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Chitosan dressing promotes healing in third degree burns in mice: gene expression analysis shows biphasic effects for rapid tissue regeneration and decreased fibrotic signaling³

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Introduction

Each year in the United States 1.1 million burn injuries require medical attention (American Burn Association, 2002). Approximately 45,000 burn injuries require hospitalization, 20,000 of these are major burns involving at least 25 percent of the total body surface, and approximately 4,500 of these people die. Complications following a severe burn may occur long after the initial incident, often when the patient is in an intensive care unit (ICU). Methods to improve wound healing and tissue repair will offer tremendous opportunities to enhance the survival rates and quality of life for burn patients, and may also help to reduce health care costs.

Chitosan is derived from the deacetylation of chitin, a naturally occurring poly $\beta(1-4)$ N-acetyl D-glucosamine (1,2) found in exoskeleton of crustacea, cuticle of insects and cell walls of fungi. Chitosan is a generic term used to describe linear polysaccharides which are composed of glucosamine and N-acetyl glucosamine residues joined by $\beta(1-4)$ glycosidic linkages (typically the number of glucosamines N-acetyl glucosamines) and which is soluble in dilute aqueous acid. The amino group on the 2-position of D-glucopyranose ring in chitosan allows for protonation, and hence solubilization of chitosan in water ($pK_a \approx 6.5$) (2). This allows the processing of chitosan into fibers and films, as well as the ability to prepare high purity chitosan for biomedical use. Chitosan has been found to have good biocompatibility for a range of biomedical applications including use as an excipient (3), hemostatic agent (4–8), anti-microbial agent (9–11), and in wound dressings (4,9,11–13).

There is substantial evidence that chitosan promotes wound healing. As early as 1970 accelerated healing was reported in rat & human subjects treated with poly-n-acetyl glucosamine (14). Improved healing has been reported in animal models such as full thickness excisional wounds in mice (9) and full thickness burn injuries in rats (15) and

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swine (12). Trials in humans have shown improved healing with chitosan based treatments in dental oral wounds (5), pressure, diabetic foot, and leg ulcers (16), and skin donor sites (17,18). However, the mechanisms by which chitosan treatments promote wound healing are not fully understood. Among those proposed are decreased inflammatory responses (9), increased expression of collagen and other extracellular matrix components (12,18–20), and the formation of granulation tissue (20). Increased collagen and extracellular matrix production suggests the presence of activated fibroblasts (21), and some growth factors have been shown to be expressed at higher levels in wounds treated with chitosan (12). Studies have also found an acceleration of re-epithelialization (17,22), increased formation of dermo-epidermal junctions (12), and improved tensile strength in the healing wound (13). Taken together these data suggest that chitosan may impact signal transduction pathways involved in the wound healing process.

In our current study we used gene-expression arrays to investigate changes in gene expression in third degree burn injuries treated with a chitosan based dressing to evaluate the potential for improved healing, examine potential mechanisms, and to search for biomarkers. The chitosan dressing was developed as a hemostatic dressing (7) and was subsequently shown to be an effective antimicrobial treatment for third degree burns in mice heavily contaminated with bacteria (23). In this study we used a chitosan lactate dressing to evaluate the effects on healing responses at three and seven days by gene-array and quantitative reverse transcriptase PCR (QRT-PCR) techniques. Previous studies with chitosan acid salt dressings suggest that antimicrobial chitosan lactate based acid salt dressings may be preferred over other acid salt type chitosan dressings for burn and wound care use due to being better tolerated on tissue (24,25). In this report we investigated mechanisms by which the chitosan dressing enhances the healing process following a severe burn by investigating the changes in gene expression at two time points post injury: early (three days) and late (seven days). Our results reveal that expression of the TGF β 1 gene shows the most dramatic changes during these two phases: its' expression is up-regulated during the injury response phase at three days followed by a decrease in expression during the latter phase of tissue modeling at seven days. Several studies have investigated the use of chitosan as a delivery system for growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) into wounds (26,27). To our knowledge this is the first demonstration of a direct effect of chitosan on the expression of a growth factor.

