* Corresponding author. Tel.: +1 504 286 4360; fax: +1 504 286 4271.
E-mail address: vedwards@srrc.ars.usda.gov (J.V. Edwards).

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protease levels. Current designs in mechanism-based wound dressings to redress the protease imbalance of the chronic wound include collagen and oxidized regenerated cellulose (Cullen et al., 2002), nanocrystalline silver coated high density polyethylene (Wright et al., 2002), bacterial cellulose and collagen (Wiegand et al., 2006), peptide (Edwards et al., 1999a) and carbohydrate derivatized cotton (Edwards et al., 1999b, 2002), ionically derivatized dressings of cotton (Edwards et al., 2001) and hydrogel polymers (Vachon and Yager, 2006). Previously proposed (Wiseman et al., 1992) and reported have been the controlled release of trace elements (Liyanage et al., 1995), antibiotics (Van Wachem et al., 1997), growth factors (Buckley et al., 1985; D'Hemecourt et al., 1998) and protease inhibitors (Edwards et al., 1999a, 2003, 2004) from wound dressings and extracoporeal biomaterials, which include materials like collagen, alginate, chitosan, carboxymethylcellulose, hydrogel polymers, hydrocolloids and polyurethane. Thus, a dressing to wound environment transfer of selective protease inhibitors is a reasonable approach that could provide a direct route to the therapeutic modulation of proteases.

Consideration of the biochemical environment of the wound is important in designing a protease inhibitor bound dressing-to-wound transfer approach with the goal of accelerating healing. Oleic acid is a potent, non-toxic and selective inhibitor of HNE (Ashe and Zimmerman, 1977). However, oleic acid is insoluble in aqueous environments and would require formulation with a carrier to facilitate elastase inhibition in the wound environment. Albumin–oleic acid formulations have been shown to lower elastase activity when released in solution from derivatized cotton wound dressings (Edwards et al., 2004). However, another potential approach to this type of treatment modality is the transfer of oleic acid from the dressing material by native albumin levels in the chronic wound. Endogenously formed oleic acid–albumin complexes that result from wound-to-dressing transfer of oleic acid could function to lower elastase activity similar to synthetic oleic acid–albumin formulations.

Albumin is a carrier of fatty acids in blood, including oleic acid. Both bovine serum albumin (BSA) and human serum albumin (HSA) have up to six primary and secondary binding sites for oleic acid at equilibrium (Spector et al., 1969). However, it is not clear whether albumin will bind more than six oleic acid mole equivalents under native conditions. Many of the studies carried out on fatty acid binding to albumin have been done under liquid phase equilibrium. Since liquid phase equilibrium conditions have a much lower binding efficiency than a liquid–solid equilibrium process, it was anticipated that wound dressing materials could transfer significant amounts of oleic acid to albumin. Albumin levels in the chronic wound environment vary considerably (Trengove et al., 1996; James et al., 2000) and it is not known whether this protein would be present in sufficient concentrations to transport an adequate amount of oleic acid from dressing material to inhibit elevated HNE levels. The present study examines the efficiency of cotton dressing materials to transfer oleic acid in the presence of albumin and various wound dressing models formulated with oleic acid, and for their ability to lower HNE levels under simulated chronic wound conditions at varying albumin concentrations.

2. Materials and methods

2.1. Chemicals

Water was deionized and processed through a Millipore MilliQ Plus system such that the resistance of the water was greater than 18.2 Ω. BSA, oleic acid, porcine pancreatic elastase, and elastase substrate (*N*-(methoxysuccinyl)-Ala-Ala-Pro-Val-4-nitroanilide) were obtained from Sigma (St. Louis, MO). The CG substrate (Suc-Ala-Ala-Pro-Phe-pNA) was obtained from BACHEM, Torrance, CA. HNE and CG were obtained from Athens Research (Athens, GA). All reagents obtained were of the highest purity. HPLC grade acetonitrile was used as a solvent and was supplied by EM Science. For cotton chromatography, the cotton used was pre-ground to pass through an 80-mesh size sifter.

2.2. Preparation of oleic acid-treated cotton, hydrogel, and hydrocolloid dressings

Cotton gauze was treated with oleic acid by applying solutions of oleic acid in acetonitrile (0.5–6.5 mg) to 50 mg swatches of cotton. The oleic acid-treated cotton gauzes were either lyophilized to dryness or dried under a constant stream of nitrogen. Hydrogel (Curagel, Kendall, Mansfield, MA) and hydrocolloid (Duoderm, ConvaTec, Princeton, NJ) dressings were cut into 1 cm^2 pieces (∼0.10–0.2 g). Liquid oleic acid was placed in acetonitrile and the mixture was applied to the dressings at a concentration of 1.6–3.4 mg oleic acid/sample and allowed to evaporate. The resulting swatches were employed in the elastase and other assays.

