

Differential Expression of Matrix Metalloproteinase-1 *in Vitro* Corresponds to Tissue Morphogenesis and Quality Assurance of Cultured Skin Substitutes¹

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Objective. To determine if matrix metalloproteinase-1 (MMP-1) was involved in the premature degradation of the dermal component in cultured skin substitutes (CSS) prepared with cells from burn patients.

Methods and results. CSS 645 and 647 were prepared from clinical human fibroblasts (HF) and keratinocytes (HK) that demonstrated premature degradation of collagen-glycosaminoglycan sponges *in vitro*. The control CSS were prepared from clinical HF and HK, CSS 648, and a pre-clinical cell strain, CSS 644 that did not degrade the sponges. Surface electrical capacitance measures surface hydration and was significantly higher for CSS 647 from days 9 through 14. MTT (3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide) conversion, an indicator of cellular viability was significantly lower for the 6-mm punch biopsies from CSS 645 and 647 at day 15 as compared to control CSS. MMP-1 protein levels measured by ELISA were significantly higher in medium from HF 645 and 647 than controls on the day of CSS inoculation. At day 14 of incubation, the mean MMP-1 concentration was significantly elevated in the medium from CSS 645 and 647 *versus* the controls, CSS 644 and 648. Western blots, and casein zymography demonstrated the presence of the latent and active forms of MMP-1 in the HF and CSS media, respectively.

Conclusion. MMP-1 was significantly higher in the media from two of the four HF strains and CSS after a 24 h incubation period. Elevated MMP-1 coincided

with premature degradation of the dermal substitute *in vitro*, and reduced numbers of CSS that met quality assurance standards for clinical transplantation. © 2005 Elsevier Inc. All rights reserved.

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INTRODUCTION

Cultured skin substitutes (CSS) are used to close burn wounds and restore the major function of the skin by reestablishing the epidermal barrier. Wound healing involves both the removal and replacement of injured tissue, and the long-term remodeling of the skin. Matrix metalloproteinases (MMPs) are a family of enzymes involved in degradation of the extracellular matrix of the skin and other tissues. Structurally, the MMPs have a catalytic domain with a zinc-binding site, a signal peptide and a propeptide [1]. There are three members of the collagenase subgroup including MMP-1 (collagenase-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3). The collagenases are responsible for breaking down fibrillar collagen types I, II, III, VII, VIII, and X in the extracellular matrix. The collagenases have substrate preferences, with MMP-1 degrading type III collagen more effectively than the other collagen types [1]. The latent or inactive form of MMP-1 is released into the extracellular space where proteolytic enzymes convert it to the active form. Several activators of the latent form of MMP-1 include trypsin, plasmin and stromelysin [1, 2].

MMP-1 is required for the growth of the epidermis and skin appendages during development and is expressed in basal keratinocytes and dermal fibroblasts beginning about the third month of gestation [1]. Ex-

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pression of the MMP-1 protein decreases during human development, and cannot be detected in the normal adult epidermis. However, if the skin is injured, MMP expression is activated as part of the complex process of tissue repair and regeneration [3]. The expression of MMPs in the skin is induced by growth factors and cytokines, such as, epidermal growth factor, platelet derived growth factor-BB, interleukin-6 and interleukin-1 β [1, 4–5]. Recently, Ghahary *et al.* (2004) [6] reported that stratifin, a factor released by keratinocytes can stimulate collagenase mRNA in human fibroblasts. Conversely, transforming growth factor- β , insulin-like growth factor-1, and endothelin-1 down regulate MMP-1 synthesis by dermal fibroblasts [7–9]. Negative regulation of MMP activity also results from the synthesis of tissue inhibitor of metalloproteinases (TIMPs) by fibroblasts and keratinocytes that bind to active MMPs in the extracellular space [1, 10]. In burn wounds, collagenase activity and TIMPs are both elevated as compared to normal, uninjured skin suggesting that tissue repair and remodeling are well-regulated to restore homeostasis [11]. An imbalance between the production of MMPs and TIMPs can result in skin disease. For example, recessive dystrophic epidermolysis bullosa has been linked to elevated MMPs, and in the most severe cases, the TIMP levels are decreased resulting in blister formation [12]. Hypertrophic scarring observed in the post-burn patient has been related to suppression of MMP-1 levels and elevated insulin-like growth factor-1 [8, 13].

Previous studies from this laboratory have reported the regulation of keratinocyte proliferation and barrier formation in skin substitutes by addition of vitamin C to the culture medium [14], generation of pigmentation by addition of selective cultures of epidermal melanocytes to CSS [15], formation of vascular analogs by addition of dermal microvascular endothelial cells to CSS [16], and successful grafting of autologous CSS for closure of full-thickness burns [17–19], reconstructive surgery [20], or chronic wounds [21]. In selected patients, it was observed that CSS *in vitro* demonstrated premature degradation of the collagen and chondroitin-sulfate substrate during the maturation phase of the CSS incubation. The biopolymer substrates of those grafts deteriorated to the extent that fibroblasts attached directly to the underlying culture support, and those CSS often failed to meet quality assurance standards. The current investigations compared the MMP-1 secreted into the culture media by two human keratinocyte and fibroblast strains that demonstrated this abnormal CSS phenotype, to two control strains that did not. The results of these studies showed that the CSS with the abnormal phenotype released significantly elevated MMP-1 concentrations into the culture medium.

