Journal of Surgical Research **128,** 79–86 (2005)
doi:10.1016/j.jss.2005.03.018

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

Viki B. Swope, D.V.M.* and Steven T. Boyce, Ph.D.,*,†,[2]

*Department of Surgery, University of Cincinnati College of Medicine; and †Research Department, Shriners Hospitals for Children, Cincinnati, Ohio

Submitted for publication December 13, 2004

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.**

Key Words: **cultured skin substitutes; fibroblasts; keratinocytes; matrix metalloproteinase-1; proteases; wound healing; burns; skin grafts; biopolymers.**

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-

[1] These studies were supported by grants from the National Institutes of Health (GM50509), and Shriners Hospitals for Children (8670 and 8450).

[2] To whom correspondence and reprint requests should be addressed at Research Department, Room 422, Shriners Hospital for Children, 3229 Burnet Avenue, Cincinnati, OH 45229. E-mail: boycest@email.uc.edu.

0022-4804/05 $30.00

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-1β [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 × 9 cm), packaged into peel-packs, and sterilized by gamma-irradiation at ~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 ($0.5 \times 10^6/cm^2$) were inoculated onto the collagen-GAG substrate and cultured at 37°C and 5% CO_2. 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 μg/ml insulin, and 0.5 μg/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 ($1.0 \times 10^6/cm^2$) 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 (~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 (~20% and ~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 ± 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 ± SEM.