I concur with this review. M. Serabian 12/29/11

FOOD AND DRUG ADMINISTRATION
Center for Biologics Evaluation and Research
Office of Cellular, Tissue and Gene Therapies
Division of Clinical Evaluation and Pharmacology/Toxicology
Pharmacology/Toxicology Branch

BLA NUMBER:	STN #125400.000
DATE PHARM/TOX MODULE RECEIVED BY CENTER:	May 13, 2011
DATE REVIEW COMPLETED:	October 04, 2011; amended December 08, 2011; amended December 27, 2011
PRODUCT:	Apligraf® (oral) –b(4)--
SPONSOR:	Organogenesis, Inc.
PROPOSED INDICATION:	For the treatment of surgically created gingival and alveolar mucosal surface defects in adults
PHARM/TOX REVIEWER:	Patrick Au, Ph.D.
PHARM/TOX SUPERVISOR:	Mercedes Serabian, M.S., DABT
DIVISION DIRECTOR:	Wilson Bryan, M.D.
OFFICE DIRECTOR:	Celia Witten, Ph.D., M.D.
PROJECT MANAGER:	Terrolyn Thomas, MS, MBA

Formulation and Chemistry:
Apligraf® (oral) is a bi-layered tissue construct consisting of allogeneic epidermal keratinocytes (---b(4)--------), fibroblasts –b(4)---------- and bovine type I collagen. The upper layer is made of living human keratinocytes (derived from neonatal foreskin), which are organized into a well differentiated cornified layer similar to human skin. The supporting lower layer is constructed of bovine-derived collagen, human extracellular matrix proteins, and human neonatal dermal fibroblast cells. The upper and lower layers adhere as one bi-layered unit, making up approximately ---b(4)--------- respectively, of the construct. The construct is supplied as a single circular disk that is approximately 75 mm in diameter and 0.75 mm thick. The clinical product is stored on a semi-permeable synthetic membrane, which separates it from a semi-solid agarose nutrient medium below, and an air layer of carbon dioxide above, in a transparent plastic container.

Abbreviations:

ALI- air-liquid interface
----b(4)----------
----b(4)----------

CSO-cotton seed oil
DC-dendritic cell
DE-dermal equivalent
----b(4)----------

EGF-epidermal growth factor
ELISA-enzyme-linked immunosorbent assay
FGF-fibroblast growth factor
----b(4)----------

---b(4)----------
HDF-human dermal fibroblast
HES-Hematoxylin-Eosin-Saffron
HK-human keratinocyte
HLA-human leukocyte antigen
HUVEC-human umbilical vein cell
IFN-γ-Interferon-γ
IGF-insulin-like growth factor
IHC- immunohistochemistry
IL-interleukin
ISH-*in situ* hybridization
---b(4)----------
--b(4)----------
---b(4)----------
MMP-matrix metalloproteinases
mRNA- messenger ribonucleic acid
MTG-Masson Trichrome-Goldner
NaCl-sodium chloride
PA- percutaneous absorption
PBL- peripheral blood lymphocyte
PBMC-peripheral blood mononuclear cell
PBS-phosphate buffered saline
PDGF-platelet derived growth factor
PHA- Phytohemagglutinin
RT-PCR-reverse transcriptase polymerase chain reaction
SCID-severe combined immunodeficiency disease
TEM-transmission electron microscopy
TGF-transforming growth factor
TIMP- tissue inhibitors of matrix metalloproteinases
TNF-tumor necrosis factor
VEGF-vascular endothelial growth factor

Application History:
BLA submission: May 13, 2011

Cross-references:
IDE # G050122
IDE #G070012
IDE #G070178
PMA #P950032 – Sponsor: Organogenesis, Inc.; Product: Apligraf®; Indication: venous leg ulcers and diabetic foot ulcers; Approved on 5/22/98 and 6/20/00

Table of Contents

INTRODUCTION

To maintain the health and function of the oral cavity, an adequate quantity and quality of functionally appropriate soft and hard tissue must be present. Oral mucosal deformities (also referred to as "mucogingival deformities") can involve both hard and soft tissues. Deformities to the oral mucosa may be developmental or acquired, and often require surgery to correct them. Developmental causes of oral mucosal deformities can include high frenum attachments, muscle pulls, and buccal or lingual tooth eruptions. Acquired deformities may arise from periodontal disease, trauma, surgery, loss of teeth, neoplasms, or overly aggressive tooth brushing. The goal of surgery is to restore tissue with site-appropriate form and function to the defect. The current treatment for soft tissue repair involves the use of autografts (e.g., free gingival graft), which restore mucosal function (e.g., increased keratinized tissue). However, such procedures result in pain at graft donor site and poor aesthetic quality (texture and color mismatch) (*1*).

The sponsor-proposed label indication is: *"Apligraf (oral) is indicated for the treatment of surgically created gingival and alveolar mucosal surface defects in adults. The product is applied over a vascular wound bed to regenerate site-appropriate oral mucosal tissues."*

Apligraf®, which is identical to Apligraf® (oral), was approved in 1998 and 2000 (PMA #P950032) for the treatment of venous leg and diabetic foot ulcers. Apligraf® was developed to mimic the architecture and function of skin and it was originally thought to function as a substitute for skin. However, subsequent investigation revealed that the cellular components do not persist long term (*2*); Apligraf® is currently believed to function to stimulate wound healing by secondary intention, which relies upon growth factors and cytokines. Secondary healing occurs when wounds are not anatomically reapproximated and wounds heal through formation of granulation tissue and re-epithelialization (*3*).

According to the sponsor there are numerous similarities in the tissue structure and wound healing process of oral mucosa and cutaneous wounds (*4*), the sponsor's position is that Apligraf® would similarly stimulate oral mucosal healing by secondary intention. However, per the BLA the exact mechanism by which Apligraf® achieves oral mucosal wound healing has not been fully characterized. Per the sponsor briefing package for the 11/17/11 advisory committee meeting, the stated mechanism of healing is multi-modal: Apligraf® does not vascularize, integrate, or persist at the implanted wound site. Rather, the sponsor contended that the production of growth factors and cytokines by the cells and the stratum corneum barrier provided upon application of the product to the wound site, contribute to enhanced wound healing by secondary intention.

The proposed package insert (PI) submitted by the sponsor states that Apligraf® (oral) is to be applied over a vascular wound bed to regenerate 'site-appropriate' oral mucosal tissue. Additional applications within a treatment cycle have not been established using Apligraf® (oral). Apligraf® (oral) is supplied as a circular disk approximately 75 mm in diameter and 0.75 mm thick and is stored on a semi-permeable polycarbonate membrane

within a plastic ring, which separates the product from the gel medium (agarose), in a sterile transparent shipping tray. The plastic container is sealed in a polybag containing an air atmosphere supplemented with approximately 10% carbon dioxide at the time of packaging. Each unit of Apligraf® (oral) is intended for single-use and should not be applied to more than one patient or after more than 15 minutes has elapsed after opening the sterile tray. However, the same unit of Apligraf® (oral) ----- ---b(4)----------
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References:

(1). Griffin TJ, Cheung WS, Zavras AI, Damoulis PD. Postoperative complications following gingival augmentation procedures. J Periodontol. 2006 Dec;77(12):2070-9.
(2). Griffiths M, Ojeh N, Livingstone R, Price R, Navsaria H. Survival of Apligraf in acute human wounds. Tissue Eng. 2004 Jul-Aug;10(7-8):1180-95.
(3). Franz MG. Chapter 6. Wound Healing. In: Doherty GM, ed. *CURRENT Diagnosis & Treatment: Surgery*. 13th ed. New York: McGraw-Hill; 2010
(4). Sciubba JJ, Waterhouse JP, Meyer J. A fine structural comparison of the healing of incisional wounds of mucosa and skin. J Oral Pathol. 1978 Aug;7(4):214-27.

