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Laminin Modified Infection-Preventing Collagen Membrane Containing Silver Sulfadiazine–Hyaluronan Microparticles

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Abstract: The newly developed laminin modified infection-preventing collagen membrane consists of a 3 component laminate, comprising 2 outer collagen layers and a central laminin layer. The 2 outer collagen layers (dense and porous layers) were fabricated by air-drying and freeze drying, respectively, and the laminin layer was formed by a straightforward liquid coating method. In addition, hyaluronan based microparticles containing silver sulfadiazine (AgSD) were incorporated into the 2 collagen layers (AgSD content 50 $\mu g/cm^2$). Laminin coated collagen surfaces did not promote fibroblast attachment but showed a retarded fibroblast proliferation rate and an increased rate of collagen synthesis versus pure collagen surfaces. In an animal study, a laminin coating on a nonmedicated collagen membrane significantly increased both wound size reduction and vessel proliferation 7 days after application versus polyurethane film. Interestingly, the laminin coated AgSD medicated collagen membrane demonstrated higher wound size reduction and vessel proliferation and lower inflammation than the polyurethane control, suggesting that the laminin AgSD medicated collagen membrane substantially improves dermal wound healing. **Key Words:** Collagen membrane—Silver sulfadiazine—Hyaluronan microparticles—Laminin—Wound healing.

A large number of collagen-based membranes have been introduced to facilitate damaged skin regeneration (1,2). Several bilayered membranes consisting of a silicon and collagen sponge have been developed as dermal dressings and have resulted in the successful reconstruction of dermis when applied to a clean wound (3–5). Despite these benefits, they have not been used universally due to a perceived increase in related infections. (6,7). To overcome this limitation, antibiotics such as silver sulfadiazine (AgSD), gentamycin, tobramycin, and amikacin have been applied in combination to these membranes (8–10). AgSD has been applied to skin wounds for a long time as Silvadine cream and has been reported to control effectively *Pseudomonas aeruginosa*, *Candida albicans,* and other bacteria related to skin infections (10,11). However, due to its cellular toxicity, AgSD must be released into wounds at a controlled level, for example, to bone marrow and fibroblasts (11). To enable the sustained release of AgSD, hyaluronan (HA) microparticles were prepared and incorporated into a collagen membrane. HA, like collagen, has physical and biological properties that make it attractive as a tissue regenerative biomaterial (2,12). The combination of collagen and glycosaminoglycans such as HA and chondroitin-6-sulfate has an extensive history in a wide variety of biomedical applications, particularly as skin healing promoters and/or dressings (2,5).

Current strategies for tissue regenerative wound dressings have focused on the development of implantable matrices that mimic natural tissue (2). Skin consists of three layers: epidermis, dermis, and a basement membrane intercalated between the two. Laminin is a large basement membrane-specific glycoprotein that mediates the attachment of epithelial cells to a matrix and also has been shown to alter the shape of certain cells and to promote matrix deposition under integrin mediation (13). Although many reports have demonstrated that laminin promotes epithelial cell adhesion and migration, studies on interactions between laminin and fibroblasts are rare (14,15).

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In this study, AgSD impregnated HA microparticles were fabricated and incorporated into 2 collagen layers of a laminin modified collagen membrane which consisted of a 3 component laminate (2 outer collagen layers and a central laminin layer). This study was undertaken to investigate the effect of laminin on fibroblast adhesion, proliferation, and collagen synthesis in vitro and to evaluate the wound healing effect of this membrane versus conventional materials in order to prove its suitability as a wound dressing material.

MATERIALS AND METHODS

Preparation of silver sulfadiazine–impregnated hyaluronan microparticles

HA microparticles were prepared by gelling HA with calcium chloride as previously described (16). Type I atelocollagen was extracted from bovine tail skin using pepsin and salt precipitation and HA (sodium salt molecular weight 1,200–1,500 kilodaltons) as supplied by the Hanwha Group (Taejon, Korea). Briefly, 3% (wt/wt) of HA solution and 3% (wt/wt) collagen solution were mixed at a weight ratio of 3:1. $CaCl_2$ was then added to a concentration of 10% (wt/vol), followed by AgSD (Hanwha Group) to a weight ratio of total polymer (HA and collagen) to AgSD of 7:3. A methanol and acetone (3:1, vol/vol) solution was added to the resulting mixture, and the final concentration of $CaCl_2$ was adjusted to 5% (wt/vol). The resulting gel was incubated under vacuum for 24 h at 37°C and then freeze dried at −70°C. The dried gel was granulated into microparticles using a micro mill (MICRO-MILL, Scienceware, Pequannock, NJ, U.S.A.) and sieved to obtain a particle size of 75 to 150 μm. The HA microparticles were then divided into 2 groups, namely, AgSD nonmedicated HA microparticles (HAM) and AgSD medicated HA microparticles (AgSD-HAM).