Materials and Methods

Dressing Materials

Hyclone Hypure water for injection; DL Lactic Acid (J.T.Baker Product 0196-01 Lot E13667 (USP Assay C₃H₆O₃ 90.0%)); FMC Biopolymer NovaMatrix P B Lot R-06-015 (shrimp derived, DDA[†] 85%, Mw[§] 367 kda, Mn 177 kda); FMC Biopolymer NovaMatrix UP B Lot 1353881 (shrimp derived, DDA[†] 88%, Mw[§] 365 kda, Mn 179 kda). [†]Percentage degree of deacetylation (DDA) was determined using FTIR and the method of Miya (28,29). [§]Number average (Mn) and weight average (Mw) molecular weights were determined by gel permeation chromatography (GPC) relative to known Mw of monodisperse polyethylene oxide standards.

Dressing Preparation

Chitosan solutions containing either NovaMatrix P B or NovaMatrix UP B chitosan were prepared in screw-capped polypropylene bottles (1 L) at room temperature. The solutions were prepared from 2.0% w/w chitosan and 1.0% w/w lactic acid and 97.0% w/w water. The chitosan was dispersed in the water with agitation followed by addition of the lactic acid from a serological pipette. The chitosan mixtures were placed at room temperature on bottle

rollers (> 2h) to allow for dissolution of the chitosan. Aliquots (70 g) of the chitosan solutions were poured into the Teflon™ coated square (11 cm × 11 cm × 1 cm) cavities of 8 cavity aluminum molds. The molds were placed inside a 4 shelf (50 cm × 25 cm each) Virtis 25 EL freeze dryer with shelf temperature of −40°C. Freezing induced phase separation of the ice and chitosan lactate at −40°C was conducted (3 hrs.) followed by freeze drying to remove the ice by sublimation (72 hrs). The freeze-drying resulted in consistent chitosan lactate porous matrices (10 cm × 10 cm × 0.6 cm) with residual moisture less than 5% w/w. The matrices were compressed at 80 °C at a compression rate of 0.1 mm/s between horizontal heated platens into chitosan sheets (10 cm × 10 cm × 0.15 cm). After compression, the sheets were conditioned at 80 °C for 15 minutes. The sheets were cut to 4.0 cm × 4.0 cm × 0.15 cm dressing test pieces and vacuum-sealed in individual foil pouches labeled according to chitosan PB or UPB. The pouched dressings were sterilized by gamma irradiation dose between 11 & 19 kGy. Note that a NovaMatrix PB chitosan based dressing was used in the three day timepoint while a NovaMatrix UP B chitosan based dressing was used in the seven day timepoint. The difference between NovaMatrix P B chitosan is that it is intended for external wound-care use while the NovaMatrix UP B chitosan is intended for internal wound-care or pharmaceutical applications. Apart from this intended use difference, the physical and chemical characteristics of the two chitosans used in this study can be considered substantially the same. This can be justified by percentage differences in degree of deacetylation and molecular weight parameters between NovaMatrix P B and UP B chitosans of less than 4%. A carboxymethylated cellulosic fiber wound dressing (30), AQUACEL® Hydrofiber, was used as a control. The primary chemical difference between dressings is the presence of glucosamine functionality in the chitosan dressing which is absent in the AQUACEL® dressing. An adhesive Tegaderm™ polyurethane film covering was used to attach dressings to the burn wounds and also as a control wound covering.

Animal experiments

All animal experiments were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital. NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23, Revised 1985) have been observed. Female BALB/c mice aged 6–8 weeks were shaved on the back and depilated with Nair (Carter-Wallace Inc., New York, NY) the day before the experiment. Mice were anesthetized with an IP injection of ketamine/xylazine cocktail (90 mg/kg ketamine, 10 mg/kg xylazine). A 1cm × 1 cm brass block heated to 100°C was placed against the epidermal area on the back for up to 40 seconds to create a full thickness burn injury (down to but not through the panniculus carnosus). This is equivalent to a third degree burn in humans. In initial experiments mice were divided into groups of three and left untreated or had burns covered with the chitosan dressing and sacrificed three days after the administration of the burn (n=3 mice per treatment group). In the second, longer study, treatments were: (a) covering the burn with Tegaderm™ only, (b) covering the burn with AQUACEL® and Tegaderm™, and (c) covering the burn with chitosan dressing and Tegaderm™ (n=3 for the expression arrays, n=6 for the QRT-PCR analysis) (Figure 1).