Preclinical Studies

Apligraf®, which is identical to Apligraf® (oral), was approved in 1998 and 2000 (PMA #P950032) for the treatment of venous leg ulcers and diabetic foot ulcers. In addition to the preclinical studies that supported the PMA two additional studies were conducted and submitted for BLA review:

1. Preclinical evaluation of Apligraf applied to full thickness wounds in Swiss Nude mice (PCLR_101219_IVV7_CFR_1) for short (7-28 days) and long (3-6 months) periods of time

2. Apligraf Periodontal Preclinical Dressing Evaluation (NCLR-0254-001)

Note: ‘Apligraf®’ is also presented as ‘Apligraf’ in this review.

Summary of Pharmacology Studies

The following list includes the pharmacology studies that were conducted initially in support of Apligraf®, and were submitted to the PMA. Each study is briefly described below.

(1). Cytokine and receptor analysis of Graftskin (Apligraf) by RT-PCR (ITS97001)
(2). Response of Apligraf® (Graftskin) to Physical Injury *In Vitro* (RR-0128)
(3). Effect of Growth Factors Secreted from Bioengineering Living Tissue on the Migration and Proliferation of Human Skin Cells
(4). Morphological Development and Maturation of Graftskin (Apligraf) *In Vitro*
(5). A *In-vitro* Wound Model Using Apligraf™ (RDR-022)
(6). -----b(4)----------

(7). Evaluation of Graftskin (Apligraf) Composite Grafts on Full-Thickness Wounds on Athymic Mice
(8). Tissue Remodeling and Cellular Persistence in Skin Construct Implants
(9). Remodeling of a Bioengineering Living Skin Construct Grafted onto Nude Mice
(10). Lack of Rejection of Graftskin (Apligraf) on hu-SCID Mice (ITS97005)
(11). Preclinical evaluation of Apligraf over short (7-28 days) and long (3-6 months) full thickness wounds in Swiss Nude mice (PCLR_101219_IVV7_CFR_1)
(12). Immunohistological characteristics of Apligraf® (RD-2000-02026)
(13). Effect of Graftskin (Apligraf) Development on Graft Performance and Barrier Function Formation *In Vivo* (RDR-024)
(14). *In vitro* Neutral Allograft Study
(15). Expression of AB Blood Group Antigens on Apligraf™ (RDR-031)
(16). Molecular Typing of Apligraf™ Cells For Rh Blood Group Antigens (RDR-029)
(17). *In vitro* Allograft Tolerance Studies
(18). *In vitro* analysis of the relative roles of costimulatory receptor ligand interactions in the T cell alloantigen response to Graftskin™ (Apligraf) cellular components (ITS97003)
(19). Analysis of the role of inflammatory/costimulatory cytokines on the alloantigenicity of the cellular components of Graftskin™ (ITS97004)
(20). Characterization of Primary and Secondary Allogeneic T cell Responses after Priming with HLA-Matched Professional and Non-Professional APC (RR-0076)
(21). Graftskin (Apligraf): Determination of cell purity in human keratinocyte and dermal fibroblast cell banks (ITS97002)
(22). Determination of Residual Bovine Serum Proteins in Apligraf (NCLR-0105-001)
(23). Apligraf Periodontal Preclinical Dressing Evaluation (NCLR-0254-001)
(24). Graftskin (Apligraf) Compatibility with Antimicrobial Agents *In Vitro* Studies
(25). Investigation of *in vitro* tolerability by the human skin equivalent Apligraf® of exposure to antibiotics/antiseptics for 24 hours (001974)
(26). Graftskin (Apligraf) Compatibility with Antimicrobial Agents *In Vivo* Animal Study

Pharmacology Studies

1. **Cytokine and receptor analysis of Graftskin (Apligraf) by RT-PCR,** Study# ITS97001; conducted by Organogenesis; non-GLP; 1997

   The purpose of this study was to determine the cytokine and cytokine receptor mRNA profiles of Apligraf and its component cells (HKs and HDFs).

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b(4)

[b(4)]

Conclusion: The cytokine and receptor mRNA expression profiles of Apligraf were established. Apligraf expressed many of the cytokines associated with normal human skin.

Comments:

- The passage number for the HKs and HDFs was not provided.
- The study did not incorporate normal human skin as a control for comparison.

- -----b(4)----------

- The RT-PCR assay used was non-quantitative and the cutoff densiometric value of 0.05 appears to be arbitrary. Therefore, the results of the study did not suggest comparable levels of cytokine expression between Apligraf and normal human skin.

References:

(1). Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol*. 2007;96:41-101.

2. **Response of Apligraf® (Graftskin) to Physical Injury *In Vitro,*** Study# RR-0128; conducted by Organogenesis; non-GLP; 1998

   The purpose of this study was to determine the *in vitro* wound healing response of Apligraf.

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   [b(4)]

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   - ----b(4)--- ----------

2 Pages determined to be not releasable:

b(4)

----------**b(4)**-- ----------

[b(4)]

Conclusion: Per the sponsor, the study showed that Apligraf was able to respond to physical trauma with a healing response of keratinocyte migration, re-epithelialization, and keratinization. The cytokine expression profile was consistent with normal wound healing.

Comments:

- The sponsor did not specify the location on the –b(4)---- Apligraf at which the biopsies were taken. The gene expression of cells in close proximity to the margin of cell migration would likely differ from cells distant to the wound margin.

- The methods section in the study protocol did not specify that RT-PCR analysis would be performed for ---b(4)--- ----------.

- The RT-PCR used was non-quantitative, thus the trends in the temporal profiles of gene expression may not be accurate.

- ---b(4)-- ----------

3. **Effect of Growth Factors Secreted from Bioengineering Living Tissue on the Migration and Proliferation of Human Skin Cells,** Study# None; conducted by ------b(6)-----.; non-GLP; 1999

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4. **Morphological Development and Maturation of Graftskin (Apligraf) *In Vitro***
   Note: Please see the CMC review memo authored by M. Lee.

5. **A *In-vitro* Wound Model Using Apligraf®**, Study# RDR-022; conducted by Organogenesis; non-GLP; 1997

   The purpose of this study was to determine the ability of Apligraf to re-epithelialize after physical trauma.

   Test article: Apligraf (Lot no. ----b(4)----------

   ---b(4)---- ----------

   ---b(4)--- ----------

   Conclusion: According to the study report, complete re-epithelialization was observed after b(4) days in culture in each of the three experiments. Per the sponsor, these results suggest that Apligraf is capable of responding to physical trauma in a manner similar to normal human skin.

   Comments:
   - The sponsor did not provide sufficient data in the study report to allow for independent verification by this reviewer.

   References:

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1 Page determined to be not releasable:

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7. Evaluation of Graftskin (Apligraf) Composite Grafts on Full-Thickness Wounds on Athymic Mice, Study# None; conducted by the –b(4)----------and Organogenesis; non-GLP; 1992

The purpose of this study was to assess Apligraf for: 1) its ability to take, to integrate with host tissue, and persist for 60 days in an animal model and 2) to compare to split-thickness human –b(4)----------skin grafts on the same model.