Preparation of the laminin modified collagen membrane

Type I atelocollagen was dissolved in 0.001 *N* HCl solution at 4°C and adjusted to pH 7.4. The resulting fibrous precipitate was centrifuged at 3,000 × *g* for 15 min, and the concentrated precipitate was used as a reconstituted atelocollagen fiber. This precipitate was poured onto a polystyrene Petri dish (5 cm in diameter) and air-dried at 4°C for 24 h to form a dense collagen membrane. To produce the laminin modified collagen membrane, the dense membrane was coated with 2 ml of laminin (50 μg/ml, Sigma Chemical Co., St. Louis, MO, U.S.A.) under air blowing at 4°C. Both fabricated membranes were then crosslinked with ultraviolet (UV) irradiation (wavelength 254 nm), placed in a UV chamber (Daeil DBO231S, Seoul, Korea) and exposed to 8 10 W UV bulbs for 4 h at 4°C under N_2 purging.

Preparation of an infection-preventing bilayered membrane

The infection-preventing bilayered membrane was fabricated by combining the air-drying and the freeze-drying methods, described previously with some modification (17). A schematic diagram is presented in Fig. 1. Briefly, 5 mg of HA microparticles were mixed with 5 ml of reconstituted atelocollagen fiber. The mixture then was poured onto a polystyrene Petri dish (5 cm in diameter), and then fabricated into a dense membrane. This dense membrane was coated with 2 ml of laminin (50 μg/ml, Sigma) under air blowing at 4°C. For trilayered membrane casting, 5 ml of a mixture of HAM or AgSD-HAM plus the reconstituted atelocollagen fiber was poured onto the laminin coated dense membrane and quickly frozen at −70°C in a deep freeze (Forma Scientific, Inc., Marjetta, OH, U.S.A.) and lyophilized at −50°C for 24 h using a freeze dryer (Labconco Co., Kansas City, MO, U.S.A.). The fabricated laminate then was crosslinked by UV irradiation (wavelength 254 nm). Figure 2 represents the fabrication schematically.

Cell culture

Primary cultures of fetal dermal fibroblasts were obtained by the sequential enzymatic treatment of fetal skin using the method described by Linge et al. (18). Fetal skin was dissected aseptically from a therapeutically aborted fetus obtained in May 1998 at the Department of Obstetrics and Gynecology, Severance Hospital, Seoul, Korea. After receiving permission from the Medical Resource Committee at Yonsei University College of Medicine, the epi-

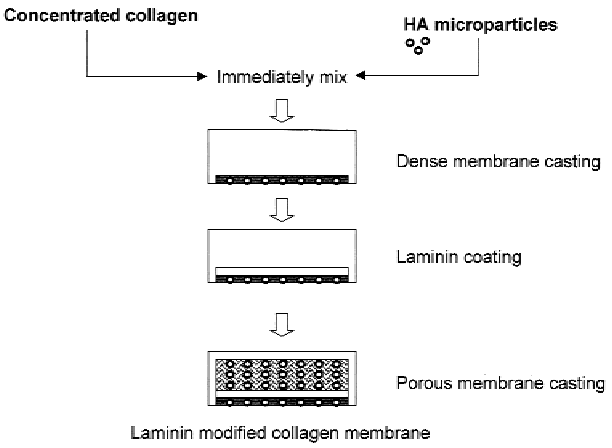

FIG. 1. Shown is the fabrication process of the laminin modified collagen membrane.

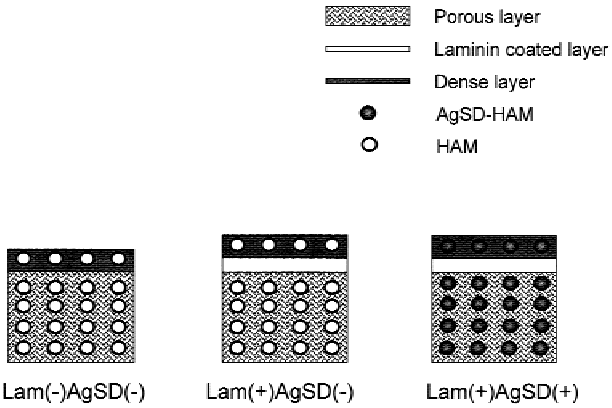

FIG. 2. The schematic presentation is of the collagen wound dressing membrane (HAM: AgSD nonmedicated HA microparticle, AgSD-HAM: AgSD medicated HA microparticles, Lam; laminin).