Tissue preparation and RNA isolation

At the time of sacrifice, tissues (wounded skin, distal unwounded skin, liver, brain and spleen) were rapidly removed and placed into RNA stabilizing solution (RNAlater™) and stored at −80°C. RNA was isolated from tissue samples by first grinding in liquid nitrogen followed by extraction with Trizol (Invitrogen). Total RNA was isolated using spin columns (Qiagen or Omega Scientific) according to the manufacturers' directions.

Gene Array Hybridization and Analysis

RNA samples were reverse transcribed into cDNA using oligo(dT) primers (Pharmacia) in the presence of ^{32}P labeled CTP to incorporate radioisotope label into the samples. Mouse 17k cDNA microarray and gene annotation information were from the National Institute on Aging (NIA) (<http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html>). Pre-hybridization, and hybridization of the labeled samples with the 17K NIA arrays were conducted according to NIA's protocols (<http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html>). Digital images of the hybridized arrays were created using a Typhoon-Image scanner and processed using ArrayProbe Analysis to generate quantified raw data that were subsequently processed for background subtraction. Data were normalized against the median value of all genes in a given array, and the values of repeated spots were pooled for all individual genes in the same array. Global gene expression profiles were compared between the wounded treated and untreated mice, and control mice using the 17k microarray, containing 16,820 different cDNA clones, representing 11,852 'unique' putative genes (<http://www.ncbi.nlm.nih.gov/unigene>), covering 70% of the expressed mouse genome determined by UniGene (31). Based on information provided by the National Institute on Aging, 3,226 of these genes have at least some putative function annotation, including the suggested gene symbol, gene product, and putative function description. The Significance Analysis of Microarrays (SAM) software was used to determine significant changes in gene expression in microarray datasets, using a modified t-test, T-statistics (32). SAM calculates a relative difference score (score(d)), based on the standard deviation of the replicates for a specific gene. The greater the score (d) above a threshold value, the more the difference in the levels of a gene between two groups is significant. We determined that a score(d) of 1.7 to be comparable to a significant probability (p) value of $p = 0.05$. For microarray datasets the p-value is referred to as the false discovery rate (FDR). The percentage of genes identified by chance is thus provided by the FDR, and corresponds to the q-value. The q value is expressed as the percentage difference of false significant genes over the total number of significant genes, and is dependent on a threshold delta value (Δ) and the whole set of genes on the microarray (see SAM manual at <http://www-stat.stanford.edu/~tibs/SAM/>)(33).

Quantitative reverse transcriptase PCR (QRT-PCR)

For QRT-PCR, cDNA was generated from 0.5 μg total RNA (three day samples) or 1 μg total RNA (seven day samples) using a cDNA synthesis kit (Quanta Biosciences) comprising a mixture of random and oligo d(T) primers and reverse transcriptase enzyme, according to the manufacturers directions. QRT-PCR for specific targets was carried out using an Mx3005P thermal cycler, using Perfecta SYBR green FastMix, low ROX (Quanta Biosciences) as the detection system. Table 1 shows the oligo sequences used. Briefly, 1 μl of template cDNA was combined with 0.1 μM primers and SYBR Green FastMix in a total volume of 20 μl . Cycling parameters were set for annealing at 60°C, extension at 72°C and melting at 95°C, 30 seconds each step, for 40 cycles with fluorescence data captured during the extension phase of each cycle. A standard curve was generated for each primer set. cDNA standards were created for skin and liver by mixing equal volumes of cDNA from each experimental sample. A five fold dilution series of cDNA standards was set up ranging from 625ng/reaction to 1 ng/reaction. The threshold cycle (Ct) data from these standards were used to generate efficiencies for each primer set to allow calculation of changes in gene expression to be calculated taking into account the efficiency of the reactions at different template concentrations according to the method of Pfaffl (34). We chose to normalize changes in target gene expression to a 'housekeeping' gene, a gene whose expression is not significantly changed over the course of the experiment (35), in this case Rpl13. For each oligo pair tested, the expression was normalized to Rpl13, as detailed above. Data were further normalized to the expression in the TegadermTM only treatment for the three day analysis, and to the uncovered wound in the seven day analysis..