MATERIALS AND METHODS

Cell Culture and CSS Preparation

Human keratinocytes (HK) and fibroblasts (HF) were isolated simultaneously from either surgical discard tissue (strain #644) or skin obtained from burn patients (strains #645, #647, #648). The cells were grown in selective growth media and cryopreserved at passage 0 (primary culture) providing a stock of cells for cultured skin substitutes [22, 23]. Acellular biopolymer substrates were prepared as previously described [24] from comminuted bovine hide collagen, and chondroitin-6-sulfate (GAG), except without chemical cross-linking with glutaraldehyde [25]. Briefly, bovine collagen powder was solubilized in 0.5 M acetic acid, and co-precipitated with GAG to yield a final concentration of 0.6% wt/vol. The co-precipitate was cast into sheets, frozen, lyophilized, cross-linked by vacuum dehydration at 105°C for 24 h, cut into squares (9 \times 9 cm), packaged into peel-packs, and sterilized by gamma-irradiation at \sim 25 Kg. For cell culture, the polymer substrates were rehydrated in Hepes-buffered saline solution, and changed into culture medium for inoculation of cells. For inoculation, substrates were placed on top of N-terface mesh, a cotton pad and a steel lifting platform. Then HF (0.5 \times 10⁶/cm²) were inoculated onto the collagen-GAG substrate and cultured at 37°C and 5% CO₂. The HF culture medium consisted of Dulbecco's modified eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 10 ng/ml epidermal growth factor, 5.0 μ g/ml insulin, and 0.5 μ g/ml hydrocortisone [14]. On the following day, the collagen-HF substrates were rinsed and incubated overnight in the CSS serum-free culture medium [26]. HK (1.0 \times 10⁶/cm²) were inoculated on the lifted collagen-HF substrates ($n = 3$ per cell strain) in the CSS culture medium (incubation day 0), and the CSS culture medium was replaced daily. Biopsies for histology were collected on days 7 and 14 for light microscopy. The clinical CSS were assessed for quality assurance (QA) by surface electrical capacitance (see below), and histologic examination according to criteria previously determined [27]. CSS that did not meet these standards failed QA, and were not grafted. Clinically, the CSS performed well with patient 648 (\sim 3% failed QA). However, the CSS phenotype for patients 645 and 647 was variable with thin, collapsed areas of the dermal substitute, and high rates of QA failure (\sim 20% and \sim 60%, respectively). Based on these observations, the strains 645 and 647 were chosen for analysis in these studies. A strain derived from surgical discard (#644), and a contemporary clinical strain (#648) performed well *in vitro*, and served as control CSS.

Surface Electrical Capacitance (SEC) Measurement

Skin surface hydration is measured by capacitance and is inversely proportional to the electrical impedance [28, 29]. SEC measurements were collected using the NOVA Dermal Phase Meter (DPM 9003; NOVA Technology, Portsmouth, NH) from the CSS grafts *in vitro*. On culture days 7, 9, 12, and 14, 10-serial measurements at 1-s intervals were taken from four sites on each CSS *in vitro* (12 values per group) and the SEC values are expressed in DPM units as mean \pm SEM.

MTT Viability Assay

On day 15, 6-mm punch biopsies were collected ($n = 4$ per CSS; 12 per group). The biopsies were incubated with 1 ml of 0.5 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO) for 3 h at 37°C. MTT was cleaved to a formazan by-product by mitochondria from viable cells [30]. One milliliter of 2-methoxy-ethanol released the MTT-formazan reaction product from the cells after 3 h of incubation on a shaking platform and was measured at 590 nm on a microplate reader (Cambridge Technologies, Watertown, MA). The values represent the mean optical density \pm SEM.

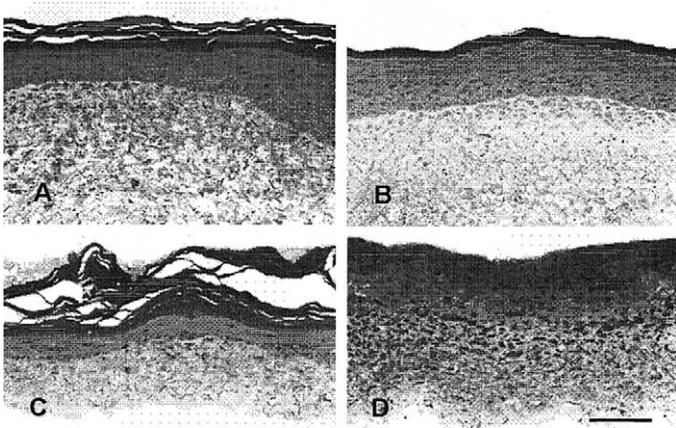


FIG. 1. The histologies were prepared in glycolmethacrylate and stained with methyl blue. The CSS cross-sections are from day 14 post-HK inoculation representing CSS 644 (A), CSS 648 (B), CSS 645 (C), and CSS 647 (D). Scale bar = 0.1 mm.

MMP-1 ELISA

MMP-1 concentrations were measured with a human metalloproteinase-1 Biotrak ELISA (Amersham Biosciences Corp., Piscataway, NJ). Twenty-four hour incubation media samples ($n = 3$ per strain) were collected from HF and HK on the day of inoculation and from CSS on days 7 and 14. Duplicate 100 μ l samples were assayed and normalized to ng per 10^5 cells or per cm^2 of CSS. This ELISA measures total human MMP-1 including free MMP-1 and MMP-1 bound to the specific inhibitor TIMP-1, but does not measure MMP-1 complexed to the non-specific inhibitor α -macroglobulin. The assay does not cross-react with TIMP-1, MMP-2, 3, or 9. The values represent the normalized MMP-1 concentration \pm SEM.