dermal layer was striped off, minced, and digested at 37°C by an enzyme mixture containing 0.1% collagenase (Type IA, Sigma) and 0.25% trypsin in 0.02% EDTA (Sigma) with magnetic stirring. The cells then were collected by centrifugation for 10 min at 400 × *g*, and the cell pellets resuspended in 35 mm tissue culture dishes at a density of 4×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (Sigma). Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Confluent monolayers were propagated by trypsinization (0.25% trypsin, 0.02% EDTA) and replated at 1:2 dilutions. Fibroblasts in passages 5 to 9 were used for experimentation.

Cell attachment

Membrane specimens used in the cell attachment studies were divided into 3 groups: tissue culture plate (TCP, made of polystyrene) used as the control, collagen dense membrane (COL), and laminin coated collagen dense membrane (LAM-COL). Oval membrane specimens were prepared with a diameter of 1.6 cm, and these were thoroughly washed with phosphate-buffered saline (PBS) solution before being placed on the tissue culture plates (24 wells). Fibroblasts (1.2×10^5) were seeded onto each specimen and the bottom of the TCP, as a control, and cultured for 3 h. Unattached cells were removed by washing with PBS solution. After incubation in 0.5 ml of enzyme mixture (0.1% collagenase and 0.25% trypsin in 0.02% EDTA) for 20 min, adherent cells were removed from the membrane which then was washed thoroughly twice with 0.5 ml of PBS solution. Cells in the enzyme and PBS solutions were centrifuged together for 10 min at 400 × *g* and then resuspended in fresh PBS. An aliquot of the resulting cell suspension was counted using a Neubauer hemacytometer (Sigma).

Cell proliferation

COL and LAM-COL were placed on the bottom of the TCP (24 wells) and fixed with an inert, silicone-based, vacuum grease to prevent them from floating in the growth media. Fibroblasts were plated onto the membranes at a plating density of 2.5×10^4; the medium was changed every 24 h. Cell proliferation on each specimen was determined after 1, 3, 5, and 7 days. Unattached cells were removed by washing with PBS solution. Attached cells were removed from the membrane by incubating in 0.2 ml of mixed enzyme solution for 20 min. Membranes then were washed with 0.2 ml of PBS solution, and the cells in the enzyme solution and in the PBS solution were centrifuged together for 10 min at 400 × *g* and resuspended in fresh PBS solution. An aliquot of the resulting cell suspension was counted using a hemacytometer.

Collagenous protein synthesis

The amount of total collagenous protein produced by the fibroblasts over 1, 3, 5, and 7 days was determined by radiolabeling all synthesized proteins with ^{3}H-proline and by digesting selectively collagenous proteins with collagenase (19). Fibroblasts (2.5×10^4) were plated on the membranes and the control wells, and the medium, which contained ^{3}H-proline, was changed every 24 h. For radiolabeling proteins, cells were cultured in complete media containing 1 μCi/ml [2,3-^{3}H]proline (NEN Life Science Products Inc., Boston, MA, U.S.A.) for 1, 3, 5, and 7 days. After incubation, the proteins were extracted with 0.5 *M* acetic acid containing pepsin (2,800 U/mg, Sigma) for 4 h at room temperature. Aliquots of the protein extract solutions (0.2 ml) were added to 1.5 ml microcentrifuge tubes with 0.1 ml of HEPES buffer (60 μmol, pH 7.2) containing 25 μg of purified collagenase (105 U/ml, Type IA, Sigma) and 0.25 μmol of $CaCl_2$. The tubes then were incubated at 37°C with shaking for 90 min, and an equal volume (0.3 ml) of a 10% trichloroacetic acid solution was added to stop the reaction. Tubes were placed in an ice bath for 20 min and centrifuged at 1,500 × *g* for 5 min at 4°C. Two tenths of a milliter of the supernatant solution, which contained the digested collagenous proteins, was transferred to counting vials containing 1 ml of scintillation cocktail. The precipitates were resuspended in 0.3 ml of 5% trichloroacetic acid solution and centrifuged for 5 min. Superna-

tants were counted on a scintillation counter (LSC, 1450 MicroBeta TRILUX; Wallac, Turku, Finland) to detect the presence of radioactive proline.