Species: --b(4)------- mice, 24 mice/group (-----b(4)----------); age and sex of the mice were not provided in the study report

Test article: Apligraf (Lot no. G100-08-09)

Methods: A 2 x 2 cm full-thickness skin section was excised from the dorsum of 24 mice and Apligraf pieces were sutured to the wound bed. Split-thickness –b(4)--- skin –b(4)-- was sutured to the wound bed of another 24 mice. Mice (6/group) were euthanized at 6, 15, 30, and 60 days post-surgery. At each time point, grafts were photographed and macroscopically examined for evidence of wound contraction, areas of graft loss, and presence of vascularization. Tissue biopsies were collected for histological analysis.

Results: Minimal wound contraction in the graft sites was observed in both study groups at all time points evaluated. Morphologic analysis showed that Apligraf and -b(4)-- remained intact in all animals, suggesting graft adherence. Junctions between the murine skin and the Apligraf –b(4)--- grafts were contiguous, suggesting integration with host tissue. The dermis of the –b(4)- grafts was highly vascularized beginning from 6 days post-surgery. Small blood vessels penetrating the dermal matrix of Apligraf from the wound margin were detected at 6 days post-surgery, with their number and size increasing as the study progressed. Transmission electron microscopy (TEM) analysis confirmed the presence of the ultrastructural features of a differentiated epidermis in both graft types. Immunohistochemistry (IHC) staining showed the persistence of HKs and the presence of a fully formed basement membrane in the Apligraf and the –b(4)- grafts at all time points evaluated.

Conclusion: According to the study report, the graft integration and persistence of Apligraf and –b(4)- following application to full-thickness wounds of nude mice were comparable.

Comment:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

8. Tissue Remodeling and Cellular Persistence in Skin Construct Implants, Study# None; conducted by Organogenesis; non-GLP; 1999

The purpose of this study was to characterize the *in vivo* tissue maturation, remodeling, and cellular persistence of Apligraf in a full-thickness wound in athymic mice.

Species: Athymic mice, 6-8 weeks old; the number, strain and sex were not provided in the study report

Methods: An approximate 3 cm^2 full-thickness excision wound was created on the central dorsum of athymic mice, and pieces of Apligraf were cut to the size of the wound and applied to the wound bed. At each time point (not provided), grafts were collected and processed for light microscopy and TEM. Formalin fixed paraffin embedded sections were used for H&E staining, trichrome staining and IHC to assess morphology, matrix remodeling and cellular persistence. Antibodies specific for human involucrin and human vimentin proteins were used to detect Apligraf keratinocytes and fibroblasts, respectively. Sections fixed and processed for TEM analysis were used to assess basement membrane (BM) maturation. The barrier function was determined using a percutaneous absorption (PA) assay (i.e., measuring permeability rate of tritiated water) according to the method of –b(4)----------

Results: Engraftment was successful for approximately 95% of the animals. There was no macroscopic evidence of bleeding or an inflammatory reaction. Histological evaluation revealed no inflammatory infiltrates in the body of the graft. The basement membrane of Apligraf, which was immature prior to grafting, was comprised of a thin, discontinuous lamina densa with numerous gaps. By 14 days post-graft, the basement membrane structure was formed, with all of the expected morphological features apparent. Barrier function improved significantly during the first 7 days post-graft. The barrier on the grafts was 10-20-fold less permeable than that of Apligraf in culture. At day 14 post-graft, the PA values were in the range of the values for normal human skin. From the histology staining, the graft showed proper stratum-specific expression of epidermal differentiation markers and approximately the same vascular density as the surrounding mouse skin. The grafted keratinocytes and fibroblasts persisted for at least 1 year after grafting. The human fibroblasts, like the keratinocytes, did not migrate away from the graft. The only intermixing of the human graft with mouse cells occurred when the mouse cells migrated into the graft.

Conclusions: According to the study report: 1) both keratinocytes and fibroblasts persisted for at least 1 year after grafting of Apligraf onto the backs of athymic mice; 2) matrix remodeling was a slow and steady process that was similar to the process

for uninjured skin; and 3) rapid maturation of normal barrier function and basement membrane was demonstrated.

Comments:

8. Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer

9. The study report did not specify the animal sacrifice time points.

10. The study report did not define the meaning of a "successful" engraftment of Apligraf.

11. The study report did not specify the range of PA values for normal human skin

12. The study report stated that there were no inflammatory infiltrates in the body of the graft; however, it did not report whether this finding was also observed at the graft margin.

13. Data to support the species specificity of the anti-involucrin and anti-vimentin antibodies were not provided.

References:

------b(4)--- ----------

9.Remodeling of a Bioengineering Living Skin Construct Grafted onto Nude Mice, Study# None; conducted by the ----b(4)-- ----------; non-GLP; 1999

The purpose of this study was to investigate the progressive remodeling of Apligraf and the presence of Apligraf cells at the wound site over time in the athymic mouse model, and to determine the cellular and the matrix components of the graft from 0-365 days post-graft using –(b)(4)---.

Species: Athymic mice, 6-8 weeks old; the number, strain and sex were not provided in the study report

Methods: An approximate 3 cm^2 full-thickness excision wound was created on the central dorsum of athymic mice, and pieces of Apligraf were cut to the size of the wound and applied to the wound bed. Grafts were harvested at 7, 14, 28, 56, 100, 180, 232, and 365 days after grafting. Tissue samples were fixed and processed for histology and electron microscopy analyses. Species-specific antibodies (murine, bovine, human) were used to label extracellular matrix proteins or cell markers. Antibodies used were: (1) extracellular matrix proteins: type IV and V collagens and

(2) cell markers: human involucrin (keratinocytes), human vimentin (fibroblasts), CD31 (endothelial cells), and α-Smooth Muscle Actin (myofibroblasts).

Results: The harvested grafts stained positive for human collagen type IV (weak/normal staining) and type V (weak/strong staining) at all time points; for blood vessels (weak/normal staining) at most time points and myofibroblasts (normal staining) at only two early time points (Table 1). Human vimentin and human involucrin positive cells were detected in the harvested grafts out to 365 days after grafting.

Table 1: Staining of Matrix Protein and Cell Markers

DAYS POST GRAFTING	COLLAGEN IV (HUMAN)	COLLAGEN IV (MURINE)	VESSELS	COLLGEN V	ENDOTHELIAL CELLS	MYO-FIBROBLASTS
O	+	-	-	+/-	-	-
7	+	-	+/-	+	-	+
14	+	-	-	++	+/-	+
28	+	-	+	++	+	-
56	+	+/-	+	++	+	-
102	+	+/-	+	++	+	-
180	+	-	+/	++	+/-	-
232	+/-	-	+/	+	+	-
365	+/-	-	+/	+/-	+/-	-

+++: very strong staining; ++: strong staining; +: normal staining; +/-: weak staining or incomplete; -: no staining

Note: Only the center of the harvested graft was evaluated.

Note: Only the center of the harvested graft was evaluated

Conclusions: Per the study report, although human cells were still present and biologically active at the wound site as long as one year post-application on the backs of athymic mice, cell presence progressively decreased with time. No signs of abnormal skin development or hyper-inflammation were found. Types IV and V human collagen followed the same pattern. Types I and b(4) bovine collagen also progressively decreased and were replaced by host tissue. Endothelial cells progressively infiltrated into the graft over time, but the myofibroblasts followed an opposite pattern; therefore, there was no development of a hypertrophic scar.

Comments:

14. Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

15. Data to support the species specificity of the antibodies used were not provided.

10.Lack of Rejection of Graftskin (Apligraf) on hu-SCID Mice, Study# ITS97005; conducted by Organogenesis; non-GLP; 1997

The purpose of this study was to determine if Apligraf would be rejected by unprimed human lymphocytes in the hu-SCID mouse model.