Results

For the gene array analysis of the three day study each treatment group consisted of three mice. There was insufficient RNA obtained from each of the individual animals to run each individual sample on the microarray. Therefore, the RNA isolated from the three mice in each group was combined into a single sample before being reverse transcribed into cDNA. Subsequently three arrays were hybridized for each sample, thus giving three technical replicates for one biological sample. Although we would have preferred to analyze each animal individually we are primarily interested in differences between the control and treated groups and not between individual animals. By hybridizing to three individual arrays we controlled for variability in the sensitivity of the arrays rather than biologic variability. At this early time point in the healing of a large burn wound (three days after wounding) we did not find differences in gene expression between the untreated burn and the chitosan treated burn at an FDR of less than 0.05 (data not shown). However there were subtle variations in expression observed on the original heat maps (data not shown) that encouraged us to carry out additional analysis of the data with a higher FDR of 0.1. The results of this second, less stringent analysis generated the gene lists that are shown in table 2. The mRNAs corresponding to several genes likely to be involved in wound healing and tissue reconstruction were up-regulated in the chitosan treated burn compared with untreated. Those gene products most likely to be involved in the wound healing process were chosen for further study: type III Collagen (alpha 1 chain) (Col3a1), and Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase) alpha 1 polypeptide (P4ha1) both showed increased expression in the chitosan treated animals. Type III Collagen is an important component of the provisional matrix that is produced in the early stages of wound healing (36), and P4ha1 is involved in the organization of collagen fibrils (37). Some other genes of likely interest in wound healing from table 2 are Cyclin D2 (Ccnd2), Importin 8 (Ipo8) and Regulator of G-protein signaling protein 2 (Rgs2); all three of these genes are involved in the signaling pathways needed for cell replication and tissue reconstruction (38–40), suggesting that these activities were elevated in the chitosan treated burn compared to untreated. Among the genes that showed decreased expression in the chitosan treated wounds are Adam9 (A disintegrin and metallopeptidase domain 9) and Kif3a (Kinesin family member 3A). These two genes are involved in the dynamic equilibrium of extracellular matrix (41) and microtubule cytoskeletal reorganization during normal cell maintenance (42).

We used quantitative reverse transcriptase PCR (QRT-PCR) to validate the changes in mRNA levels observed in the gene array data. Archived RNA samples isolated from the skin taken three days after wounding were used to generate cDNAs from each individual animal. To normalize changes in gene expression, the mRNA level corresponding to the gene of interest was compared to the amount of mRNA corresponding to a 'housekeeping' gene, a gene whose expression is not significantly changed over the course of the experiment (35). We tested three genes often used for this purpose, Gapdh (glyceraldehyde-3-phosphate dehydrogenase), β -actin and Rpl 13 (ribosomal protein L13). In these studies Rpl13 was found to have the lowest level of variation between samples (data not shown), thus all data presented was normalized to the expression of Rpl13. Figure 2A shows QRT-PCR data examining mRNA levels of Col3a1, Kif3a and Adam9 and confirms the changes observed on the gene array. Data for the other genes examined by QRT PCR did not show any significant changes.

The expression of collagen is under the control of both Transforming Growth Factor β 1 (TGF β 1) and Plasminogen Activator Inhibitor1 (PAI1) now known as SERPINE1(43,44). In the gene array dataset we did not detect changes in the expression of either of these genes. However, by QRT-PCR we found that the mRNA corresponding to the gene for TGF β 1

was about twice as high in the chitosan treated wound compared with the untreated wound (Figure 2B). We did not detect any changes in levels of mRNA corresponding to the gene for Pai-1 (data not shown).