Evaluation of wound dressing in animals

Three types of specimens were investigated, i.e., laminin uncoated/AgSD nonmedicated, laminin coated/AgSD nonmedicated, and laminin coated/AgSD medicated membranes (hereafter Lam[–]AgSD[–], Lam[+]AgSD[–], and Lam[+]AgSD[+], respectively). Guinea pigs weighing 200 to 400 g were anesthetized with ketamine (Yuhan Co., Seoul, Korea) by intraperitoneal injection, and the dorsal hair was removed. After disinfecting the skin with Betadine (Hyundai Pharmaceutical Ind. Co., Seoul, Korea), 2 full thickness round shaped skin defects (2 cm in diameter) were prepared by excising the dorsal skin. The excised wounds produced were covered with commercial polyurethane (PU) film (Tegaderm; 3M, St. Paul, MN, U.S.A.) as a control, Lam(–)AgSD(–), Lam(+)AgSD(–), or Lam(+)AgSD(+). A sterilized elastic band was used to fix the specimens. Three, 7, and 14 days after implantation, the guinea pigs were sacrificed, and the wound sizes measured. Percentage wound size reduction was calculated according to the following formula:

$$Cn = ([So - Sn]/So) \times 100 \quad (1)$$

where Cn = wound size reduction (%) on 3, 7, and 14 days after implantation; So = initial wound size (cm); and Sn = wound size 3, 7, and 14 days after implantation (cm).

Tissue specimens from animal wounds were obtained by excising the wound with a surrounding rim of normal guinea pig skin and the underlying panniculus carnosus, fascia, and muscle. For histological examination, the tissue specimens were fixed in 10% neutral-buffered formalin solution, embedded in paraffin wax, sectioned at 3 μm, and stained with hematoxylin-eosin in the conventional manner. The resulting healing effects were investigated histologically under an optical microscope. The degree of inflammation, new vascularization, and fibroblast proliferation of the wound tissues were classified on a 4 point scale (0–3) after the numbers of each cell type in 10 visual fields per animal had been counted (Table 1).

Statistical analysis

All results were analyzed using the Student's *t*-test or one-way analysis of variance followed by Tukey's honestly significant difference (HSD) tests for multiple comparisons and are expressed as means ± standard error. The Pearson correlation test was performed. A value of $p < 0.05$ was accepted as statistically significant.

TABLE 1. *Indices of inflammation, new vascularization, and fibroblast proliferation for histological examination of tissue and their classification*

Classification	Inflammation (number of infiltrated leukocytes)	New vascularization (number of lumen including red blood cells or lumen lined with endothelial cells)	Fibroblast proliferation (number of proliferated fibroblasts)
0	0–10	0–10	0–100
1	11–100	11–20	101–500
2	101–500	21–30	501–1,000
3	>501	>31	>1,001

All the numbers were counted within the visual area (0.5 mm in diameter) after optical microscope examination (magnification ×400, n = 10).

RESULTS

Cellular interaction with the laminin coated collagen membrane

When a suspension of fibroblasts was seeded onto the TCP, COL, or LAM-COL, the percentages of attached cells were found to be 45.6%, 77.6%, and 83.1% at 2 h (Fig. 3). The numbers of cells attached to the COL and LAM-COL surfaces were similar and significantly higher than the control TCP ($p < 0.05$). In addition, the kinetics of the attachment process to these 2 substrates (COL and LAM-COL) was almost identical.

Fibroblast proliferation was observed on Days 1, 3, 5, 7, and 9 (Fig. 4). The absolute number of cells attached to TCP was significantly less than the num-

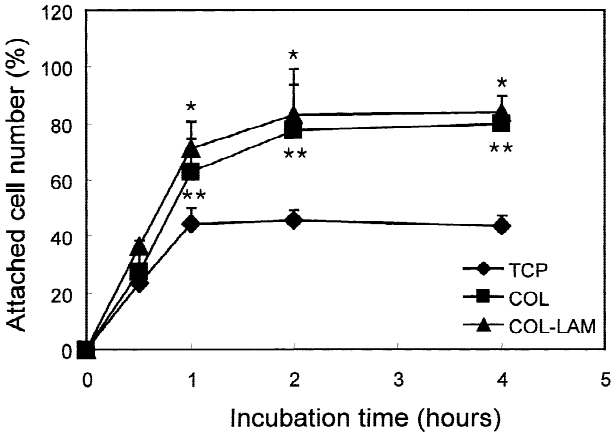

FIG. 3. The graph shows adhesion of fibroblasts onto noncoated and laminin coated dense collagen membranes after 30, 60, 120, and 240 min (control: TCP). Each point represents the mean ± SD cells (n = 5). *, **: COL and COL-LAM groups were significantly different compared to the control group, respectively ($p < 0.05$) (TCP: tissue culture plate, COL: dense collagen membrane, LAM-COL: laminin coated dense collagen membrane).