Species: SCID male mice (--b(4)----------); 4-6 weeks old, 7-13 mice/ group, 21.4-31.4 g

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human volunteers. To generate hu-SCID mice, SCID mice were intraperitoneally injected with the PBMCs at 3-5 x 10^7 cells/mouse. At 3-5 weeks post-cell injection, serum was collected and analyzed for human IgG levels by –b(4)--. Only mice with circulating levels of human IgG ≥250 μg/ml were subsequently grafted with either human skin or with Apligraf. In some study groups, Apligraf was pre-treated with 250 U/ml of IFN-γ before applying to the mouse. For grafting, a 2 x 2 cm full-thickness wound was created on the right posterior lateral dorsum and human skin or fully differentiated Apligraf (--b(4)---------- were then grafted onto the wound and held in place with sutures. Both SCID and hu-SCID mice were grafted. Animals were sacrificed 14-32 days after grafting. At necropsy, skin grafts and spleen were removed. b(4) was performed with human specific involucrin antibodies and mouse anti-human HLA-ABC, HLA-DR, and CD45 monoclonal antibodies. DNA was extracted from the spleens of the mice to evaluate engraftment of human cells. Detection of human cells was performed using –b(4)-----. Production of cytokines (IL-10, TGF-β1, IL-6, IL-8 and TNF-α) by the pre- and post-implanted Apligraf in the SCID mice was analyzed by b(4) analysis.

Table 1: Study Design

STUDY ARTICLE	ANIMAL TYPE	NUMBER OF ANIMALS PER GRROUP
Experiment 1		
Human Epidermis	SCID	12
Human Epidermis	Hu-SCID	7
Apligraf	SCID	11
Apligraf	Hu-SCID	8
Experiment 2		
Apligraf-not treated with IFN-γ*	SCID	11
Apligraf-not treated with IFN-γ*	Hu-SCID	10
Apligraf-treated with IFN-γ*	SCID	13
Apligraf-treated with IFN-γ*	Hu-SCID	9
*Apligraf was treated with 250 units/mL of recombinant IFN-γ		

Results: The Apligraf had a slightly higher rate of graft survival as compared to the human skin graft following application on the SCID mice (11/11 versus 9/12, respectively). This difference was magnified in the hu-SCID mice; only 2/7 of the

human skin grafts persisted at 14 days, while 7/8 of the Apligraf grafts persisted at 28 days (Table 2). b(4) analysis using antibodies against human involucrin showed the presence of human epidermal cells on the Apligraf grafts at day 28-30.

Table 2. Graftskin is not rejected by hu-SCID mice

Type of Graft	Animal Type[1]	Graft Survival[2] (% survival)
Human	SCID	9/12 (75)
Human	hu-SCID	2/7[3] (28)
Graftskin	SCID	11/11 (100)
Graftskin	hu-SCID	7/8[3] (88)

HLA-class II expression was induced by pretreating Apligraf with IFN-γ. The graft survival rate of IFN-γ-treated Apligraf on hu-SCID mice (9/9) was equivalent to untreated Apligraf on hu-SCID mice (10/10). No IL-10 expression was detected in Apligraf by RT-PCR pre-graft or at day 14 post-graft on SCID mice, which suggests that IL-10 does not play a direct role in the survival of Apligraf following grafting.

Conclusion: Per the study report, Apligraf was not rejected when transplanted on hu-SCID mice even when the graft was pre-treated with IFN-γ to induce HLA-class II expression. In contrast, the human skin graft was rejected in this model.

Comments:

- The manufacturing lots of Apligraf used in the study were not specified in the study report.

- It's uncertain whether injection of PBMCs could lead to long term engraftment in SCID mice as the number of hematopoietic stem cells in PBMCs is low. To achieve long term engraftment, human CD34+ cells are generally used (*1*).

- Data to support the human specificity of the anti-involucrin antibody were not provided.

- In the study report, the methods section is poorly written and contains multiple gaps.

- The sponsor stated that IgG levels of ≥250 μg/ml at 3-5 weeks post-injection of the human PBMCs were found to correlate with the presence of human cells in the spleen of the hu-SCID mice. These data were not provided.

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

References:

(1). Manz MG, Di Santo JP. Renaissance for mouse models of human hematopoiesis and immunobiology. *Nat Immunol*. 2009 Oct;10(10):1039-42.

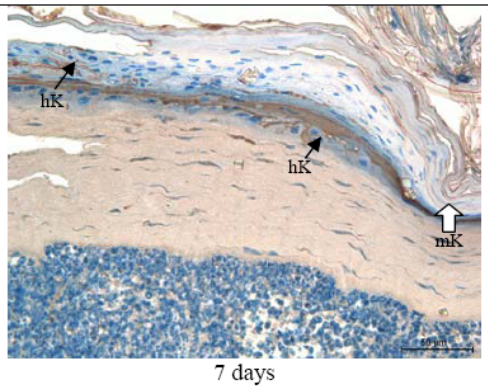
11.Preclinical evaluation of Apligraf® over short (7-28 days) and long (3-6 months) time periods by grafting onto full thickness wounds in Swiss Nude mice, Study# PCLR_101219_IVV7_CFR_1; conducted by Organogenesis; non-GLP; 2010

The purpose of this study was to evaluate the performance of Apligraf in a nude mouse full-thickness wound model. In addition, cell migration from the construct was examined by IHC and *in situ* hybridization (ISH).

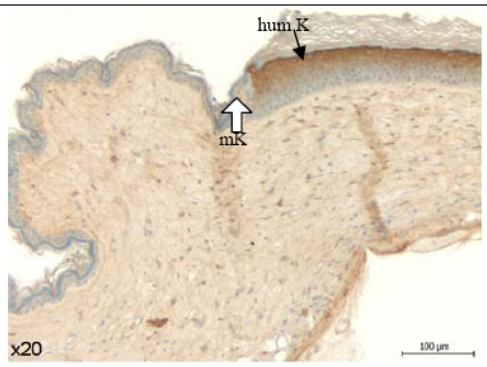
Species: Swiss nude female mice (-b(4)----------), 4-7 mice/group; the age and weight were not provided in the study report

Methods: Apligraf was applied to full-thickness wounds that were created on the dorsum of each mouse. A total of 4 animals/time point was sacrificed at 7, 14, and 28 days and 3 months. A total of 7 mice were sacrificed at 6 months. The grafts were excised for histological analysis with Hematoxylin-Eosin-Saffron (HES) and Masson Trichrome-Goldner version (MTG) stains. IHC was performed using antibodies against human involucrin (for human keratinocytes) and human vimentin (for human fibroblasts). Cell persistence was analyzed using ISH targeting the Alu repetitive sequences in human DNA.

Results: At 7 and 14 days post-grafting, MTG staining showed inflammatory cells within the hypodermis, which decreased over time. There was also progressive remodeling of the grafted Apligraf and progressive integration into the host tissue. Differentiated human keratinocytes and human keratinocytes were detected within the graft at all time points. Human keratinocytes positive for involucrin were not observed to migrate beyond the borders of the graft construct (Figure 4b).



7 days



6 months

Figure 4b. Human involucrin immunostaining on Apligraf® grafted onto Swiss nude mice: observation of the mouse / Apligraf® junction at 7 days and 6 months. The arrow (➔) shows the human keratinocytes (hum K) positive for Involucrin. The mouse keratinocytes (mK, ⇨) appear negative for the involucrin staining.