We were also interested in investigating the healing of burn wounds at a later stage of healing. To do this we chose a time-point seven days after wounding. The choice of this time point was informed by previous studies of excisional wounds where after eight-tonine days the presence of the rigid chitosan bandage began to inhibit healing through prevention of contraction in the rodent (9). In the seven day study additional treatments of the wounds were included in the protocol: Tegaderm™ to cover the wound to help prevent infection, and to prevent the damaged tissue from drying out; and a treatment group with AQUACEL®, a moist wound dressing used in the treatment of burns (45,46). Tegaderm™ is a breathable, transparent dressing that covers and protects the wound site, and was also used to tether the non-adhesive AQUACEL to the wound area. We included a wound covered with Tegaderm™ alone to control for effects of the chitosan dressing that could be due simply to the wound being covered as opposed to being exposed. A treatment arm in which the burn area was covered with AQUACEL® held in place with Tegaderm™ was included to compare the chitosan treated wounds with a treatment currently in use for burn treatment in the clinic. As AQUACEL® is non-adhesive the Tegaderm was needed to attach it to the mice, therefore to maintain comparable conditions, Tegaderm was then also used for the no treatment control and for the chitosan bandage. mRNA from three individual animals per treatment group were hybridized with the gene arrays.

The expression array data showed that in many cases genes were expressed at similar levels in the AQUACEL® and chitosan dressing treatments (data not shown) suggesting that the chitosan dressing may have benefits similar to that of the AQUACEL® treatment. For instance, the mRNA for type I collagen (Col1a2) at seven days was lower in both the wounds treated with AQUACEL® and chitosan compared to the Tegaderm™ only treatment. In other cases gene expression was different between the chitosan treatment and the AQUACEL® treatment. The mRNAs for Cyclin E2, which promotes cell cycle progression (47) and the junction cell adhesion molecule 1 (Jam-1), required for cell-cell adhesion (48), were both found at higher levels in the chitosan treated wounds than in the AQUACEL® treated mice (data not shown). These data suggest that the chitosan dressing had positive effects on cell growth and proliferation in the wound, and these effects were elevated compared to the AQUACEL® treatment.

Pathway analysis of the changes in gene expression in the chitosan treated burns at seven days is shown in Figure 3. Mitogen activated protein kinase 8 (Mapk8), also known as Jun N-terminal kinase (Jnk) is a downstream component of many growth factors and cytokines (49), and in turn affects the activity and expression of many other gene products, e.g. the expression of type I collagen (50). Lower levels of Mapk8 and Col1a2, one of the alpha chains required for type I collagen synthesis were observed in the chitosan treated wound. We validated the changes in mRNA levels of these two genes by QRT-PCR, and confirmed that they were present at lower levels in the chitosan treated samples (Figure 4A and B). We also used QRT PCR to examine the mRNA levels of genes we found to be differentially regulated in the three-day study. At this later time point in healing, we found that mRNA levels for TGFβ1 were decreased in the chitosan treated wounds (Figure 4C), as was expression of type III collagen (Figure 4D), although the latter result was not statistically significant. We did not find any differences in the levels of Kif3a or Adam9 mRNA at this time point (data not shown).

Both our gene chip data and the PCR validation showed high levels of variability, most likely due to small number of mice in each group. Table 3 shows the QRT-PCR results for

three individual mice per group taken from the seven-day study (data were generated in one group of three mice and similar results were obtained in an additional group of three mice for each condition tested). The levels of mRNA for each gene being tested were normalized to the amount of Rpl13 present in each sample and compared to the average amount in the uncovered wounds. This analysis shows that the variability in gene expression is most likely due to variations in the overall response of the individual animals. For instance in the Tegaderm™ only treatment, mouse 1 and mouse 3 had very similar levels of expression of the different genes, while mouse 2 showed lower expression. In the chitosan treated group mice 2 and 3 show a more similar pattern while mouse 1 had elevated expression for several of the genes tested.