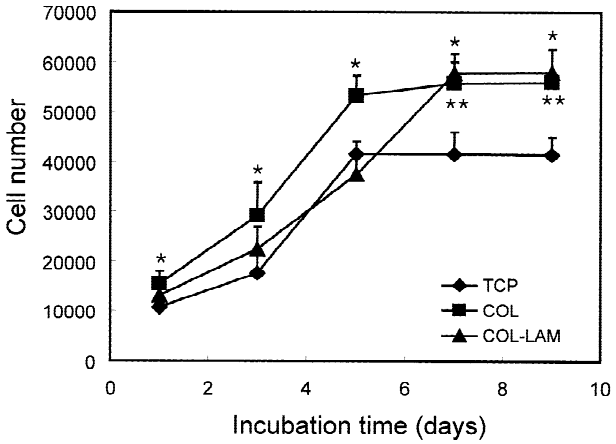

FIG. 4. The graph shows fibroblasts growth on noncoated and laminin coated dense collagen membranes over periods of 1, 3, 5, 7, and 9 days (control: TCP). Each point represents the mean ± SD of the number of attached cells (n = 4). *, **: COL and COL-LAM groups were significantly different from the control group, respectively ($p < 0.05$) (TCP: tissue culture plate, COL: dense collagen membrane, LAM-COL: laminin coated dense collagen membrane).

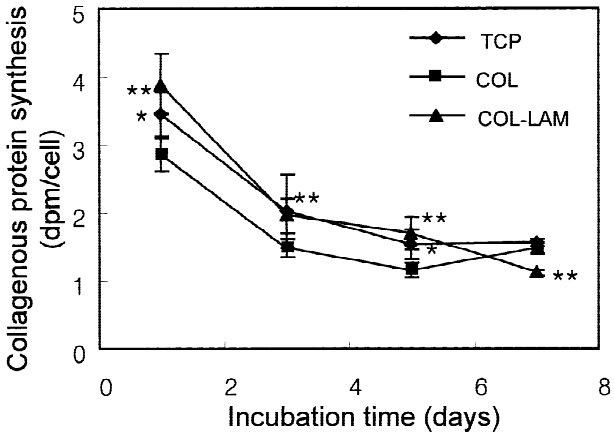

FIG. 5. The graph shows collagenous protein synthesis by fibroblasts cultured on noncoated and laminin coated dense collagen membranes for 1, 3, 5, and 7 days (control: TCP). Each point represents the mean ± SD dpm/cell (n = 6). *, **: TCP and COL-LAM groups differed significantly from the COL group, respectively ($p < 0.05$) (TCP: tissue culture plate, COL: dense collagen membrane, LAM-COL: laminin coated dense collagen membrane).

ber attached to COL, but no significant difference between the surfaces was observed in terms of cellular growth rate. The slopes of the graphs for the TCP and COL groups were significant and positively correlated ($r = 0.962$). In contrast, fibroblasts grown on LAM-COL reached confluency more slowly than cells grown on both the TCP and COL surfaces ($r = 0.274$ and $r = 0.305$). The proliferated numbers of cells attached to the control TCP was 10.9×10^3, 17.6×10^3, 41.5×10^3, 41.6×10^3, and 41.6×10^3 on Days 1, 3, 5, 7 and 9, respectively. The corresponding values on the COL surface were 15.5×10^3, 29.0×10^3, 53.3×10^3, 55.8×10^3, and 56.5×10^3 cells and on the LAM-COL surface were 13.1×10^3, 22.5×10^3, 37.5×10^3, 57.8×10^3, and 57.9×10^3.

Collagenous protein synthesis by fibroblasts cultured on these substrates also was determined on Days 1, 3, 5, and 7. The amount of collagen synthesized by fibroblasts on the control TCP and LAM-COL surfaces was significantly greater than that produced by fibroblasts on the COL surface with increasing culture time but was decreased on Day 7. The collagenous protein produced by fibroblasts on the TCP was 3.46, 2.02, 1.54, and 1.57 disintegrations per minute (dpm)/cell on Days 1, 3, 5 and 7 whereas on the COL surface, this was 2.86, 1.49, 1.15, and 1.48 dpm/cell and on LAM-COL 3.90, 1.96, 1.71, and 1.13 (Fig. 5).