2.3. Assessment of HNE and CG activity in the presence of oleic acid-treated cotton gauze, hydrogel, and hydrocolloid

The inhibition of HNE and CG was assessed with oleic acid-treated dressings. The oleic acid-treated dressings were prepared as described above. Samples treated with oleic acid were submerged in 1 ml of buffer, HNE (∼0.1 units/ml) or CG (0.3 μg/ml) and varying concentrations of BSA. The samples were allowed to incubate at room temperature for 2 h in the enzyme solutions, whereupon the dressing samples were centrifuged in syringes for 5 min at 4000 rpm to collect all incubation liquor. The assays with HNE were conducted in pH 7.6 buffer composed of 0.1 M sodium phosphate, 0.25 M NaCl. Two hundred microliter aliquots were removed from each incubation liquor sample. Sixty microliter aliquots of substrate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (0.867 mM) were added to initiate the reaction. The assays with CG were conducted in 160 mM Tris–HCl buffer, pH 7.4 with 1.6 M NaCl. Two hundred microliter aliquots were removed from each incubation liquor sample. Fifty microliter aliquots of substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (1 mM) were added to initiate the reaction. Kinetic activity of the enzymes at 37 °C was followed in a Bio-Rad Microplate Reader (Hercules, CA) with a 96-well format, by spectrophotometric measurement of the release of

p-nitroaniline at 405 nm from the enzymatic hydrolysis of the substrates (Nakajima et al., 1979).

2.4. Assessment of oleic acid released from the dressings

Oleic acid-treated samples were incubated in 1 ml of buffer for 2 h and 200 μl aliquots from each tube were diluted to 1 ml followed by addition of 1 ml of 0.15N acetic acid, and internal standard, heptadecanoic acid. A Bligh extraction (Bligh and Dyer, 1959) was completed as follows: 7.5 ml of chloroform/methanol (1:2) was added to the formulation and the solution was vortexed three times at 5 s intervals then centrifuged for 5 min at 4000 rpm, and the supernatant was transferred into a glass tube. The residue was combined with chloroform (2.5 ml), vortexed as above and centrifuged for 5 min at 4000 rpm whereupon the liquid supernatant was added to the glass tube and 2.5 ml of a 0.88% KCl solution was added. The glass tube was vortexed three times at 5 s intervals and centrifuged for 5 min. The lower chloroform layer was transferred to a brown 12 ml vial and the solvent was evaporated to dryness in an argon stream by heating to 45 °C. The dried oleic acid was silylated with 0.3 ml trimethylsilylimidazole (Pierce, Rockford, IL). It was heated at 45 °C for 30 min and injected into a Varian 3600 CX gas chromatograph (Sugar Land, TX) with a dimethylpolysiloxane column DB-1 (J & W Scientific), 30 m × 0.32 mm i.d., and helium as a carrier (1.577 ml/min, 16 psi) with a split ratio of 48:1. Quantitation of peak areas was completed with heptadecanoic acid as internal standard using Turbochrom 4.12 software (PE Nelson, Norwalk, CT).

An HPLC method was utilized to analyze oleic acid and albumin simultaneously. The HPLC method allowed quantification of albumin and oleic acid concentrations. The peak areas obtained from a UV detector were used to quantify both analytes. A large pore reversed phase column (Hi-Pore RP-318, 250 mm × 4.6 mm, Bio-Rad Laboratories, Hercules, CA) was used with a linear acetonitrile gradient against water and a continuous flow rate of 1.0 ml/min. The HPLC system was a Bio-Rad Series 500 HRLC. The detector was a Gilson 118 UV/vis detector that was set to either 210 nm or 280 nm for the detection of oleic acid and albumin, respectively. Eluents were either water, or acetonitrile, or a linear gradient from water to acetonitrile. Some analyses were carried out with a low-dead volume connector in place of a column when only one component was present. In those cases, the only components used were oleic acid and acetonitrile. Samples containing oleic acid were injected into the HPLC system and oleic acid was quantified by absorbance at 210 nm. A calibration curve was generated from at least 5 calibration points. The correlation coefficient (R^2) was always greater than 0.95, but typically 0.99 or better.

2.4.1. Raman spectrometer

Raman spectroscopy was performed using a Raman 950 (Nicolet Analytical Instruments, Madison, Wisconsin), equipped with a neodymium yttrium vanadium oxide (Nd:YVO_4) excitation laser. The wavelength of the excitation laser was 1065 nm (9394 cm^{-1}). The collection geometry was 180° from the incident laser. The Raman 950 uses a Michelson interferometer for detection of all wavelengths simultaneously.

2.5. Cotton chromatography

Poly-prep chromatography columns were obtained from Bio-Rad Laboratories (Hercules, CA). These columns measured 0.8 cm × 4 cm and were loaded with approximately 400 mg ground 80-mesh (0.18 mm) cotton fibers. The fibers had been previously prepared by adding either 8.5 mg/ml or 26.2 mg/ml oleic acid in acetonitrile dropwise to the 80-mesh cotton until each column contained 26–34 mg oleic acid/g cotton. After each drop of acetonitrile (and oleic acid) was added, the cotton was dried under nitrogen. The amount added was carefully monitored by the volume of solution added so that no acetonitrile eluted through the column. Untreated cotton samples were prepared in exactly the same manner except the acetonitrile did not include oleic acid. In order to measure the displacement of oleic acid from the columns, two types of control columns were used. In one type of control, the effect of saline solution (0.15 M NaCl in 5 mM Tris, pH 7.4) was tested with oleic acid-treated cotton as the stationary phase. In the second type of control BSA was eluted over a cotton column which was not treated with oleic acid. Both types of eluent were collected and analyzed by HPLC as described above.

The eluted samples were collected and analyzed by HPLC to assess BSA and oleic acid content as described above. This was done to determine the amount of oleic acid displaced by albumin. A typical chromatogram is shown in Fig. 1. Injection volume was 20 μl and a refrigerated autosampler was used (operated at 25 °C), to carefully control injection volumes.