Discussion

These data are the first demonstration that a chitosan dressing is capable of directly promoting wound healing at the molecular level and suggests a mechanism of action. Most importantly, our results reveal that chitosan causes a biphasic TGFβ1 response with increased mRNA levels present during the early injury response followed by down-regulation during the tissue re-modeling phase. This biphasic regulation of TGFβ1 message level may be used as a biomarker for efficacy tests of therapies in the healing of burn and other wounds. Interestingly, the TGFβ1 response to the chitosan treatment controls signaling pathways necessary for the two phases of tissue post-burn regeneration. The early phase of healing is largely composed of regeneration of the lost tissue and collagen matrix production is therefore necessary. In the later remodeling phase over-production of collagen would lead to scar production (51,52). Thus, a bi-phasic TGFβ1 signaling regulation for the collagen production suggests a pathway with initial robust tissue reconstruction accompanied by minimal later scar formation in the healing of burns.

The increased mRNA levels of TGFβ1 during the early post-injury phase is concordant with its downstream effector functions, i.e. the increase of collagen expression, specifically collagen III, which is an important component of the provisional matrix that is produced in the early stages of wound healing. Conversely we observed decreased mRNA for Adam9 and Kif3a. The increased mRNA levels for type III Collagen and decreased levels for Kif3a and Adam9 were confirmed by QRT-PCR analysis of the samples. Increased type III collagen mRNA indicates the production of a transitional extracellular matrix in the wound bed. Under normal conditions the production and degradation of the matrix are under strict control. During tissue reconstruction the repair process requires that the equilibrium be out of balance while the matrix regenerates and reorganizes. Adam9 is a protease that breaks down components of the matrix (41), while Kif3a controls microtubule cytoskeletal reorganization during normal cell maintenance (42). As such, these two genes are involved in the dynamic equilibrium of extracellular matrix. During the early stages of healing, their downregulation may promote tissue regeneration.

TGFβ1 has many roles in promoting wound healing and the control of inflammation. The amount of active protein is regulated at many levels post-translationally, therefore it is perhaps not surprising that we did not identify any changes in mRNA levels on the gene arrays. However, analysis of TGFβ1 message by QRT-PCR showed a two-fold increase in the chitosan treated wounds compared to the untreated burn. While modest, this increased mRNA level could potentially result in much elevated activity of TGFβ1 and we propose that the likely increased expression of TGFβ1 protein in the chitosan treated wound may be responsible for the observed increase in type III collagen mRNA. However, continued elevation of TGFβ1 signaling would be detrimental in the long term because it is strongly linked with the development of scar tissue (51,52). Thus the decreased mRNA levels for TGFβ1 in the chitosan treated wounds at seven-days suggests that the chitosan treatment

may encourage scarless healing. In addition, the bi-phasic change in message for TGF β 1 *i.e.* acute up-regulation followed by down-regulation in the latent phase, could be used as a biomarker for functionally heightened tissue regeneration and reconstruction caused by the chitosan dressing.

When the lead genes identified by the array analysis at three days were grouped according to their functional family classification we observed that gene families involved in regulation of the cell cycle and development, formation of cytoskeleton, and extracellular matrix are all found to be up-regulated in the chitosan treated burn. In contrast, fewer representatives of these lead gene families were found to be down-regulated in the chitosan treated burn condition. Taken together, these data suggest that at this early stage of healing, three days after the burn, the chitosan dressing may provide a head-start for tissue repair by jump starting the processes of reconstruction.

To further investigate the effects of the chitosan dressing at a later, more restorative phase of healing, we carried out a second study where the mice were treated for seven days after the burn injury. In this longer treatment phase we included a standard of care treatment, AQUACEL® and a condition where the wound was covered with an inert covering, Tegaderm™, which was also used to hold the AQUACEL® and chitosan treatments in place. The microarray analysis showed that, in many cases, changes in gene expression were similar in the AQUACEL® and chitosan treatments, suggesting that the chitosan dressing may have benefits similar to that of the AQUACEL® treatment. For instance, the expression of type I collagen (Col1a2) is lower in the wounds treated with AQUACEL® and chitosan compared to the Tegaderm™ only treatment. While increased expression of collagen type I is important in wound healing, prolonged expression can result in the development of scar tissue (53). Thus, a decrease in collagen type I expression at this later stage of wound healing could result in decreased scar formation. In other cases expression is altered in the chitosan treatment compared with the AQUACEL® treatment. Cyclin E2, which promotes cell cycle progression (47) and the junction cell adhesion molecule 1 (Jam-1), required for cell-cell adhesion (48) are both expressed at higher levels in the chitosan treated wounds than in the AQUACEL® treated mice. These data suggest that the chitosan based dressing has positive effects on cell growth and proliferation in the wound, and these effects may be elevated compared to the AQUACEL® treatment.