In vivo animal study

In order to evaluate the effect of the laminin modified collagen membrane on wound healing, 3 types of collagen based membranes (BMs) were investigated, i.e., Lam(−)AgSD(−), Lam(+)AgSD(−), and Lam(+)AgSD(+). Figure 6 shows the wound size reductions (%) 3, 7, and 14 days after implantation. The presence of a laminin layer did not increase significantly wound size reduction in the AgSD nonmedicated groups versus the control PU film at 3, 7, and 14 days after implantation, except for Lam(+)AgSD(−) at 7 days, whereas the AgSD medicated group [Lam(+)AgSD(+)] showed a significantly higher percentage of wound size reduction

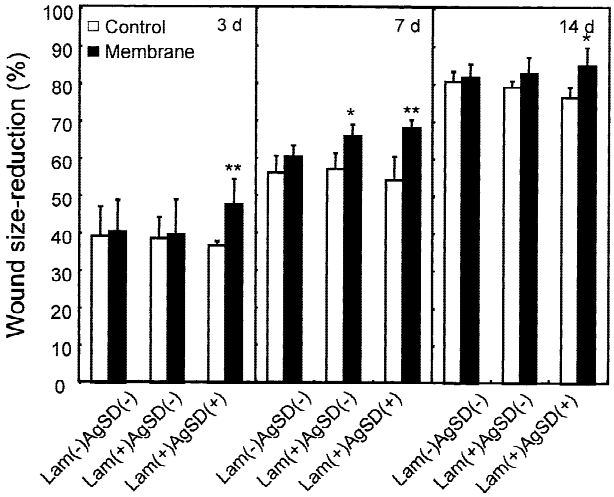

FIG. 6. Shown is wound size reduction (%) of wound surface areas covered by, PU film (as a control), Lam(−)AgSD(−), Lam(+)AgSD(−), and Lam(+)AgSD(+). *, **: significantly different versus the control group ($p < 0.05$ and $p < 0.01$, respectively) [Lam(−)AgSD(−): laminin uncoated/AgSD nonmedicated, Lam(+)AgSD(−): laminin coated/AgSD nonmedicated, Lam(+)AgSD(+): laminin coated/AgSD medicated bilayered collagen membranes (n = 4)].

than the control PU film 3, 7, and 14 days after implantation (Fig. 6).

Figure 7 shows histological results of dorsal skin wounds after 7 and 14 days. As shown in Figs. 7A and 7B, the wound surfaces of PU and Lam(−)AgSD(−) films were covered by a thick crust with acute inflammatory exudates. Lam(+)AgSD(−) had a thinner crust and a greater level of vascularization than the control PU film; however, many inflammatory cells were observed (Fig. 7C). In the case of Lam(+)AgSD(+) (Fig. 7D), the crust was much thinner and the infiltration of inflammatory cells less prominent than in the other cases. Also in this case, keratinocyte migration was observed at the wound margin in the epidermis, and the underlying dermis showed more prominent fibroblast and vessel proliferation than in the control PU and the AgSD nonmedicated groups (Table 2). Fourteen days after implantation of the PU (Fig. 7E) and Lam(+)AgSD(−) (Fig. 7F) membranes, fibroblast proliferation and an increased level of collagen were noted in the dermis, but inflammation had not disappeared, and the epidermis had not been restored completely although re-epithelization from the wound margin was observed (Fig. 7G). However, in the case of Lam(+)AgSD(+), the skin defects were completely healed (Fig. 7H).

DISCUSSION

This work was undertaken to develop a wound dressing that facilitates skin regeneration and prevents infection. To develop a dressing material that