The fibroblast-populated matrix of Apligraf stained for vimentin prior to grafting; these vimentin positive fibroblasts were still present within the graft at 6 months. ISH for the human Alu sequence showed that human cells were present within the graft up to at least 6 months. A significant number of human cells were not detected in the immediate surrounding host tissues.

Conclusions: Per the study report, implantation of Apligraf in a full-thickness wound in Swiss nude mice resulted in graft survival and integration into the host tissue for the 6-month study duration. The results obtained by IHC showed the presence of human keratinocytes and human fibroblasts in the graft at 6 months post-Apligraf application. Analysis using IHC and ISH showed that the grafted human cells generally remained within the grafted site.

Comments:

- The Hematoxylin-Eosin-Saffron (HES) staining data were not included in the study report.

- The staining for human involucrin and human vimentin was heterogeneous among samples that were collected at different time points.

- Only representative images of ISH analysis were provided in the study report.

- Although the report stated that no human cells migrated into the surrounding tissue, based on the data provided, this reviewer has difficulty in drawing such an unequivocal conclusion. This conclusion is significantly dependent on how the margin between the graft and host tissue was defined, as a distinctive border is not always so apparent. A more reasonable conclusion would be that few human cells migrated from the graft to the surrounding host tissue.

12. Immunohistological characteristics of Apligraf®, Study# RD-2000-02026; conducted by –b(4)----------; non-GLP; 2000

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References:

(1). ----b(4)----------

(2). ---b(4)----------

13.Effect of Graftskin (Apligraf) Development on Graft Performance and Barrier Function Formation *In Vivo,* Study# RDR-024; conducted by Organogenesis; non-GLP; 1995

The purpose of this study was to determine the timing and extent of normal barrier function of Apligraf once engrafted onto nude mice----b(4)---------- of Apligraf to survive once engrafted.

----------b(4)----------

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14. ***In vitro*** **Neutral allograft study,** Study# None; conducted by Organogenesis, Inc.; non-GLP; 1992

The purpose of this study was to evaluate the ability of keratinocytes and fibroblasts to stimulate allogeneic lymphocytes.

---b(4)----------

----b(4)--- ----------

Conclusion: Per the study report, fibroblast and keratinocytes ----b(4)----------
---------- did not stimulate the proliferation of allogeneic PBMCs.

Comments:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

- The report did not specify the time point at which the ---b(4)---------- was added to the –b(4)--------.

15. **Expression of AB Blood Group Antigens on Apligraf™,** Study# RDR-031; conducted by Organogenesis; non-GLP; 1998

The purpose of this study was to determine if the cells contained in Apligraf express AB blood group antigens.

----b(4)---- ----------

----b(4)--- ----------

---**b(4)**--- ----------

[b(4)]

Conclusion: Per the study report, Apligraf expresses at least the type b(4) blood group antigen, which was limited to the upper layers of the epidermis. ---b(4)----------
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Comments:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

- Blood group antigen expression appears to be heterogeneous depending on a particular cell bank. Examining a larger number of lots of Apligraf manufactured from different cell banks may provide additional information on the frequency at which specific blood group antigens are found.

- The type b(4) blood group antigen was only observed in the epidermis. The functional significance of this finding is uncertain as antibodies may not have access to the epidermis.

References:

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----b(4)----------

16. **Molecular Typing of Apligraf™ Cells For Rh Blood Group Antigens,** Study# RDR-029; conducted by the –b(4)----------non-GLP; 1998

The purpose of this study was to determine the Rh blood group type of Apligraf donors using DNA isolated from donor fibroblasts (HDF).

-----b(4)----------

[b(4)]

Conclusion: Per the study report, all Apligraf cell donors tested were found to be Rh positive (RhD) using the molecular typing technique. The potential for Apligraf to elicit seroconversion in Rhesus negative women of childbearing age following exposure to the product has not been established.

Comment:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

17. In vitro **Allograft Tolerance Studies,** Study# None; conducted by Organogenesis, Inc.; non-GLP; 1995

The purpose of this study was to determine the ability of keratinocytes to indirectly regulate T-cell proliferation through the production of soluble factors.

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----b(4)----- ----------

----**b(4)**----- ----------

Conclusion: Per the study report, the HKs were capable of indirectly inhibiting T-cell proliferation with soluble factors under certain condition (i.e., in the presence of -b(4)----

Comments:

- Only summary data were provided in the study report, and the results of the experiments using Apligraf were not reported, thus the results and conclusions could not be independently verified by this reviewer.

- In the study report, the methods section is poorly written; much of the experimental details were not included.

- It's not clear why incorporation of –b(4)---------- was evaluated in the last --b(4)----------. Assessment of cell proliferation between days –b(4)---- are thus unknown.

- It's uncertain whether this *in vitro* finding translates to *in vivo*.

***18. In vitro* analysis of the relative roles of costimulatory receptor-ligand interactions in the T cell alloantigen response to Graftskin™ (Apligraf) cellular components,** Study# ITS97003; conducted by Organogenesis, Inc.; non-GLP; 1997

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19. **Analysis of the role of inflammatory/costimulatory cytokines on the alloantigenicity of the cellular components of Graftskin (Apligraf)™**, Study# ITS97004; conducted by Organogenesis; non-GLP; 1997

The purpose of this study was to evaluate the potential for proinflammatory cytokines (IL-1α, IL-6, and IL-12) to stimulate alloantigen presentation by HKs and HDFs to induce a T-cell response.

----b(4)--- ----------

---b(4)----- ----------

[b(4)]

Conclusion: Per the study report, the alloimmune response of HKs and HDFs was not affected by the addition of IL-1α, IL-6, or IL-12, as assessed by the –b(4)- assay.

Comments:

- A detailed methodology was not provided in the study report; the method only stated that the –b(4)-assay was performed per internal SOP.

- The three cytokines were added to separate wells. It is not known whether addition of various combinations of these cytokines would elicit a different response.

20. **Characterization of Primary and Secondary Allogeneic T cell Responses after Priming with HLA-Matched Professional and Non-Professional APC,** Study# RR-0076; conducted by Organogenesis Inc.; non-GLP; 2000

The purpose of this study was to determine the ability of HLA-matched sets of human keratinocyte, dermal fibroblast, and dendritic cells to prime and re-stimulate allogeneic T-cells.

---b(4)---- ----------

----b(4)----------

Conclusion: Per the study report, HKs and HDFs did not prime allogeneic T-cells nor did they activate T-cells primed by HLA-matched DCs.

Comments:

- The –b(4)- concentration and the cell numbers used in the –b(4)- assay were not provided.

- Based on the description provided in the study report, it appears that the DCs were HLA-matched to the HKs and HDFs, but this was not explicitly stated (i.e., whether the HKs, HDFs and DCs were derived from the same individual

tissue donor). The responder T-cells were not HLA-matched to the HKs, HDFs, or DCs.

- The results section in the study report contained a limited methodology and limited discussion of the study results. Thus, the study results and conclusions could not be independently verified by this reviewer.

- The study did not evaluate the potential for indirect allorecognition (*1*).

References:

(1). Hornick P. Direct and indirect allorecognition. Methods Mol Biol. 2006;333:145-56.

21. **Graftskin (Apligraf): Determination of cell purity in human keratinocyte and dermal fibroblast cell banks,** Study# ITS97002; conducted by Organogenesis, Inc.; non-GLP; 1997

The purpose of this study was to determine the cell purity of Apligraf cell populations (HKs and HDFs) by flow cytometry –b(4)----

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22. **Determination of Residual Bovine Serum Proteins in Apligraf,** Study# NCLR-0105-001; no date provided

Note: Please see the CMC review memo authored by M. Lee.