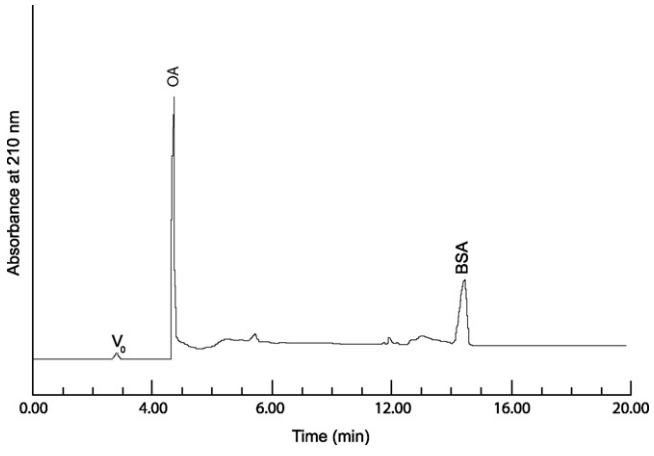

Fig. 1. Chromatogram of the separation of oleic acid (OA) and bovine serum albumin (BSA) by high-pressure liquid chromatography (HPLC). Separation was achieved with a Bio-Rad HPLC gradient system using a Bio-Rad Hi-Pore RP-318 column, eluting with a linear gradient from water to 50% acetonitrile (in water). Calibration curves were generated for each analyte. The correlation coefficient (R^2) for each calibration curve was greater than 0.95. This analytical technique was used to determine that the binding ratio of OA to BSA could be as high as 27:1.

3. Results

3.1. Oleic acid transfer from wound dressing material to wound fluid

This study assesses the potential to introduce oleic acid into an aqueous environment consistent with the protein composition of wound fluid. Oleic acid uptake by albumin from cotton wound dressing fibers into the wound environment was evaluated by way of a transfer mechanism criterion for the liquid–solid system. The uptake process employs albumin to remove oleic acid from the cotton matrix. The putative function of oleic acid is to lower elastase activity through inhibition in the chronic wound, thus, promoting healing by prompting redress of the protease imbalance.

Oleic acid was applied to standard cotton gauze, a hydrogel, and a hydrocolloid dressing to assess the potential of each substrate to inhibit elastase activity typically found in the chronic wound. Albumin is a natural carrier of oleic acid, and it is also the most concentrated protein found in the chronic wound. Median albumin levels in chronic wound fluid of 22 g/l are on average about one-half that of serum levels, and chronic wounds with albumin levels of less than 20 g/l rarely heal (James et al., 2000; Trengove et al., 1996). The hydrogel and hydrocolloid dressings were compared with cotton to assess the relative capacity of these three different types of dressing materials to release oleic acid in wound fluid and inhibit elastase activity.

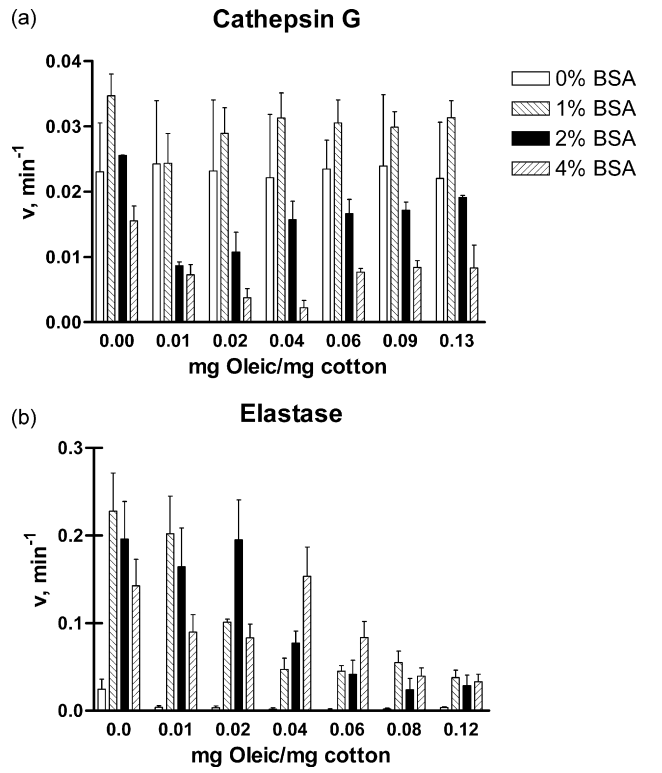

Fig. 2. (a) A plot of cathepsin G activity (*y*-axis) vs. oleic acid-treated cotton gauze (*x*-axis). (b) Plot of elastase activity (*y*-axis) vs. oleic acid-treated cotton gauze (*x*-axis).

3.2. Oleic acid inhibition of cationic serine proteases

A comparison dose response analysis of the effect of oleic acid-treated cotton on CG and HNE activity in the presence of 1, 2, and 4% albumin is shown in Fig. 2. CG is a cationic serine protease also elevated in the chronic wound. Some activation of CG was apparent at 1% albumin, which is consistent with a previous observation of increased HNE activity at low levels of albumin concentration (Edwards et al., 2004). The inhibition of CG in 2% albumin is greatest at 0.01–0.02 mg of oleic acid/mg cotton. However, CG activity increases at oleic acid doses up to 0.13 mg/g cotton. The inhibition of CG in 4% albumin is greatest at 0.042 mg of oleic acid/mg cotton, and CG activity increases at higher does up to 0.13 mg/mg cotton.