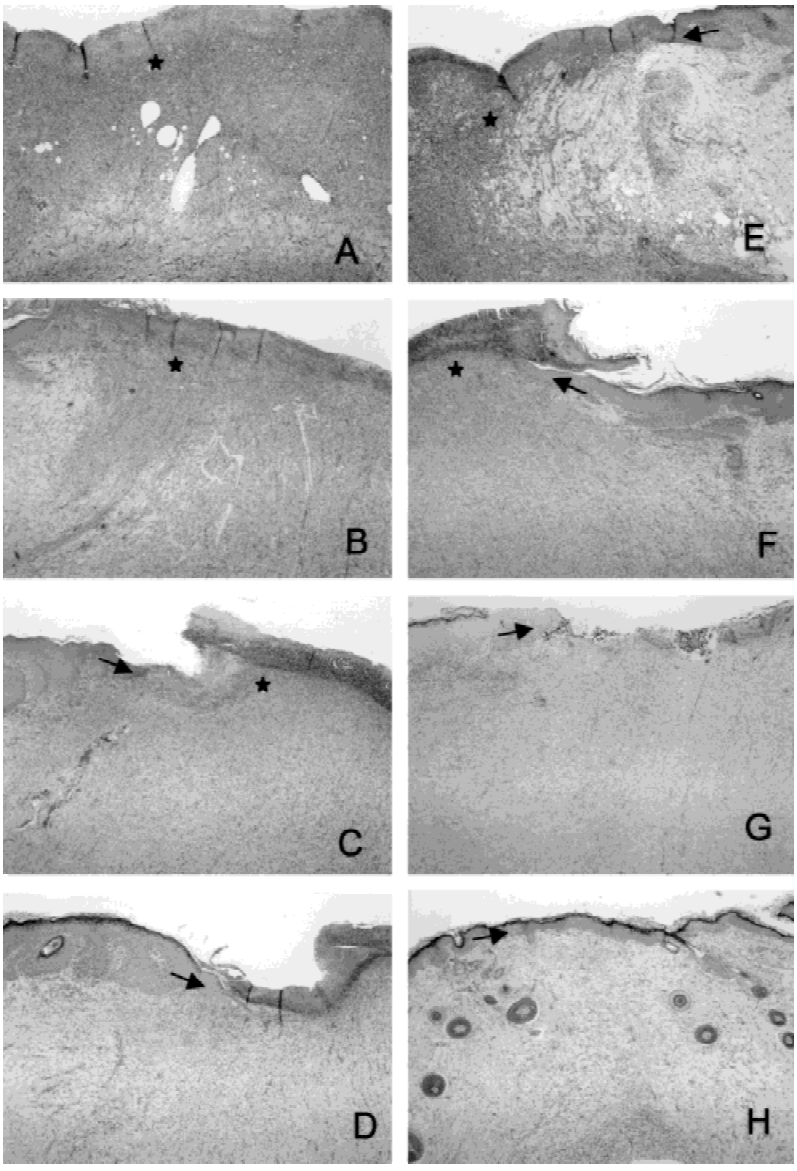

FIG. 7. The histological photographs are of wound surfaces covered with PU film (as control) 7 days after implantation **(A)** and Lam(−)AgSD(−) **(B),** Lam(+)AgSD(−) **(C),** and Lam(+)AgSD(+) **(D)** 7 days after implantation (all magnification ×40). Also shown are PU film (control) 14 days after implantation **(E)** and Lam(−)AgSD(−) **(F),** Lam(+)AgSD(−) **(G),** and Lam(+)AgSD(+) **(H)** 14 days after implantation (magnification ×40). Arrows indicate epithelization sites and stars indicate inflammatory cells. (Lam(−)AgSD(−): laminin uncoated/AgSD nonmedicated, Lam(+)AgSD(−): laminin coated/AgSD nonmedicated, Lam(+)AgSD(+): laminin coated/AgSD medicated collagen 2 layer membranes).

TABLE 2. *Histological examination of tissue after 7 and 14 days*

	Days	Control	Lam(–) AgSD(–)	Lam(+) AgSD(–)	Lam(+) AgSD(+)
Inflammation	7	2.4 ± 0.5	2.3 ± 0.6	2.1 ± 0.5	1.3 ± 0.3
	14	1.3 ± 0.5	1.6 ± 0.7	1.3 ± 0.5	0.8 ± 0.4
New vascularization	7	1.0 ± 0.5	1.0 ± 0.6	1.7 ± 0.6	2.1 ± 0.1
	14	1.5 ± 0.5	1.5 ± 0.7	1.5 ± 0.5	2.0 ± 0.1
Fibroblast proliferation	7	1.0 ± 0.5	1.0 ± 0.6	1.7 ± 0.6	2.1 ± 0.6
	14	1.7 ± 0.4	1.9 ± 0.1	2.2 ± 0.1	2.5 ± 0.5

These values were based on indices expressed in Table 1.

mimics natural skin, we selected the extracellular matrix (ECM) components collagen, HA, and laminin as biomaterials and designed a 3 component laminated membrane, comprising 2 outer collagen layers and a central laminin layer.

The interaction between cells and biomaterials may influence directly their activities; for example, the adhesion, proliferation, and differentiation of fibroblasts are related to their ability to bind molecules of the ECM. The integrins α1β1, α2β1, and α3β1, which are expressed by fibroblasts, are major collagen receptors. Moreover, these species have different functions; for example, α1β1 appears to regulate collagen synthesis whereas α2β1 regulates collagenase expression (20).