Western Blot Analysis

Proteins from HF 644, 645, 647, and 648 were extracted using the Mammalian Cell Lysis Kit (Sigma Chemical Co.). Twenty-five micrograms of protein were separated by SDS-PAGE on a 10% gel under denaturing and reducing conditions with 50 mM dithiothreitol. The proteins were transferred to a nitrocellulose membrane, blocked, and incubated with 1 μ g/ml mouse anti-human-MMP-1 (Ab-1; Calbiochem, LaJolla, CA) overnight on a shaking platform. The proteins were detected using the Western Breeze Chemiluminescent Immunodetection Kit (Invitrogen Life Technologies, Carlsbad, CA). This antibody detects the latent (57/52 kD) and active (46/42 kD) forms of MMP-1 and does not cross react with human MMP-2, MMP-3, MMP-8, MMP-9, or MMP-13. Positive control purified pro-MMP-1 was included on the gel (100 ng, 50 ng, and 25 ng).

MMP-1 Zymography

Twenty-four hour CSS media samples were concentrated using Centricon YM-3 centrifugal filter units by a factor of 7 ($n = 2$ CSS media samples per group). A 12% Zymogram casein gel (Invitrogen Life Technologies) was loaded with 25 μ l of each concentrated CSS media sample and electrophoresed using Tris-glycine SDS running buffer. The gel was equilibrated in a renaturing buffer containing 2.7% Triton X-100 for 30 min. Subsequently, the gels were incubated overnight at 37°C with a developing buffer containing calcium necessary for enzyme activity (Invitrogen Life Technologies). The gel was stained with Coomassie Blue and the protease activity was visualized as clear bands against the blue casein background. A control gel represented a titration of pro-MMP-1 (100-10 ng/lane) and pro-MMP-1 (100-10 ng/lane) activated with 50 μ M

p-aminophenylmercuric acetate ("APMA"; Calbiochem, San Diego, CA) for 2 h at 37°C. The APMA promotes the cleavage of the pro-MMP-1 into its active form [31, 32].

Statistical Analysis

The SEC, MTT, and MMP-1 ELISA data were analyzed by one-way ANOVA (Sigma Stat). Significance was determined on all pairwise multiple comparisons by Tukey's Test ($P < 0.05$).

RESULTS

Cultured skin substitutes are evaluated for quality assurance using histologies and surface electrical capacitance. Histologies were taken at 14 days post-HK inoculation from the CSS prepared for this study (Fig. 1). In CSS with strains 644 (Fig. 1A) and 648 (Fig. 1B), epidermal substitutes were well stratified and keratinized, and the collagen-GAG polymer was clearly evident in dermal substitutes. Premature degradation and collapse of the collagen-GAG sponge was observed in CSS 645 (Fig. 1C). The CSS prepared with HF/HK strain 647 (Fig. 1D) had a disorganized epidermal component, and no stratum corneum.

Figure 2 shows that values of surface hydration (SEC) were significantly higher (wetter) at day 7 for CSS 644 and 648 compared to CSS 645 and 647. At all other time points, the SEC for CSS 647 was elevated compared to all other groups. Elevated SEC values for CSS 647 indicates a failure to form the epidermal barrier corresponding with the histology for CSS 647 (Fig. 1D). The other three groups, CSS 644, 648, and 645

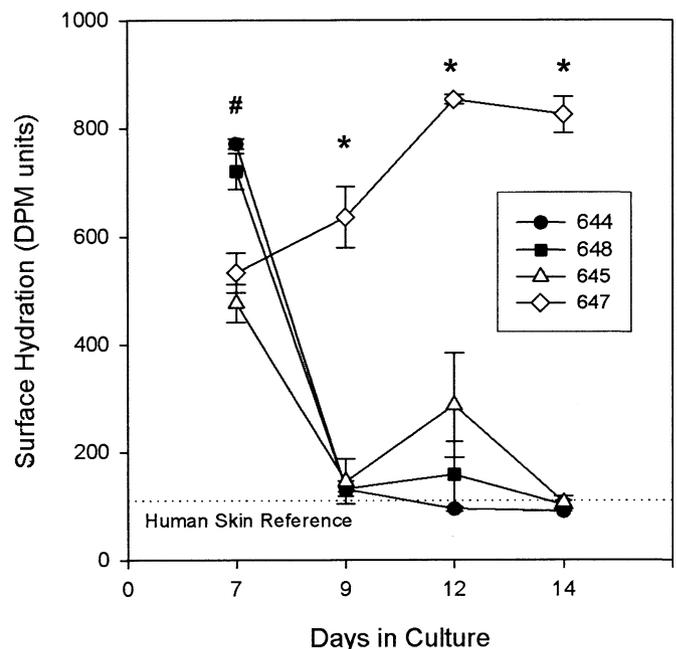


FIG. 2. Surface electrical capacitance measured the hydration of the CSS on days 7, 9, 12, and 14 ($n = 3$ per group). Statistical analysis showed that 644 and 648 were different from 645 and 647 ($P < 0.05$; #) and 647 was different from all other groups ($P < 0.05$; *).

were not statistically different from each other at days 9, 12, and 14 and were similar to normal human skin SEC values (Fig. 2, reference line).

At incubation day 15, the CSS biopsies from the two control CSS strains 644 and 648 had significantly higher mean MTT values, 0.749 and 0.639, respectively (Fig. 3). The mean MTT measurements were lower from the CSS prepared with HF/HK 645 and 647 (0.297 and 0.161, respectively) indicating the presence of less viable cells.

The MMP-1 concentrations in medium from HF 645 and 647 (14.6 and 15.2 ng/10⁵ cells, respectively) were significantly higher than from the control HF 644 or 648 (4.5 and 10.5 ng/10⁵ cells, respectively) on the day of CSS inoculation (Fig. 4A). The pre-clinical control HF 644 secreted significantly less MMP-1 into the culture medium when compared to all clinical HF strains (645, 647, and 648). Minimal MMP-1 (<1 ng/10⁵ cells) was measured in the culture media from the 4 isogenic HK strains (data not shown). The mean MMP-1 concentration from the CSS culture media on day 14 post-HK inoculation was also significantly higher from CSS 645 and 647 (273.0 and 182.5 ng/cm², respectively; Fig. 4B) as compared to control CSS 644 and 648 (103.3 and 106.1 ng/cm², respectively). The higher MMP-1 levels in HF and CSS culture media from clinical strains 645 and 647 corresponded with an increased tendency of CSS to deteriorate during maturation *in vitro*.