As has been previously shown, albumin enhances the activity of HNE, with the enhancement gradually diminishing at albumin levels from 1 to 10% (Edwards et al., 2004). As shown in Fig. 2b, HNE without albumin has almost no activity and the gauze absorbs what little enzyme is present. In the presence of 1% BSA, oleic acid inhibits at doses >0.02 mg oleic acid/mg cotton; in 2% BSA inhibition is noted at doses >0.04 mg oleic acid/mg cotton; and in 4% BSA inhibition is noted at doses of 0.01–0.02 and >0.06 mg oleic acid/mg cotton.

Hydrogel and hydrocolloid dressings treated with oleic acid were compared with cotton wound dressings and assessed in the presence of albumin solutions of 0, 2, and 4%. The elastase activities of the three oleic acid-treated wound dressings are shown in Fig. 3. The hydrogel dressing alone accelerated elastase substrate hydrolysis nearly three-fold. A greater than two-fold increase in the hydrolysis occurred at 2 and 4% albumin. At albumin concentrations of 2 and 4% there was a 75–80% (compared with untreated cotton dressing) decrease in elastase activity when oleic acid was bound to cotton in the presence of albumin. Thus, albumin uptake of 20% of the oleic acid applied to cotton gauze significantly lowered elastase activity at levels found in the chronic wound. At similar albumin concentrations the oleic acid-treated hydrogel and hydrocolloid dressings revealed a 14–50% (compared with untreated cotton) decrease in activity. In contrast oleic acid-treated cotton varying from 2 to 128 μg/mg evaluated at 0, 2, and 4% albumin concentrations shows a lowering of elastase activity within a linear dose response. The effect of oleic acid inhibition on elastase is similar with the three albumin concentrations at an oleic acid concentration of 0.13 mg oleic acid/mg cotton.

The release of oleic acid from the cotton gauze in the presence of albumin is shown in Fig. 4. The amount of oleic acid released from the cotton fibers ranged from 5 to 27 mg/g. The amount of oleic acid remaining on the cotton gauze accounts for 70–85% of the oleic acid applied to the gauze. The amount of oleic acid solubilized from the hydrogel and hydrocolloid wound dressing is shown in Fig. 4c. The amounts of oleic acid solubilized from the hydrogel and hydrocolloid wound dressings were significantly less than that with the cotton wound dressing.

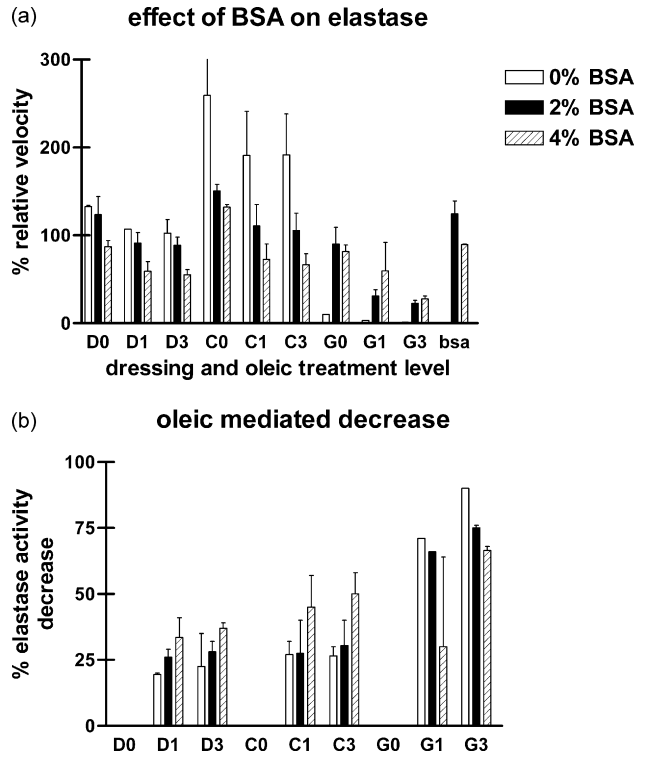

Fig. 3. A plot of percent relative velocity for a hydrocolloid (D), hydrogel (C), and cotton (G) wound dressing treated with oleic acid. The elastase activity was assessed for the oleic acid-treated wound dressings in the presence of albumin (BSA) concentrations of 0, 2, and 4%. (a) A plot of percent relative velocities for each type of wound dressing at the three albumin concentrations. (b) A plot of the percent decrease in elastase activity for each of the oleic acid (OA) treated dressings. Treatments are designated as—control (D0, C0, G0); hydrocolloid: Duoderm ($D_1 = 0.012$ mg OA/mg material; $D_3 = 0.025$ mg OA/mg material); hydrogel: Curagel ($C_1 = 0.012$ mg OA/mg material; $C_3 = 0.024$ mg OA/mg material); cotton: cotton gauze ($G_1 = 0.032$ mg OA/mg material; $G_3 = 0.069$ mg OA/mg material).

3.3. Chromatography of albumin uptake of oleic acid

Since cotton demonstrated a higher capacity for transfer of oleic acid to albumin, oleic acid-treated cotton samples were examined chromatographically to examine the mechanism of transfer of oleic acid to albumin. Chromatography provides a mobile phase/stationary phase model of solid–liquid phase equilibrium distribution between molecules, proteins and the solid phase they bind to. It was found that when oleic acid was adsorbed to 80-mesh ground cotton, oleic acid transferred to BSA in pH 7.4 saline buffer in a ratio of 27 moles of oleic acid bound per mole of BSA. These values were verified by HPLC, protein assay data, and GC/MS. An example a chromatogram of albumin and oleic acid used to quantify oleic acid/albumin binding ratios is shown in Fig. 1. Two doses of oleic acid (1.7 and 3.4 mg oleic acid/g cotton) were evaluated on cotton using 40 mg samples of cotton with each application, as described in Section 2. Based on the chromatographic results oleic acid was not displaced from cotton by saline. When BSA was included in the Tris eluent, oleic acid was detected at a concentration of approximately 1 mg/ml. Under the chromatographic conditions the transfer of oleic acid to fat free BSA took less than 1 min.