The laminins are a family of heterotrimeric molecules ubiquitously distributed in the basement membrane. They control cell behavior by interacting with the integrins α1β1, α2β1, and α3β1 expressed by fibroblasts (13). Moreover, keratinocytes attach, proliferate, migrate, and differentiate by interacting with laminin using integrins α3β1 and α6β1 (15).

The initial attachment reaction of cells in culture involves interaction between Type I collagen and the α1β1, α2β1, and α3β1 integrins on the cell's surface (20). These integrins mediate the transmembrane signal transduction that ultimately leads to changed cellular expressions (20). Our results demonstrate that fibroblast attachment is promoted on COL and LAM-COL versus the control TCP which suggests that the collagen surface supports initial attachment. Fibroblasts adhered to COL and TCP and reached confluency in 5 days whereas those on LAM-COL took 7 days to reach confluency. Laminin is reported to have the ability to bind to a variety of substances, including carbohydrates, proteoglycans, and gangliosides, and any one of these substances might participate in cellular proliferation whether this occurs via transmembrane signals or cell-substrate interactions. Such interactions also alter the shape and cellular spreading, which, in turn, could affect the synthesis of protein and DNA (21). Generally, the surrounding ECM influences cellular activities, and the reorganization of the ECM is an important aspect of many biological processes. Culturing fibroblasts in a collagenous environment leads to the change of fibroblast biosynthetic capacity, which is accompanied by a fundamental reprogramming of fibroblast morphology and metabolism and results in a downregulation of Type I collagen and the induction of matrix-degrading proteases. The downregulation of Type I collagen and the induction of collagenase occur naturally during ECM reconstruction, such as occurs during wound healing (22). Our results demonstrated that collagenous protein synthesis is downregulated by contact with a collagenous surface (COL) as compared with the control TCP surface in the earliest stage of culture (Fig. 5). However, the amount of synthesized collagen from COL had increased distinctly at 7 days. This finding suggests that the regulation of collagen synthesis seems to be related to collagenase induction by integrin-ligand binding. Sudbeck et al. reported that low levels of collagenase-1 mRNA were detected 2 h after plating keratinocytes on Type I collagen and that this expression increased progressively over the following 10 h and that thereafter the collagenase 1 mRNA level fell to levels coincident with keratinocytes at confluency. Sudbeck et al. also suggested that a low level of collagenase-1 mRNA was expressed by keratinocytes cultured on a laminin coated surface (23). Since the stimulation of collagenase-1 expression is related closely to collagen synthesis, the low level of collagenase-1 mRNA may have induced the upregulation of collagen synthesis. Accordingly, our result showed that collagenous protein synthesis was higher on laminin coated collagen surfaces than on collagen surfaces.

The efficacy of fabricated collagen based membranes was evaluated by in vivo animal testing. Conceivably, the collagen based membrane is likely to accelerate the healing process by immobilizing overflowing hemorrhagic exudates associated with a fresh wound, thus preventing the formation of mobile fluid pockets and promoting the rapid dehydration of the wound bed. In addition, coated laminin is expected to facilitate the rapid migration of keratinocytes and therefore to result in accelerated re-

epithelization. Zhang and Kramer demonstrated that keratinocytes require laminin for motility and that α3β1 integrin appears to be the primary receptor that mediates this laminin-dependent motility (15). Our study showed that membranes not containing AgSD did not promote significantly wound healing when compared with commercial wound dressings. However, a laminin coating on an AgSD nonmedicated membrane induced more rapid epithelial migration by histological examination; however, we could not conclude that it had a beneficial effect.

Because laminin coated and AgSD medicated membranes demonstrated higher wound size reduction and vessel proliferation and lower inflammation than control PU film, our results suggest that the laminin AgSD medicated collagen membrane achieved its biological effect by releasing AgSD into the wound without retarding skin regeneration and that this led to accelerated fibroblast infiltration and the formation of new capillaries. Previous reports also have demonstrated antibiotic-medication accelerated wound healing in animal skin defects (9,24). Since wound infection is known to retard the wound healing process, antibiotic dressing material would be expected to accelerate the healing of skin defects. Moreover, AgSD is known to control effectively *Pseudomonas aeruginosa*, *Candida albicans*, and other bacteria related to skin infection (9–11) although the mechanism of AgSD's antibacterial action has not been elucidated fully. Nevertheless, it is believed that the AgSD molecule dissociates and that the silver moiety becomes bound to bacterial cells, penetrates the bacterial cell wall, and subsequently is attached toDNA and prevents bacterial proliferation. The sulfadiazine moiety also has a bacteriostatic effect (25). In the present study, a combination of AgSD and laminin modified collagen membrane was shown to facilitate the dermal wound healing process.

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