Western blots demonstrated immunodetection of MMP-1 in the fibroblast lysate samples. Bands for both the latent (57/52 kD) and active (46/42 kD) forms of MMP-1 were identified by SDS-PAGE in HF 644, 647, 648 and 645 (lanes 2–5, respectively; Fig. 5). Lanes 7 to 9 correspond to a titration of the positive control pro-MMP-1 with both the latent and active forms present on the blot. These fibroblast-derived samples represent the cell-associated MMP-1.

Zymography was performed to demonstrate the MMP-1 enzyme activity secreted in the culture media from the CSS after 24 h of incubation. Concentrated media were electrophoresed on casein gels and the clear zones on the gel represent areas of caseinolytic activity (Fig. 6). Positive control pro-MMP-1 established a titration (100–10 ng/lane) of the latent (57/52 kD) and active (46/42 kD) forms of MMP-1 following activation with 50 μ M p-aminophenylmercuric acetate (Fig. 6A). Both the latent and active forms of MMP-1 were detected on the zymogram of the CSS media from all four cell strains (Fig. 6B). The more prominent bands for active MMP-1 are seen from media of CSS 645 and 647 correlating with CSS that had a higher percentage of QA failure. The higher molecular weight (60–70 kD) band of caseinolytic activity is consistent with MMP-13 and may also contribute to the CSS degradation [31].

DISCUSSION

Data from this study show a strong association between elevated expression of MMP-1, and premature degradation of the dermal component of autologous CSS used for investigational grafting of burn patients. To provide the greatest benefits to patients, it is important to reduce this degradation to a minimum, and promote maximum development of skin anatomy and physiology *in vitro*. It is expected that understanding the mechanisms of degradation will allow regulation of conditions to promote anabolic metabolism, and reduce catabolic activities. Evaluations of microscopic anatomy, epidermal hydration, cellular viability, expression of MMP-1 protein, and enzyme activity provide important indicators of how the anatomy and physiology of CSS may be regulated to provide the greatest benefits to patients.

CSS degradation *in vitro* is clearly attributable to individual differences in expression of MMP-1 among patients. In this study, samples from patients (#645, #647) with the most extreme degradation were selected specifically to emphasize the observation, and provide the greatest probability of finding a biochemical marker of the phenomenon. It is noteworthy that both of these patients were treated at hospitals outside of the city where the CSS were prepared. Consequently, the biopsies of skin used to generate the CSS were shipped by express delivery, and experienced ischemic periods up

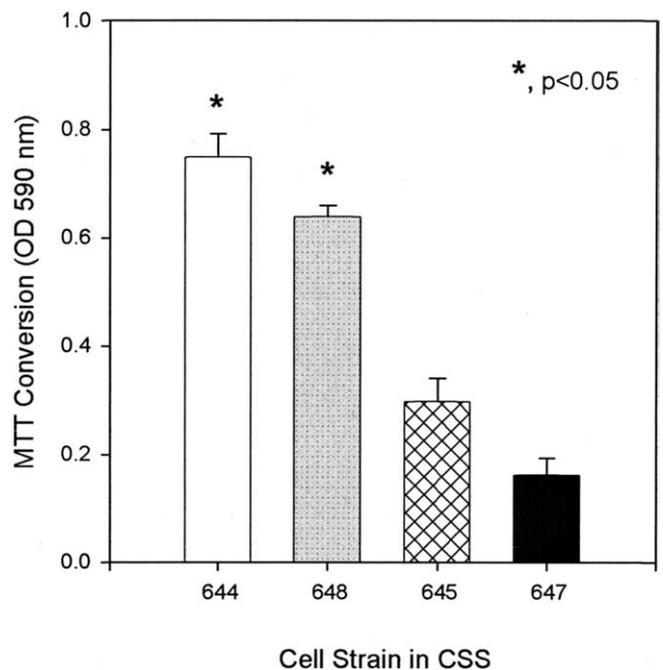


FIG. 3. On day 15, 6 mm punch biopsies of the CSS were tested in the MTT viability assay ($n = 12$ per group). The formazan reaction product was measured at 590nm. CSS 644 and 648 had statistically higher mean MTT levels when compared to CSS 645 and 647 ($P < 0.05$, *).