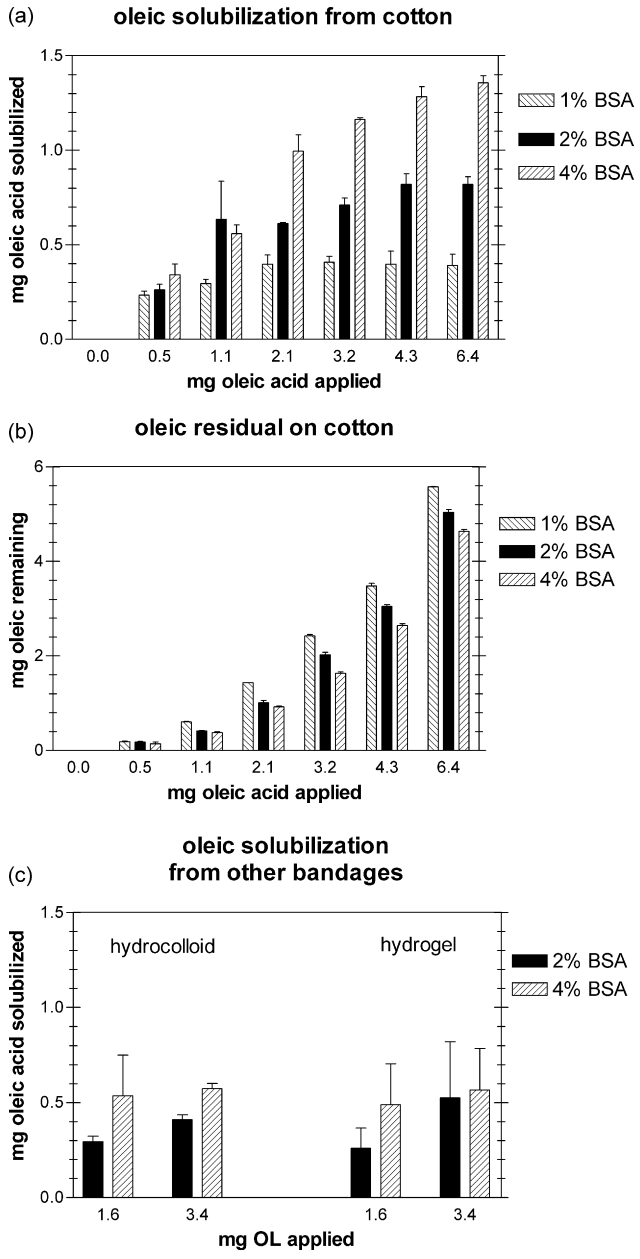

Fig. 4. (a) A plot of oleic acid solubilized (*y*-axis) vs. oleic acid applied (*x*-axis). (b) A plot of oleic acid remaining on gauze (*y*-axis) vs. oleic acid applied to gauze (*x*-axis). Samples were incubated for 2 h in 1 ml of buffer. (c) A plot of oleic acid released (*y*-axis) from hydrocolloid and hydrogel materials vs. oleic acid applied (*x*-axis).

Thus, albumin is capable of transferring significant amounts of oleic acid in an aqueous solution from a cotton dressing under conditions of protein, pH, and saline properties mimicking wound fluid. The better ability of cotton to release oleic acid compared with the other two dressing materials prompted evaluation of the conditions and mechanism of transfer required for albumin uptake from cotton fibers.

Table 1 summarizes some key values used to estimate maximal oleic acid levels that could be attained in plasma and wound fluid with oleic acid-loaded cotton. Oleic acid interacts directly with cotton in an aqueous environment and good retention of oleic acid suggests that both hydrophobic and hydrophilic

Table 1
Stoichiometric requirement for oleic acid (OA) release from cotton into wound fluid

Maximum OA:albumin	Maximum concentration of wound fluid albumin	Concentration of OA required to saturate albumin in wound fluid	Requirements for release of OA/ml wound fluid and 1 g of cotton wound dressing		18:1 found in chromatography eluant following from albumin + saline mobile phase
			Albumin	OA	
27:1	0.75 mM	20.25 mM	7.5 μmoles of albumin	200 μmoles OA or 1.3 mg/g cotton	1.5 mg/ml

The maximum oleic acid:albumin ratio was determined in this study (see Fig. 1). The concentration of albumin in plasma (0.75 mM) is the maximum found in wound fluid.