Both the gene chip and PCR data showed high levels of variability. This is most likely due to small number of mice in each group. We examined the PCR data from the seven-day study to investigate whether variability in expression was random across the samples, or whether individual mice showed consistent differences in message levels that would more likely represent biological variability. The mRNA levels corresponding to a number of genes were examined in individual mice from the seven day study (Table 3). We found that the variability in gene expression appeared to be due largely to variations in the response of the individual animals *e.g.* in the Tegaderm™ only group, mouse 1 and mouse 3 showed similar expression levels of all mRNAs tested, while mouse 2 showed lower expression at each of the genes tested, with several showing effectively the same expression as in the untreated control. These data suggest that much of the variability is due to changes in the overall healing process in the individual animals.

Conclusion

Data from this study show that the chitosan dressing had beneficial effects in burn healing. Effects of the chitosan dressing in the healing burn included increased mRNAs representing TGF β 1 and type III collagen locally during the early reactive phase of wound healing, suggesting that the pace of wound healing is accelerated. During the later restoration phase,

wounds treated with the chitosan dressing exhibited increased mRNAs corresponding to genes known to be associated with tissue reconstruction. These studies were performed in a small number of animals and thus suffered from sample-sample variability. We were also restricted to examining gene expression through RNA levels due to limited availability of the relevant areas of healing tissue in an effort to reduce heterogeneity. Nevertheless, the data presented here are promising for the use of the chitosan dressing to positively affect wound healing in addition to its hemostatic and antibacterial properties.

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Figure 1. Photographs of mice taken immediately after application of the various dressings to the burn area. (A) Tegaderm™ alone covering burn, (B) AQUACEL® dressing covered with Tegaderm™, (C) chitosan dressing before application of Tegaderm™.

Figure 2.

Validation of mRNA levels of lead genes of interest three days after wounding. mRNA levels were validated from archived RNA samples from each of the three mice per treatment group using quantitative RT-PCR. A. Fold change in expression of indicated genes three days after wounding in the chitosan treated wound compared to the untreated burn. B. TGFβ1 mRNA in chitosan treated wound vs untreated wound three days after wounding. All QRT-PCR data were normalized to the expression of Rpl13.

Figure 3.

Schematic of potential gene interactions identified from the gene chip analysis seven days after wounding. MAPK8: mitogen activated protein kinase 8, COL1A2: collagen type I alpha 2 chain, GJA1: gap junction protein alpha 1, FN1: fibronectin, CDH1: E-cadherin, CDH2: N-cadherin, PRL: prolactin, GATA2: GATA binding protein 2.

Figure 4.

Validation of mRNA levels for lead genes of interest seven days after wounding. mRNA levels were measured using QRT-PCR in the same RNA samples used for the array hybridizations (n=3) and in a second set of samples extracted from an independent set of wound samples (n=3) for a total of six samples. Data were normalized to levels of Rpl13 mRNA in each sample, and are presented as the fold change in expression compared to the untreated wound. p-values were calculated using the Students t-test. A. Mapk8, B. Col1a2, C. TGF.1, D. Col3a1.

Table 1

Primer sequences used for validation by QRT-PCR.