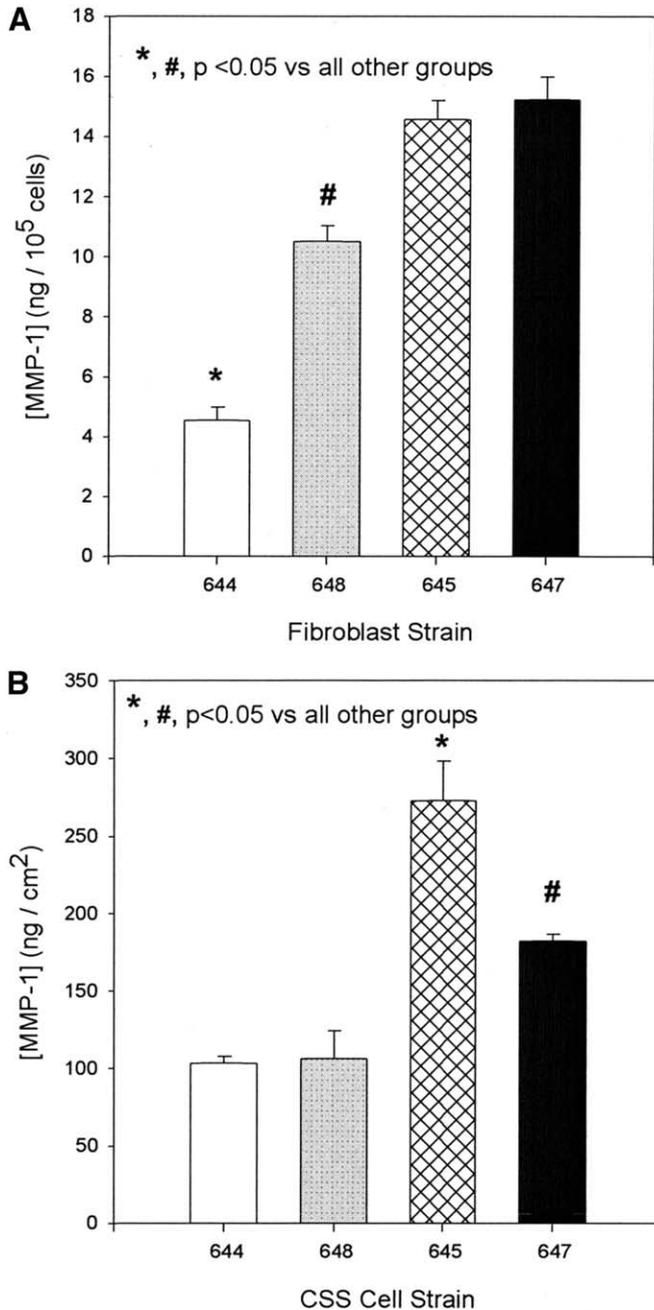


FIG. 4. MMP-1 was measured using an ELISA. Fig. 4A represents the mean MMP-1 concentration from the culture media of the human fibroblasts 644, 645, 647, and 648 ($n = 3$ per group). HF 644 (*) and 648 (#) were statistically different from all other groups ($P < 0.05$). (B) Represents the mean MMP-1 levels in the culture media of the CSS ($n = 3$ per group). CSS 645 (*) and 647 (#) were statistically different from all other groups ($p < 0.05$).

to 24 h, compared with less than 2 h for intramural patients. Hypoxia has been implicated in the induction of MMP-1 in dermal fibroblasts [33, 34]. In those studies, a three-dimensional culture method was developed to study the effects of hypoxia on dermal human fibroblasts. By 48 h of reduced oxygenation (2% O₂), the mRNA levels of pro-MMP-1 and MMP-1 were signifi-

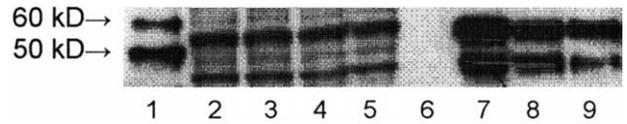


FIG. 5. Immunodetection of MMP-1 by Western Blotting. SDS-PAGE was used to show the presence of the latent (57/52 kD) and active (46/42 kD) forms of MMP-1 in the fibroblast cell lysate. Lanes 2 to 5 represent the HF 644, 647, 648, and 645, respectively. The positive control pro-MMP-1 was titrated in lanes 7 to 9 (100 ng, 50 ng, and 25 ng per lane, respectively). Lane 1 represents the molecular weight markers.

cantly elevated as compared to the HF cultured under normoxic conditions (20% O₂). Kan *et al.* (2003) [34] found that after 72 h of hypoxia, reoxygenation for an additional 72 h did not restore the MMP-1 levels to normal. This possibility for induction of MMP-1 expression may be investigated in future studies, and could be regulated by increasing the oxygen concentration in the transport medium. However, it should also be recognized that overall rates of quality assurance failures has been low (<10%) in the study of more than 75 patients over more than 10 years. Therefore, in a context of variable expression of proteases, or other degradative enzymes in the population of burn patients, another consideration is the stability of the biopolymer component to the activities of those enzymes. Analysis of cross-link density [35] in the starting materials may

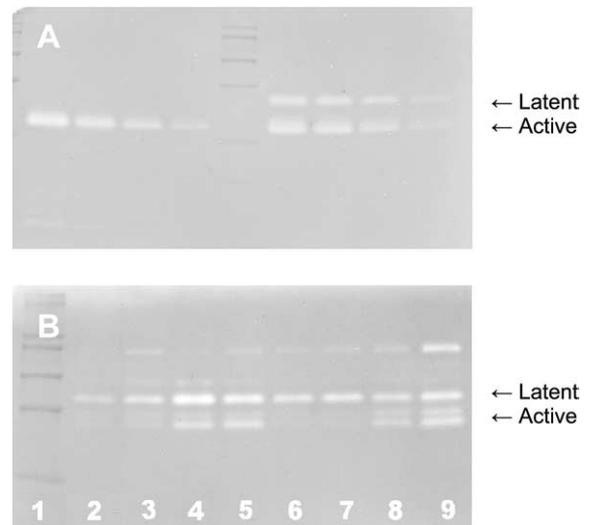


FIG. 6. Zymography of MMP-1 in the CSS culture media. (A) Represents a titration of the active form (46/42 kD) of pro-MMP-1 activated with 50uM APMA in lanes 1 to 4 (100, 50, 25, 10 ng/lane). Both the latent (57/52 kD) and active forms (46/42 kD) of the control pro-MMP-1 are present in lanes 6 to 9 (100-10 ng/lane). Figure 6B represents the MMP-1 digestion from duplicate CSS culture media samples for CSS 644 in lanes 2 and 3, CSS 647 in lanes 4 and 5, CSS 648 in lanes 6 and 7, and CSS 645 in lanes 8 and 9. All CSS media have the latent and active forms of MMP-1. CSS 645 and 647 have more prominent bands than the control CSS representing the active form of MMP-1. The higher molecular weight band may represent MMP-13. Molecular weight markers: A) Lane 5; B) Lane 1.

provide an index of polymer stability. If cross-link density is insufficient for polymer stability, the addition of chemical cross-linking [25], as with carbodiimide [35, 36] would stabilize the polymer component, and prevent premature degradation.