23. **Apligraf Periodontal Preclinical Dressing Evaluation,** Study# NCLR-0254-001; conducted by Organogenesis, Inc.; non-GLP; 2009

The purpose of this study was to determine the effects of standard periodontal dressings and anti-microbial rinses on Apligraf following application to athymic nude mice.

Species: Athymic nude mice, 4 mice/group; the sex, age, and weight were not provided in the study report

Test article: Apligraf (Lot no. GS0506.28.01.1A)

Methods: The study design included four groups: two with various periodontal dressings (Groups 1 and 2), one anti-microbial rinse (Group 3), and an Apligraf alone control (Figure 1).

Figure 1. Study Groups

GROUP	TEST ARTICLE	NUMBER OF TEST ARTICLES ANIMALS
1	Apligraf + Barricaid® Periodontal Surgical Dressing	4
2	Apligraf + Coe-Pack™ Periodontal Surgical Dressing	4
3	Apligraf + 0.12% chlorhexidine solution	4
4	Apligraf (Control)	4

A full-thickness skin wound (2 cm in diameter) was created on the posteral lateral dorsum of each mouse. Apligraf was trimmed to fit the wound size and applied to the wound bed. The (Barricaid® (Dentsply International Inc., Milford DE) and Coe-Pak™ (GC America, Alsip IL) periodontal dressings were applied for 7 days on the graft, followed by standard occlusive dressing (petrolatum gauze). For Group 3, grafts were rinsed twice a day for 1 minute/rinse with 0.12% Chlorhexidine solution, followed by one rinse of PBS for 1 minute, then application of standard occlusive dressing The Group 4 control consisted of covering Apligraf with standard occlusive dressing (petrolatum gauze). At 14 days post grafting, the animals were sacrificed and the grafts were excised. Histological evaluation of the grafts using H&E staining and IHC staining for human involucrin examined graft take, epidermal integrity, differentiation, and dermal integrity with host tissue and remodeling.

Results: One animal each in Groups 1, 2, and 4 died prior to day 7. One graft in the control group (Group 4) did not pass pre-graft acceptance criteria based on morphological analysis by histology, thus this graft was considered invalid. The day 14 graft histology showed similar morphology among the groups, showing a well-structured epidermis, well-integrated graft margins, normal cellularity and minimal inflammatory cell infiltrate. The grafts on animals in all four groups were also positive for human involucrin staining.

Conclusion: Per the study report, the Barricaid® dressing the Coe-Pak™ dressing, and 0.12% Chlorhexidine solution were compatible with Apligraf.

Comments:

- The study lacked a positive control; hence it's uncertain whether a14-day study duration was appropriate to detect a difference in graft take.
- The study report did not discuss the cause of the unscheduled deaths.
- There were only four animals were initially included in each group. This number was further reduced due to several unscheduled deaths and failed pre-graft acceptance criteria. Thus, any conclusions made from the resulting data are questionable.

24. Graftskin (Apligraf) Compatibility with Antimicrobial Agents *In Vitro* Studies, Study# None; conducted by Organogenesis, Inc.; non-GLP; 1992

The purpose of this study was to determine the effects of a variety of commonly used antimicrobial agents on the viability and morphology of Apligraf.

Test article: Apligraf (Lot nos. ---b(4)----------). All lots were between –b(4)----------

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Conclusion: Per the study report, Apligraf showed different levels of compatibility with different anti-microbial agents. Based on the study, agents such as Dakin's solution, Sulfamylon and Silvadene should be used with care as they maybe toxic to Apligraf.

Comments:

- The study report did not specify the histology scoring scale. According to the study report, the scoring system was similar to one used for manufacturing release of Apligraf
- The study report did not discuss the reasons for testing Apligraf lot(s) outside the approved shipping window time
- The study report did not specify the approved window of time for shipping
- An *in vivo* study to evaluate the objective of this study may be more relevant.

25. Investigation of *in vitro* tolerability by the human skin equivalent Apligraf of exposure to antibiotics/antiseptics for 24 hours, Study# 001974; conducted by ---b(4)-----.; non-GLP; 2000

The purpose of this study was to evaluate the tolerability of Apligraf when exposed to various commonly used antibiotics and antiseptics, including Amoxicillin/ Clavulanate, Ceftazidime, Fusidic acid, or Chlorhexidine gluconate.

Test article: Apligraf (Lot no. –b(4)----------

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Conclusion: Per the study report, clinically relevant systemic concentrations of Amoxicilin/Clavulanate, Ceftazidime and Fusidic acid were well tolerated by the Apligraf construct after 24 hours of *in vitro* exposure. Exposure of Apligraf to Chlorhexidine gluconate decreased tissue viability in a concentration-dependent manner.

Comments:

- The study report stated that the effects of antibiotic exposure on the Apligraf construct following –b(4)---hours of exposure were investigated previously. However, these time points were not further evaluated in this present study due to morphological signs of degeneration and degradation, decreased cell viability, and strong variability between replicates in both treated and untreated Apligraf. These results suggest that evaluation of this study objective under *in vitro* culture conditions may not be optimal for Apligraf.

- Only summary of the histology findings was provided, therefore, this aspect of this study could not be independently verified by this reviewer.

26. Graftskin (Apligraf) Compatibility with Antimicrobial Agents *In Vivo* Animal Study, Study# None; conducted by Organogenesis, Inc.; non-GLP; 1993

The purpose of this study was to determine the effects of commonly used antimicrobial agents on the integrity and morphology of Apligraf when applied at the time of grafting onto an animal.

Species: Athymic nude mice (--b(4)---------), 5 mice/group/time point; the sex, age, and weight were not provided in the study report

Test article: Apligraf (Lot no. G100 9207.28)

Methods: A 2 x 2 cm piece of a full-thickness skin section was excised from the dorsum of the nude mouse and a piece of Apligraf (2 x 2 cm) was sutured into place and antimicrobial agents (100 µl) were applied to the surface of the graft. The agents tested were: no agent (control), Silver sulfadiazine (1% cream), Mafenide acetate (10% cream), Silver nitrate (0.5% aqueous solution), GU irrigant (polymyxin B-neomycin solution), Dakin's solution (1/4 strength, 0.125% sodium hypochlorite solution), Polymyxin-nystatin (cream), and Gentamicin (0.5 mg/ml aqueous solution). The grafts were covered with Vaseline gauze, dry gauze, and adhesive bandage. The mice (5/group) were sacrificed at 7 and 14 days post-grafting. The excised graft was analyzed by histology to assess for integrity or damage to the graft, overall morphology, inflammatory response, and graft viability.

Results: Apligraf had different degrees of tolerability to various anti-microbial agents (Table 1).