regions of the oleic acid molecule are accommodated in the crystalline cellulose lattice of cotton. The cotton behaves as an exchange medium such that the affinity of oleic acid for the fatty acid binding site of BSA is greater than the affinity of oleic acid to cotton. The oleic acid desorbs from the cotton and is absorbed to the native BSA binding sites, yielding stoichiometric binding (Table 1) to the primary and secondary binding sites of BSA (27 sites in total) as judged by the chromatographic assessment. It has been reported that serum albumin will bind up to six molecules of oleic acid under liquid phase equilibrium (Spector et al., 1969). The chromatographic analysis suggests that higher oleic acid–albumin binding (27:1 as a ratio of oleic acid:BSA) occurs under conditions of a solid–liquid medium. The concentration of albumin in plasma is approximately 0.75 mM, which is typically the maximum concentration found in wound fluid. The cotton columns employed in this study were loaded with 17 mg oleic acid/g cotton. To saturate 1 ml of wound fluid containing the maximum level of albumin would require 20 mmoles (using the 27:1 ratio) or 5.7 mg oleic acid/g cotton. Therefore, approximately 3× the amount of oleic acid required to provide 27 moles of oleic acid per mole of albumin in a chronic wound environment containing 1 ml of plasma or serum can be made available from 1 g of oleic acid saturated cotton. In the wound environment the available albumin will be exposed to the cotton gauze but will not be eluted as in a chromatography column. The ratio of 6:1 for oleic acid:albumin binding is based on liquid–liquid equilibrium conditions when BSA is incubated in the presence of oleic acid for 36 h at 37 °C (Spector et al., 1969). Thus taken together these results suggest that if each mole of albumin absorbs only 6 moles of oleic acid rather than 27, the saturated cotton would be more than adequate to supply the wound environment with oleic acid for inhibition of elastase.

3.4. Characterization of soluble liquid–liquid equilibrium conditions with Raman spectroscopy

In the course of assessing albumin binding of oleic acid in an aqueous environment the maximum oleic acid binding to albumin was assessed in a freely soluble system. Previously we have shown that soluble, stable albumin:oleic acid formulations inhibit elastase (Edwards et al., 2004). Clarifying the relationship of the limits of albumin binding of oleic acid to its uptake from a solid phase assists in understanding the structure function properties of lipid micelle–protein interactions and the protein binding sites of oleic acid. BSA is soluble in buffer and largely soluble in acetonitrile. On the other hand oleic acid is not soluble in aqueous buffer, but is very soluble in acetonitrile. It is noteworthy in this regard that cotton did not bind oleic acid in the presence of acetonitrile. For the purpose of oleic acid binding to cotton in acetonitrile approximately 300 and 3000 ppm oleic acid was dissolved in acetonitrile and added to 20 mg cotton. On the other hand albumin:oleic acid formulations prepared under these conditions will form a suspension when placed in buffer.

Table 2
Conversion table to convert from the molar ratio oleic acid:bovine serum albumin (OA:BSA) to wt.% oleic acid (OA)

Molar ratio (OA:BSA)	Total wt.% (OA)
N/A	100
300	56.2
200	46.1
150	39.1
100	30
50	17.6

Table 2 shows conversions between molar and weight ratios of oleic acid and BSA. These conversions are useful when examining data in Fig. 5. Here, it is apparent that a change occurs at the 200:1 molar ratio in slope for the peak ratio I_{2854}/I_{1438}. This

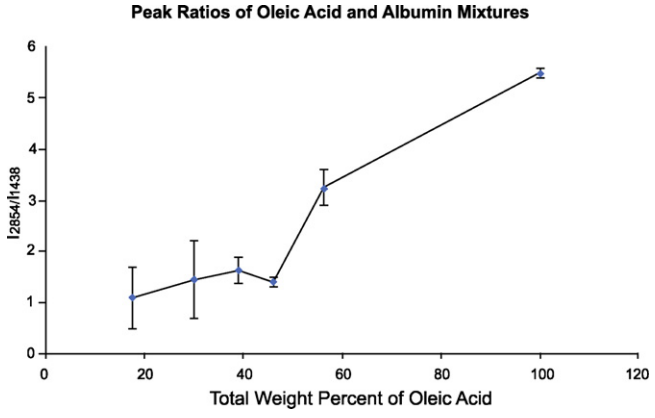

Fig. 5. The relative Raman peak heights were compared between 2852 and 1438 cm^{-1} for various mixtures of fat free BSA and oleic acid. Samples of oleic acid and BSA were prepared by mixing various amounts of BSA in 1 ml of water. After the BSA dissolved, 1 ml of 50% acetic acid:acetonitrile mixture was added with rapid stirring. Oleic acid was then added dropwise. Acetonitrile was added as necessary to dissolve all components of the desired mixtures. These samples were then dried and analyzed using a Raman spectrometer. The 2854 cm^{-1} (C–H stretching) and 1438 cm^{-1} (C–N stretching, amide III) peaks were then compared to one another.

ratio shows that the predominance of the C–H stretching motion is growing compared to the C–N stretching (amide III). In a native form of BSA, expect a molar ratio of 6 to 1 (oleic acid to BSA) or 27:1 represents a saturated state (Spector et al., 1969). One explanation for the transition in Raman spectra at a 200:1 molar ratio is that BSA has been unfolded by the preparatory procedure which involved dissolution in acetic acid and acetonitrile. Under these aqueous conditions unbound oleic acid forms micelles or liposomes with the polar groups exposed to the water and hydrophobic acyl chains aligned to one another in parallel. In the presence of increasing concentrations of acetonitrile, the structure of BSA has the opportunity to unfold, exposing more hydrophobic regions of the globular protein. The effect of unfolding and exposing the hydrophobic domains of the protein creates more binding sites for the hydrophobic acyl chains of oleic acid of the micelles formed under aqueous conditions.