The polymer component of CSS consists of both protein (mostly type I collagen), and carbohydrate (chondroitin-6-sulfate) [24]. Therefore, the degradation of the polymer component may result from either proteases, and/or glycosidases. In the present study, activity of a single protease (MMP-1) was associated with premature degradation of CSS. However, degradation or premature loss of the polysaccharide from the polymer component could also contribute to degradation of the dermal substitute of CSS. Previous studies [24] have shown that the chondroitin-6-sulfate is not cross-linked covalently to the collagen of the biopolymer component in this model, but is bound ionically. Therefore, if the chondroitin-6-sulfate is lost too rapidly from co-precipitate with collagen, then the collagen may be more subject to disaggregation. Preliminary studies with biopolymer substrates made without chondroitin-6-sulfate suggest that premature degradation is reduced. Conversely, measurement of glycosaminoglycan content (GAG/mg wet weight) of CSS over time shows that more GAG is synthesized and added to the substrate by cells, than is added to the co-precipitate from which the substrates are made (data not shown). Therefore, regulation of the degradation of the dermal component may be expected to require considerations of the concentrations of degradative enzymes released from cells in CSS, the biochemical stability of the polymer to the activities of the enzymes, and the synthesis of new extracellular matrix by cells in the grafts.

Histology of CSS showed few or no reticulations of the collagen-GAG polymer in strains 645 and 647 in which premature degradation was most pronounced. However, dense populations of fibroblasts were observed (Fig. 1). This supports the hypothesis that polymer degradation was cellular derived. In CSS 644 and 648, with low expression of MMP-1, the development of epidermal barrier as measured by surface hydration followed a typical decrease between days 7 and 14 of incubation, and reached levels of uninjured human skin (Fig. 2). CSS 645 and 647, with high expression of MMP-1, showed statistically lower hydration at day 7, and CSS 647 did not decrease in hydration which means the epithelium did not keratinize. The lower SEC of CSS 645 and 647 at day 7 is interpreted as accelerated apoptosis of keratinocytes which leads to cornification. However, if sufficient numbers of viable keratinocytes are not supporting the cornified layer, it can deteriorate prematurely with increase of SEC values as observed in CSS 647. In a condition of low cellular viability, the cornified epithelium can generate false positive SEC values as seen in CSS 645. This is

consistent with the histology of this strain that shows no formation of stratum corneum in CSS 647, and fewer layers of nucleated keratinocytes in CSS 645. Therefore, the degradative activities may affect both the polymer and cells in the cultured grafts. The inverse relationship between cell viability and premature degradation of CSS strains 645 and 647 is demonstrated by statistically lower MTT values (Fig. 3). SEC is an index of differentiated epithelium, and MTT demonstrates cell viability. In a condition of low cell viability (MTT), any positive SEC readings can be interpreted as false positive, and CSS can be predicted not to heal after transplantation. Furthermore, the numbers of samples per condition for MTT and SEC ($n = 12$) was greater than for histology ($n = 1$). Therefore, the quantitative data have greater statistical strength than the qualitative histology.

The source of released MMP-1 was shown by ELISA to be predominantly from fibroblasts, with negligible expression from keratinocytes. MMP-1 protein was highest in strains 645 and 647 before inoculation of fibroblasts onto the polymer (Fig. 4A). This suggests that the cellular secretion of MMP-1 was activated during selective culture, or may be induced by burn injury. Alternatively, the release of MMP-1 into the medium may result from cell lysis during CSS degradation. Elevated expression of proteases in burn wounds has been reported [37], and activation of MMPs in wound healing is well understood [38]. Not surprisingly, release of MMP-1 was also elevated significantly in CSS 645 and 647 in which premature degradation was observed (Fig. 4B). However, CSS 644 and 648 with normal phenotypes expressed statistically lower levels of extracellular MMP-1. There was a notable decrease of MMP-1 detection from fibroblast strain 648 compared to CSS 648 which released levels equal to control CSS 644. The decrease of secreted MMP-1 may be attributed to addition of keratinocytes that are known to down-regulate fibroblast factors, including MMP-1 [39] and TGF- β 1 [40]. Results of this study also imply that epithelial factors inhibit expression of MMP-1, and may regulate the development and stability of the CSS phenotypes. This result is also consistent with the suppression of granulation tissue and scar by unmeshed, sheet grafts [41–43].

Western blot analysis of cellular extracts (Fig. 5) confirmed that both latent (57/52 kD) and active (46/42 kD) forms of MMP-1 were expressed in the four strains of fibroblasts tested. No qualitative differences were detected in the proportions of latent and active forms of MMP-1. Together with the ELISA results (Fig. 4) these data suggest that the quantitative differences in secretion of active collagenase accounts for its contribution to premature degradation of CSS. That interpretation is supported by quantitative differences in enzyme activity are shown by zymography (Fig. 6). Medium from

CSS (strains 645 and 647) with premature degradation demonstrated the greatest amounts of active and latent MMP-1. This result clearly implicates extracellular MMP-1 as a factor associated with premature degradation and reduced quality of CSS grafts. The release of MMP-1 may result from active mechanisms of cellular secretion, or by a passive mechanism after cell lysis. Investigation of the mechanism of release is planned for future studies.