Gene/oligo name	Oligo sequence
Rpl13 forward	TTTGGCCAGTCTCCGAAT
Rpl13 reverse	TGCTTTATGGAAAATTTATTGC
Col3a1 forward	CAAAGTGGAACCTGGTTTCTTC
Col3a1 reverse	TCTAGTGGCTCCTCATCACAGA
Kif3a forward	AGACTTCCCACGTGAGGATAAA
Kif3a reverse	CTCAGGGCTCTAATTCAGGAGA
Adam9 forward	CTTGAAAAGCCTTTCTCTTCCA
Adam9 reverse	TCCACGTCAAGTGCATTAAATC
TGF β -1 forward	CCAGATCCTGTCCAACTAAGG
TGF β -1 reverse	TTTCTCATAGATGGCGTTGTTG
Col1a2 forward	GTATTGCTGGACAACGTGGT
Col1a2 reverse	GGTTGAGTTCACCTATTGAA
Mapk8 forward	GTTTGCCACAAAATCCTCTTTC
Mapk8 reverse	TATCCAGCGTATTTAGGCCTGT

Table 2

Overview of up- and down-regulated genes in the wounds treated with the chitosan dressing compared with the no treatment three days after wounding at an FDR of 0.1. n=3 animals per treatment group, RNA was pooled before being reverse transcribed and hybridized to the arrays.

Gene Bank	Symbol	Name
<i>Lower levels in chitosan treated burn v untreated burn</i>		
BG068660	Zfp280b	Zinc finger protein 280b
BQ552617	Ccdc47	Coiled-coil domain containing 47
BG086506	Snx2	Sorting nexin 2
BG072170	Naca	Nascent polypeptide-associated complex alpha polypeptide
BG064866	Prps1	Phosphoribosyl pyrophosphate synthetase 1
BQ551722	Kif3a	Kinesin family member 3A
BQ552690	Adam9	A disintegrin and metallopeptidase domain 9 (meltrin gamma)
BG085288	Ripply3	Ripply3 homolog (zebrafish)
BG088030	Rpl7	Ribosomal protein L7
<i>Higher levels in chitosan treated burn v untreated burn</i>		
BG079504	Tug1	Taurine upregulated gene1
BG080390	Rgs2	Regulator of G-protein signaling 2
BG077023	Gata2	GATA binding protein 1
BG067666	Depdc5	DEP domain containing 5
BG065769	Sepsecs	Sep (O-phosphoserine) tRNA:Sec (selenocystein) tRNA synthase
BG080039	Ubl5	Ubiquitin-like 5
BG070228	Nampt	Nicotinamide phosphoribosyltransferase
BG067718	Acacb	Acetyl-Coenzyme A carboxylase beta
BG076824	Aco1	Aconitase 1
BG081754	Fbxo38	F-box protein 38
AA407948	Peg10	Paternally expressed 10
BG085576	Col3a1	Collagen, type III, alpha 1
BG071016	Setd1a	SET domain containing 1A
AW548258	P4ha1	Procollagen-proline, 2-oxogulatarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide
BG087093	Tpm2	Tropomyosin 2, beta
BG070664	Ipo8	Importin 8
BG085601	Lpp	LIM domain containing preferred translocation partner in lipoma
BG075832	Ccnd2	Cyclin D2

Table 3

Comparison of normalized gene expressions for three animals in each treatment group at seven days after wounding. Numbers shown are the fold change in mRNA levels (normalized to Rpl13) compared to the levels in the untreated burn. Similar results were observed in a second set of three animals.

	Tegaderm only			AQUACEL			Chitosan Dressing		
	mouse 1	mouse 2	mouse 3	mouse 1	mouse 2	mouse 3	mouse 1	mouse 2	mouse 3
TGFβ-1	2.7	1.3	3.9	2.6	3.6	3.2	1.9	1.5	2.1
Adam9	2.3	1.6	2.1	1.2	2.4	1.4	2.0	1.1	1.6
Kif3a	4.0	0.9	1.3	1.6	1.2	1.0	2.8	1.0	1.0
Col3a1	2.4	0.9	4.2	2.3	1.9	3.7	1.8	1.7	1.8
Col1a2	2.4	0.5	4.1	6.0	1.9	7.6	2.8	2.6	2.1
Mapk8	1.6	0.9	1.4	1.6	1.4	1.3	1.0	0.6	1.1