Evidence from earlier work suggests that acetonitrile together with acidic conditions elongates proteins and makes them appear to be much larger than if they were globular (Goheen, 1988). Thus, with the unfolded protein model albumin would accommodate significantly more oleic acid to binding within the hydrophobic regions of the protein. These regions are typically hidden from an aqueous solvent in nature, but can become exposed when introduced to an organic solvent such as acetonitrile. Under conditions of no binding between oleic acid and albumin a linear increase in the CH stretch band as more oleic acid is added combined with a linear decrease in the amide III band as proportionally less albumin was present. However a sudden change in slope of the peak ratio (Fig. 5), representing the point where oleic acid can no longer influence the structure of the complex, is indicative of a major molecular structure change. It is probable that the structural change is due to the formation of micelles, or lipid multi-layers in which the acyl chains lie parallel to one another and the ionic headgroups are adjacent to an ion-rich region (Goheen et al., 1977). Thus, we suspect that albumin can accept much more than 27 moles of oleic acid, but only under extreme non-physiological conditions. Cotton loaded with oleic acid and placed in a wound environment would more likely be able to release no more than a maximum of 27 moles of oleic acid per mole of albumin.

4. Discussion

4.1. Chronic wound dressings

A major goal of chronic wound management is the design of wound dressings that function in sync with the biochemistry of wound healing (Shultz et al., 2003). These dressings should be designed to bring the chronic wound into balance with acute wound healing. The concept of wound bed preparation has been employed to convert chronic wounds to healing wounds so that healing can proceed uninterrupted through the phases of coagulation, inflammation, proliferation, epithelialisation and remodeling (Clark, 1996). A variety of types of moist wound dressings are employed for wound healing, and selection of an appropriate dressing for a non-healing wound is often based on the appearance of the wound bed and granulation tissue (Falanga, 1988). Cotton gauze is used for highly exudative wounds to fill cavities within the wound and avoid impaired healing and bacterial invasion. Hydrogels are used when wounds are dry, black or yellow in color and necrotic tissue is present. This condition usually requires enzymatic debridement. Hydrocolloid dressings are useful for moderately exuding wounds when the appearance of granulating tissue is red, wet or bleeding. These three dressings were selected in this study because of their varied composition and related wound applications.

One of the goals of this study has been to assess the elastase and cathepsin inhibitory effect of oleic acid when it is displaced from wound dressing materials by albumin at high, low and medium levels found in the chronic wound. The dose dependent lowering of elastase and CG activity in the chronic wound could be modulated by a non-toxic inhibitor, and it has previously been shown that albumin/oleic acid formulations offer a dose dependent lowering of elastase activity (Edwards et al., 2004). Table 3 shows the relative percent of elastase hydrolysis for solutions taken from the three oleic acid-treated dressings. The hydrogel material appeared to have an accelerating effect

Table 3
Percent of elastase activity retained in albumin solutions following incubation with oleic acid-treated dressings

Dressing type and weight of oleic acid	Percent rate of elastase substrate hydrolysis relative to buffer control (0% albumin)	Percent rate of elastase substrate hydrolysis relative to buffer control (2% albumin)	Percent rate of elastase substrate hydrolysis relative to buffer control (4% albumin)
Hydrocolloid (no oleic)	131	103	80
Hydrocolloid (1.67 mg)	107	79	48
Hydrocolloid (3.43 mg)	118	79	49
Hydrogel (no oleic acid)	309	143	129
Hydrogel (1.67 mg)	241	86	55
Hydrogel (3.43 mg)	238	86	54
Cotton gauze (no oleic)		71	74
Cotton gauze (1.67 mg)		24	27
Cotton gauze (3.43 mg)		19	24
Albumin control		110	89
Buffer	100	100	100

The three dressings: hydrocolloid, hydrogel, and cotton gauze were loaded with various amounts of oleic acid (shown in parentheses). Elastase activities are shown relative to the buffer control.

on elastase activity in the absence of albumin. This effect of accelerating enzymatic activity might improve autolytic enzyme debridement of necrotic tissue by hydrogels. The hydrogel dressing increases the rate of elastase substrate hydrolysis three-fold in the absence of oleic acid when albumin is not present, and a two-fold increase is still evident in the presence of the inhibitor. However, when the hydrogel dressing was placed in an albumin solution the accelerated hydrolysis was diminished. The hydrocolloid dressing formulated with oleic acid had a similar pattern of substrate hydrolysis acceleration, but was not as pronounced.

The wound dressings selected to study oleic acid displacement represent different types of hydrophilic and hydrophobic surfaces. The hydrogel and hydrocolloid materials are denser materials than the cotton gauze. The net effect of these higher density materials on the oleic acid formulation compared with cotton was the primary deposition of oleic acid on the surface of the hydrogel and hydrocolloid versus penetration of the fibers of the loosely woven cotton gauze. The hydrogel is a crosslinked polymer with a greater than 70% water-containing hydrophilic polymer matrix. The hydrocolloid is a semipermeable polyurethane with an adhesive layer that enhances the ability of the dressing to contain wound exudate by forming a cohesive gel. The hydrocolloid dressing utilized in this study has a hydrophobic surface character and binds oleic acid more strongly than cotton. In addition, the hydrocolloid material binds albumin and retards uptake of oleic acid into solution. This results in less elastase inhibition of the hydrocolloid bound oleic acid than for the cotton dressing. Cellulose on the other hand is also hydrophilic but the molecular structure presents a hydrophobic face within the helical crystalline lattice (Nishiyama et al., 2002). This creates some ampiphilic (both hydrophilic and hydrophobic) character and an ability to bind hydrophobic molecules, such as oleic acid. The molecular properties of both the hydrogel and cotton result in strong binding of water. Oleic acid has been used for textile finishing of cotton, and it has been studied for its interaction with textiles as a component of human sebum that comes in contact with clothes (Sontag et al., 1970). It adheres to cotton fabrics in the presence of an aqueous environment. Because of the hydrophobic properties of oleic acid, it is not adequately released from a fiber surface under aqueous conditions as are found in chronic wounds. Oleic acid remains tightly bound to cotton under aqueous conditions. At least 4% albumin is required to release 8–30 μg/mg cotton, which is 10% of the total amount of oleic acid applied to the cotton.