Based on these results, two general approaches may be considered to maintain the anatomic and physiologic integrity of CSS for grafting. Either the polymer component may be cross-linked to resist the degradative activities, or those activities may be reduced. Increase of the cross-linking of the polymer provides a measurable and predictable alternative that may confer resistance to the degradative activities. Alternatively, addition of protease inhibitors may be effective, but would require estimation of protease activities, and may also inhibit beneficial proteases in cellular metabolism. MMP-1 expression may also be reduced by decreasing the ischemia experienced by the skin tissue during transport from outside hospitals to the site of CSS preparation. Enrichment of the transport medium with greater oxygen, or addition of an oxygen-carrier, such as a perfluorocarbon solution, may reduce ischemic injury to tissue. Each approach, or a combination of the two, will be considered to maintain standards of CSS quality within optimal limits for clinical transplantation, and to maximize availability of CSS for wound closure. With introduction of procedures for more consistent composition of CSS, its efficacy and medical benefits for wound closure may be expected to increase.

REFERENCES

- Kahari, V. M., and Saarialho-Kere, U. Matrix metalloproteinases in skin. *Exp. Dermatol.* **6**: 199, 1997.
- Lohi, J., Kahari V-M., and Keski-Oja, J. Cyclosporin A enhances cytokine and phorbol ester-induced fibroblast collagenase expression. *J. Invest. Dermatol.* **102**: 938, 1994.
- Agren, M. S., Mirastschiijski, U., Karlsmark, T., and Saarialho-Kere, U. K. Topical synthetic inhibitor of matrix metalloproteinases delays epidermal regeneration of human wounds. *Exp. Dermatol.* **10**: 337, 2001.
- Endo, H., Utani, A., and Shinkai, H. Activation of p38 MAPK suppresses matrix metalloproteinase-1 gene expression induced by platelet-derived growth factor. *Arch. Dermatol. Res.* **294**: 552, 2003.
- Rutter, J. L., Benbow, U., Coon, C. I., and Brinckerhoff, C. E. Cell-type specific regulation of human interstitial collagenase-1 gene expression by interleukin-1b (IL-1b) in human fibroblasts and BC-8701 breast cancer cells. *J. Cell. Biochem.* **66**: 322, 1997.
- Ghahary, A., Marcoux, Y., Karimi-Busheri, F., Li, Y., Tredget, E. E., Kilani, R. T., Lam, E., and Weinfeld, M. Differentiated keratinocyte-releasable stratifin (14-3-3 sigma) stimulates MMP-1 expression in dermal fibroblasts. *J. Invest. Dermatol.* **124**: 170, 2005.
- Yuan, W., and Varga, J. Transforming growth factor- β repression of matrix metalloproteinase-1 in dermal fibroblasts involves smad3. *J. Biol. Chem.* **276**: 38502, 2001.
- Ghahary, A., Shen, Y. J., Nedelec, B., Wang, R., Scott, P. G., and Tredget, E. E. Collagenase production is lower in post-burn hypertrophic scar fibroblasts than in normal fibroblasts and is reduced by insulin-like growth factor-1. *J. Invest. Dermatol.* **106**: 476, 1996.
- Shi-wen, X., Denton, C. P., Dashwood, M. R., et al. Fibroblast matrix gene expression and connective tissue remodeling: Role of endothelin-1. *J. Invest. Dermatol.* **116**: 417, 2001.
- Sugita, Y., Morita, E., Tanaka, T., Nakamura, K., and Yamamoto, S. Production of tissue inhibitor of metalloproteinase-1 and -2 by cultured keratinocytes. *J. Dermatol. Sci.* **22**: 107, 2000.
- Stricklin, G. P., and Nanney, L. B. Immunolocalization of collagenase and TIMP in healing human burn wounds. *J. Invest. Dermatol.* **103**: 488, 1994.
- Bodemer, C., Tchen, S. I., Ghomrasseni, S., Segulier, S., Gaultier, F., Fraitag, S., de Prost, Y., and Godeau, G. Skin expression of metalloproteinases and tissue inhibitor of metalloproteinases in sibling patients with recessive dystrophic epidermolysis and intrafamilial phenotypic variation. *J. Invest. Dermatol.* **121**: 273, 2003.
- Ali Bahar, M., Bauer, B., Tredget, E. E., and Ghahary, A. Dermal fibroblasts from different layers of human skin are heterogeneous in expression of collagenase and types I and III procollagen mRNA. *Wound Repair Regen.* **12**: 175, 2004.
- Boyce, S. T., Supp, A. P., Swope, V. B., and Warden, G. D. Vitamin C regulates keratinocyte viability, epidermal barrier, and basement membrane formation in vitro, and reduces wound contraction after grafting of cultured skin substitutes. *J. Invest. Dermatol.* **118**: 565, 2002.
- Swope, V. B., Supp, A. P., and Boyce, S. T. Regulation of cutaneous pigmentation by titration of human melanocytes in cultured skin substitutes grafted to athymic mice. *Wound Rep. Reg.* **10**: 378, 2002.
- Supp, D. M., Wilson-Landy, K., and Boyce, S. T. Human dermal microvascular endothelial cells form vascular analogs in cultured skin substitutes after grafting to athymic mice. *FASEB J.* **16**: 797, 2002.
- Boyce, S. T., Kagan, R. J., Yakuboff, K. P., Meyer, N. A., Riemann, M. T., Greenhalgh, D. G., and Warden, G. D. Cultured skin substitutes reduce donor skin harvesting for closure of excised, full-thickness burns. *Ann. Surg.* **235**: 269, 2002.
- Boyce, S. T., Kagan, R. J., Meyer, N. A., Yakuboff, K. P., and Warden, G. D. The 1999 Clinical Research Award. Cultured skin substitutes combined with Integra to replace native skin autograft and allograft for closure of full-thickness burns. *J. Burn Care Rehabil.* **20**: 453, 1999.
- Boyce, S. T., Goretsky, M. J., Greenhalgh, D. G., Kagan, R. J., Riemann, M. T., and Warden, G. D. Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. *Ann. Surg.* **222**: 743, 1995.
- Passaretti, D., Billmire, D., Kagan, R., Corcoran, J., and Boyce, S. Autologous cultured skin substitutes conserve donor autograft in elective treatment of congenital giant melanocytic nevus. *Plast. Reconstr. Surg.* **114**: 1523, 2004.
- Boyce, S. T., Glatter, R., and Kitzmiller, W. J. Treatment of chronic wounds with cultured cells and biopolymers: A pilot study. *Wounds* **7**: 24, 1995.
- Boyce, S. T., and Ham, R. G. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* **81**(suppl 1): 33s, 1983.
- Boyce, S. T., and Ham, R. G. Cultivation, frozen storage, and