Table 1: Summary of Histology Results

TREATMENT	DAMAGE		MORPHOLOGY		INFLAMMATION		VIABILITY	
	7day	14day	7day	14day	7day	14day	7day	14day
Control	0.3	0.5	2.6	2.5	0.4	1	2.9	3
Dakin's	1.7	0.9	1.3	2.1	1.9	1.6	1.6	2.7
Maf. Acetate	2.5	3	0.4	0	2.5	3	0.7	0
Silv. Diazine	1	1.1	1.6	1.9	1.7	1.7	2.5	1.8
Silv. Nitrate	0.9	1.3	1.8	1.7	1.2	1.5	2.8	2.3
GU Irrigant	0.9	0.6	1.8	2.4	1.5	0.4	3	2.7
Gentamicin	0.5	0.7	2	2.3	0.5	0	3	2.9
Polym./Nyst.	1.6	0.9	1.3	2.1	1.8	1	1.8	2.4

All values in this table are an average score from 5 samples per treatment at each time point. Viability was subjectively scored on a scale of 0-3, with 3 being most viable. All morphology scores were subjectively rated on a scale of 0-3 with 3 being the best (least damaged) graft. Inflammatory Response scores were rated on a subjective scale of 0 (least severe) to 3 (most severe) for the 7 day biopsies and for the 14 day biopsies a more detailed description of the cell response was recorded and converted to a 0-3 range. For the damage score, an overall score of 0-3 (with split scores such as 1-2 being counted as 1.5) was assigned to each sample primarily on the condition of the epidermis, but also reflecting viability. These numerical scores were averaged by treatment group and time point, with 0 reflecting the least damage and 3 reflecting the most damage.

Conclusion: Per the study report, Apligraf tolerated different anti-microbial agents to different degrees; ranging from 'benign' (e.g., Gentamicin) to 'harmful' (e.g., mafenide acetate), based on the damage scores. According to the calculated scores,

anti-microbial agents containing mafenide acetate and Dakin's solution should not be used with Apligraf.
Note: Apligraf tolerability to each agent was determined from the damage score. The damage score was an overall score of 0-3 assigned to each sample (5/group/time point) mainly based on the condition of the epidermis, but also reflective of cell viability. This score was averaged per group and per time point; 0 = least damage and 3 = most damage.

Comments:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

- The method used to assess cell viability was not provided, the cell types evaluated were not provided, and overall assessment appeared to be subjective.

- Only histology analysis was performed. It's unclear whether the anti-microbial agents affected the bioactivity of Apligraf, thus affecting wound healing.

Summary of Pharmacokinetic Studies

The following list includes the pharmacokinetic studies that were conducted with Apligraf:

(1). Evaluation of Graftskin (Apligraf) Composite Grafts on Full-Thickness Wounds on Athymic Mice
(2). Tissue Remodeling and Cellular Persistence in Skin Construct Implants
(3). Remodeling of a Bioengineering Living Skin Construct Grafted onto Nude Mice
(4). Preclinical evaluation of Apligraf over short (7-28 days) and long (3-6 months) full thickness wounds in Swiss Nude mice (PCLR_101219_IVV7_CFR_1)

Pharmacokinetic Studies

1. **Evaluation of Graftskin (Apligraf) Composite Grafts on Full-Thickness Wounds on Athymic Mice**, Study# None; conducted by the ---b(4)---------- and Organogenesis, Inc.; non-GLP; 1992

   Note: This study was reviewed in the Pharmacology Studies section of this BLA review (Study No. 7).

2. **Tissue Remodeling and Cellular Persistence in Skin Construct Implants,** Study# None; conducted by conducted by Organogenesis; non-GLP; 1999

Note: This study was reviewed in the 'Pharmacology Studies' section of this BLA review (Study No. 8).

3. **Remodeling of a Bioengineering Living Skin Construct Grafted onto Nude Mice,** Study# None; conducted by the ----b(4)---------- non-GLP; 1999

   Note: This study was reviewed in the 'Pharmacology Studies' section of this BLA review (Study No. 9).

4. **Preclinical evaluation of Apligraf over short (7-28 days) and long (3-6 months) full thickness wounds in Swiss Nude mice,** Study# PCLR_101219_IVV7_CFR_1; conducted by Organogenesis; non-GLP; 2010

   Note: This study was reviewed in the 'Pharmacology Studies' section of this BLA review (Study No. 11).

Summary of Biocompatibility Testing

The following list includes the biocompatibility tests that were conducted:

(1). General Safety Test
(2). Cytotoxicity (Reference: ---b(4)----------
(3). Sensitization (Reference: ---b(4)----------
(4). Intracutaneous Reactivity or Irritation (Reference: ---b(4)----------
(5). Systemic Toxicity (Acute + Subacute) (Reference: ----b(4)----------
(6). Sub-chronic Toxicity (Reference: ---b(4)----------
(7). Hemocompatibility (Reference: ----b(4)---------- ----------

Biocompatibity Testing

1. **General Safety Test,** Study# None; conducted by –b(4)------.; GLP; 1992

   The purpose of this test was to determine the toxicology potential of Apligraf when injected into guinea pigs and mice by measuring weight change and clinical signs of toxicity over the course of 7 days.

   Species: Guinea pigs (n=3), <400 g and albino mice (n=3), <22 g; the age and sex of the animals were not provided in the report

   Test article: Apligraf (Lot no. 160107)

   Test samples: 1. Spent culture medium from Apligraf

2. Supernatant from Apligraf –b(4)----- homogenization and centrifugation

3. Pelleted Apligraf homogenate

Methods: Test materials were diluted 1:20 with PBS and mixed well. The guinea pigs were injected intraperitoneally with 5.0 ml of the diluted test substance; a third control guinea pig was similarly injected with PBS. Two mice were injected intraperitoneally with 0.5 ml of test article solution, a third control mouse was similarly injected with PBS. Animals were observed for clinical signs of toxicity for 7 days after injection.

Results: All animals survived the test period and none exhibited any adverse clinical signs during the 7-day observation period. All animals exhibited weight gains.

Conclusion: Per the report, the test article meets the requirements of this test.

Comments:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

- The study report did not clearly define the term test substance or test article solution. A likely interpretation is that the three test samples were mixed together in PBS and the mixture were then injected into the guinea pigs and albino mice.

2. **Cytotoxicity (Tissue Culture-Agar Diffusion Test),** Study# 91GT-1313; conducted ---b(4)-------.; GLP; 1992

The purpose of this test was to detect the response of a mammalian monolayer cell culture to readily diffusible components of Apligraf applied to the surface of agar overlaying the cell monolayer.

Test article: Apligraf (Lot no. 9112.03/9112.24)

Methods: The assay was based on that described in ---b(4)---------- monolayer of L929 mouse fibroblast cells was propagated and the liquid medium was replaced by agar. The agar was stained with a vital dye, neutral red. A known positive control (natural rubber) and a negative control (silicone) were placed on the surface of the agar in separate Petri dishes. One positive and one negative control were prepared by passage of the L292 cells to verify the proper functioning of the test system. After application of the test and control articles, the Petri dishes were placed in a 37°C incubator supplied with 5% CO_2 for 48 hours. The extent of decolorization was evaluated at 0, 24, and 48 hours. Apligraf (referred to as Living Skin Equivalent in the test report) was applied directly to the surface of the agar. Duplicate samples of the test article (with a minimum surface area of 100 mm^2) were placed into individual Petri dish wells. The response of the cell monolayer was evaluated by the extent of

decolorization of the red stained monolayer under and around the test article when the Petri dish was viewed against a white background. Loss of color of the stained L929 cells was considered a physiologically significant reaction of the cells. The reactivity grade was assigned based on the size of the decolorized zone (Table 1).

Table 1: Reactivity Grade

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under the test article
1	Slight	Zone limited to area under the test article
2	Mild	Zone extends less than 0.5 cm beyond the test article
3	Moderate	Zone extends 0.5 to 1.0 cm beyond the test article
4	Severe	Zone extends greater than 1.0 cm beyond the test article but does not involve the entire plate
5		Zone involves the entireplate

Results: No biological reactivity (Grade 0) was observed with the L929 cells out to 48 hours post-exposure to the test article (Table 2).