When elastase and CG inhibitory profiles were compared, different types of activity were apparent. Elastase inhibition increased to a threshold of 0.128 mg/mg cotton fiber. This inhibitory level corresponds to a 0.01–0.028 mg oleic acid/mg fiber released into solution with 1–4% albumin. In contrast, CG inhibition by oleic acid increased from 0.01 to 0.042 mg oleic acid/mg cotton, and then decreased at higher oleic acid loading values.

Cotton appears to be a physiologically suitable carrier for oleic acid in the wound environment and can transfer as much as 27 moles of oleic acid to each mole of albumin. The 27:1 ratio reflects five times more oleic acid absorbed than albumin absorbed under previously reported liquid–liquid equilibrium conditions (Spector et al., 1969). Thus, formulation of oleic acid on a cotton gauze dressing may be a more efficient means of controlling elastase activity than adding oleic acid in the liquid state. The measurements used to derive this relationship were not conducted in vivo, and represent a simulated condition focusing on the dressing/wound fluid interface rather than the dynamic wound environment. On the other hand, if an analogy is drawn between cotton as a chromatographic stationary phase with the liquid from the wound serving as the mobile phase, the albumin bound oleic acid could be eluted either away from or towards the wound area. Under these conditions the available concentration for cationic protease uptake of oleic acid might differ from the concentration range of oleic acid applied in the incubation of the cotton dressing in the albumin buffer of this study. However, stronger affinity between albumin and oleic acid than between cotton and oleic acid occurs due to the native oleic acid binding sites that reside in albumin. Thus, though relative affinity of oleic acid for other proteins and lipids in the microenvironment of the wound is not yet known, we suggest that since the liquid–liquid equilibrium results in a considerably lower oleic acid:albumin ratio (6:1) than the albumin–cotton uptake conditions for oleic acid (27:1) the oleic acid–albumin complex will migrate towards the wound bed. Other wound components with affinity for oleic acid may become acceptors of excess oleic acid. However, the dynamic equilibrium in a moist wound environment would favor transfer of oleic acid from cotton to albumin in the direction of the wound bed because there is usually at least 1–4% albumin in the wound environment. However, with heavily exuding wounds excess absorption back into the wound dressing may be favored where saturation conditions of the dressing occur.

This study outlines the feasibility of using oleic acid-incorporated cotton wound dressings in varying concentrations of albumin present in wound fluid. However, considerable variation of physiological albumin and other biochemical markers in wound fluid has been documented, and shown to change with healing (James et al., 2000). In addition complications from protein loss in burn wound patients have been shown where protein levels in serum are significantly lower as compared to physiological levels, while wound fluid protein levels are elevated and remain high (Lehnhardt et al., 2005). The protein profiles in burn patients contrast differently with studies comparing albumin in the chronic wounds versus the healing wounds (James et al., 2000), which show that median albumin concentrations in wound exudate range from approximately 17 g/l (chronic) to 25 g/l (healing). On the other hand total protein ranges in the healing and chronic wound are 44 and 30 g/l, respectively. These levels of albumin and total protein in wound fluid have previously been shown to be half the levels present in serum (Trengove et al., 1996). The reduced levels of human serum albumin in chronic wound fluid have been attributed in part to oxidation of albumin (Moseley et al., 2004). Thus considerable uncertainty in physiological albumin levels exists in the non-healing and healing wound. To address the range of albumin and total protein concentrations found in the chronic and healing wounds we have employed albumin concentrations from 10 to 40 g/l in this study. However, the relevance of the range of

albumin concentrations used in this study to the in vivo efficacy of oleic acid-incorporated cotton still needs to be demonstrated.

The results of this study demonstrate that the oleic acid-bound cotton wound dressing lowers elastase levels by 60–80% when albumin levels representative of those found in the chronic wound are 1% or higher. On the other hand, the elastase-lowering effect of hydrogel and hydrocolloid materials treated with oleic acid resulted in a 20–40% decrease in activity. Thus, the potential exists to modulate elastase activity in the chronic wound with oleic acid treated wound dressings by way of albumin's uptake mechanism to release the non-toxic inhibitor in the wound environment and inhibit destructive serine protease levels. The rapid desorptive and easily formulated properties of cotton with oleic acid suggest its potential as a good carrier for delivery and transfer of ampiphilic molecules as oleic acid. The oleic acid desorbs from the cotton and is absorbed to the native BSA binding sites, yielding stoichiometric binding to the primary and secondary binding sites of BSA (27 sites in total) as judged by the chromatographic assessment. It is probable that native HSA (not defatted) would undergo a similar exchange process by transferring bound fatty acids that have a high affinity for albumin from cotton. Indeed cotton may be a valuable carrier for numerous therapeutic drugs. Future studies will explore the potential of medicinally dosed cotton columns to examine the exchange of hydrophobic molecules representative of therapeutic compounds that would be advantageous with wound and blood protein oriented therapies.

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