- clonal growth of normal human epidermal keratinocytes in serum-free media. *J. Tiss. Cult. Meth.* **9**: 83, 1985.
24. Boyce, S. T., Christianson, D. J., and Hansbrough, J. F. Structure of a collagen-GAG dermal skin substitute optimized for cultured human epidermal keratinocytes. *J. Biomed. Mater. Res.* **22**: 939, 1988.
 25. Harriger, M. D., Supp, A. P., Warden, G. D., and Boyce, S. T. Glutaraldehyde cross linking of collagen implants in vitro inhibits degradation in cultured skin substitutes grafted to athymic mice. *J. Biomed. Mater. Res.* **35**: 137, 1997.
 26. Chen, C. S. J., Lavker, R. M., Rodeck, U., Risse, B., and Jensen, P. J. Use of a serum-free epidermal culture model to show deleterious effects of epidermal growth factor on morphogenesis and differentiation. *J. Invest. Dermatol.* **104**: 107, 1995.
 27. Boyce, S. T. Fabrication, quality assurance, and assessment of cultured skin substitutes for treatment of skin wounds. *Biomed. Eng. J.* **20**: 107, 2004.
 28. Boyce, S. T., Supp, A. P., Harriger, M. D., Pickens, W. L., Wickett, R. R., and Hoath, S. B. Surface electrical capacitance as a noninvasive index of epidermal barrier in cultured skin substitutes in athymic mice. *J. Invest. Dermatol.* **107**: 82, 1996.
 29. Supp, A. P., Wickett, R. R., Swope, V. B., Harriger, M. D., Hoath, S. B., and Boyce, S. T. Incubation of cultured skin substitutes in reduced humidity promotes cornification in vitro and stable engraftment in athymic mice. *Wound Rep. Regen.* **7**: 226, 1999.
 30. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65**: 55, 1983.
 31. Varani, J., Hattori, Y., Chi, Y., *et al.* Collagenolytic and gelatinolytic matrix metalloproteinases and their inhibitors in basal cell carcinoma of skin: Comparison with normal skin. *British J. Cancer* **82**: 657, 2000.
 32. Brennan, M., Bhatti, H., Nerusu, K. C., Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin. *Photochem. Photobiol.* **78**: 43, 2003.
 33. Yamanaka, M., and Ishikawa, O. Hypoxic conditions decrease the mRNA expression of pro α 1(I) and (III) collagens and increase matrix metalloproteinases-1 of dermal fibroblasts in three-dimensional cultures. *J. Dermatol. Sci.* **24**: 99, 2000.
 34. Kan, C., Abe, M., Yamanaka, M., and Ishikawa, O. Hypoxia-induced increase of matrix metalloproteinase-1 synthesis is not restored by reoxygenation in a three-dimensional culture of human dermal fibroblasts. *J. Dermatol. Sci.* **32**: 75, 2003.
 35. Lee, C. R., Grodinsky, A. J., and Spector, M. The effects of cross-linking of collagen-glycosaminoglycan scaffolds of compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. *Biomaterials* **22**: 3145, 2004.
 36. Pek, Y. S., Spector, M., Yannas, I. V., and Gibson, L. J. Degradation of a collagen-chondroitin-6-sulfate matrix by collagenase and by chondroitinase. *Biomaterials* **25**: 473, 2004.
 37. Dasu, M. R., Spies, M., Barrow, R. E., and Herndon, D. N. Matrix metalloproteinases and their inhibitors in severely burned children. *Wound Rep. Reg.* **11**: 177, 2004.
 38. Young, P. K., and Grinnell, F. Metalloproteinase activation cascade after burn injury: a longitudinal analysis of the human wound environment. *J. Invest. Dermatol.* **103**: 660, 1994.
 39. Jones, J. L., Shaw, J. A., Pringle, J. H., and Walker, R. A. Primary breast myoepithelial cells exert an invasion-suppressor effect on breast cancer cells via paracrine down-regulation of MMP expression in fibroblasts and tumour cells. *J. Pathol.* **201**: 562, 2003.
 40. LePoole, I. C., and Boyce, S. T. Keratinocytes suppress transforming growth factor beta-1 expression by fibroblasts in cultured skin substitutes. *Br. J. Dermatol.* **140**: 409, 1999.
 41. Archer, S. B., Henke, A., Greenhalgh, D. G., and Warden, G. D. The use of sheet autografts to cover extensive burns in patients. *J. Burn Care Rehabil.* **19**: 33, 1998.
 42. Staley, M., Richard, R., Warden, G. D., Miller, S. F., and Shuster, D. B. Functional outcomes for the patient with burn injuries. *J. Burn Care Rehabil.* **17**: 362, 1996.
 43. Kagan, R. J., and Warden, G. D. Management of the burn wound. *Clin. Dermatol.* **12**: 47, 1994.