Table 2: Decolorization Measurement

		Test Article		Natural Rubber Positive Control		Silicone Negative Control		Media/Blank Negative Control	
DISH	HOURS	Zone Size (cm)	Grade	Zone Size (cm)	Grade	Zone Size (cm)	Grade	Zone Size (cm)	Grade
A	0	0.0	0	0.0	0	0.0	0	0.0	0
	24	0.0	0	0.5	3	0.0	0	0.0	0
	48	0.0	0	1.1	4	0.0	0	0.0	0
B	0	0.0	0	0.0	0	0.0	0	0.0	0
	24	0.0	0	0.5	3	0.0	0	0.0	0
	48	0.0	0	1.1	4	0.0	0	0.0	0

Conclusion: Per the report, Apligraf met the requirements of this test.

Comment:

- Only summary data were provided in the study report, thus the results and conclusions could not be independently verified by this reviewer.

3. **Sensitization (Kligman Maximization Study),** Study# 91G-1310; conducted by ---b(4)----.; GLP; 1992

The purpose of this test was to evaluate the allergenic potential or sensitizing capacity of Apligraf.

Species: -b(4)--- guinea pigs, 14 males and 16 females, 26-67 days old, 300-500 g

Test article: Apligraf (Lot no. 9112.03/9112.24)

Methods: The test article (120 cm^2) was extracted in 20 ml of –b(4)- 0.9% sodium chloride (NaCl) and cotton seed oil (CSO) at 70ºC for 24 hours. The negative controls were the extraction vehicles (NaCl and CSO); the positive control was dinitrochlorobenzene (DNCB). A primary irritation study was conducted using one animal for each extract (Table 1). On intact skin site on each animal, a 0.1 ml of the test article (CSO or NaCl extract) was applied under -b(4)- chambers at concentrations of 100% and 75% or 50% and 25% (diluted with CSO or NaCl). Primary skin irritancy was scored at 24, 48, and 72 hours according to the method of Draize (Primary Irritation Phase)(Table 2).

Table 1: Study Design

	NaCl Extract	
(1)	Experimental	(5 males/ 5 females)
(2)	Negative Controls	(1 male / 1 female)
(3)	Positive Controls	(1 male / 1 female)
(4)	Primary Irritations	(0 male / 1 female)
	CSO Extract	
(1)	Experimental	(5 males/ 5 females)
(2)	Negative Controls	(1 male / 1 female)
(3)	Positive Controls	(1 male / 1 female)
(4)	Primary Irritations	(0 male / 1 female)

Table 2: Draize Scale for Scoring Skin Reactions

	Value
Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Total possible erythema score = 4	
Edema Formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges are well defined by definite raising)	2
Moderate edema (raised approximately 1mm)	3
Severe edema (raised more than 1mm and extending beyond area of exposure)	4
Total possible edema score = 4	

For the sensitization study, the animals were intradermally injected on day 0 with 0.1 ml of the test article extract, control extract or extract mixed with Freund's complete

adjuvant (Table 3). Each extract was injected in pair with a total of 6 injections per animal. in On day 7, the skin was pretreated with 10% sodium lauryl sulfate for 24 hours and a filter paper saturated with the test article or control extract was then applied topically (remaining in place for 48 hours) (Induction Phase). On day 21, filter paper saturated with the test article or control extract was topically applied to the flank and secured in place for 24 hours (Challenge Phase). Skin reactions (erythema and/or edema) on the flank were assessed at 1 hour, 24 hours and 48 hours according to the scoring system of Kligman (Table 4). Any animal showing skin reaction at 24 hours or 48 hours of score 2 or greater for erythema and edema was considered positive sensitization (Table 2).

Table 3: Dosing Regimen

Experimental Group (NaCl or CSO):
(1) 0.1 ml FCA 1:1 with NaCl or CSO
(2) 0.1 ml test article extract (NaCl or CSO)
(3) 0.1 ml test article extract (NaCl or CSO) 1:1 with FCA

Negative Control Group (NaCl or CSO):
(1) 0.1 ml FCA 1:1 NaCl
(2) 0.1 ml NaCl or CSO
(3) 0.1 ml NaCl or CSO 1:1 with FCA

Positive Control Group (NaCl or CSO):
(1) 0.1 ml FCA 1:1 NaCl
(2) 0.1 ml 0.1% DNCB in 95% EtOH
(3) 0.1 ml 0.1% DNCB in 95% EtOH 1:1 with FCA*

* DNCB (Dinitrochlorobenzene) dissolved in 95% Ethanol and suspended in FCA to a final concentration of 0.1%

Table 4: Kligman Scoring System

Using the Scoring System of Kligman, the allergic potential of a test article is classified as follows:

Sensitization Rate (%)	Grade	Class
0-8	I	Weak
9-28	II	Mild
29-64	III	Moderate
65-80	IV	Strong
81-100	V	Extreme

The test results were interpreted based upon the percentage sensitization observed.

Results: All animals showed body weight gains. No adverse clinical signs were observed in any animal. For the primary irritation study, the test article extracts were designated as a nonirritant (score of 0). For the sensitization study, animals did not exhibit skin reaction to the test article extracts (score of 0).

Conclusion: Per the report, for the primary irritation study, the Apligraf extracts did not elicit a skin reaction, based on Draize scoring. For the sensitization study, based on the scoring system of Kligman, Apligraf has weak allergenic potential.

4. **Intracutaneous Reactivity or Irritation (Primary Skin Irritation Study),** Study# 91G-1315; conducted by –b(4)----------; GLP; 1991

   The purpose of this test was to evaluate the potential of Apligraf (referred to as Living Skin Equivalent or LSE in the experimental report), to produce primary dermal irritation after a single intracutaneous injection in albino rabbits.

   Species: New Zealand white rabbits (----b(4)----------), n=3/sex, 10-12 weeks old, weight 2.0 to 3.0 Kg

   Test article: Apligraf (Lot no. 9112.03)

   Methods: Each animal was applied topically with the test article on two sites, one abraded skin and one intact skin. Test article (~7cm^2) was applied under a gauze patch and was kept in contact with the skin for 24 hours. Animals were observed for signs of erythema and edema at 24 and 72 hours post application of the test article and scored according to the Draize Scale for Scoring Skin Reactions. A Primary irritation index (PDII) was calculated based on the skin reaction score for the test and untreated control sites.

   Results: All of the test animals exhibited a gain in body weight during the study. No overt signs of toxicity were evident in any of the animals during the course of the study. No signs of erythema or edema were present at the test sites at 24 hour and 72 hour post-test article administration. The Primary irritation index was calculated as 0 for both test article and control sites.

   Conclusion: The test article was considered as a non-irritant.

5. **Systemic Toxicity (Acute + Subacute),** Study# 91G-1311; conducted by –b(4)----------.; GLP; 1992

   The purpose of this test was to determine the biological response of animals following systemic injection of the Apligraf extract.

   Species: 1. –b(4)---- female mice (----b(4)----------), 34-41 days old, 17-23 g

   2. –b(4)---------- white rabbits (--b(4)----------), 10-12 weeks old, 2-3 kg

   Test article: Apligraf (Lot no. 9112.03/9112.24)

   Methods: The test article (120 cm^2) was extracted in 20 ml of appropriate medium at 70ºC for 24 hours.

Systemic injection test: --b(4)---------- mice (5 mice/group) were injected intravenously and intraperitoneally with the test article extract (Table 1). The animals were observed immediately post-injection and at 4, 24, 48, and 72 hours after injection for adverse effects.

Table 1: Study Design

Table 1: Study Design

Test Article or Control Article Extracts	Dosing Route	Dose/Kg	Injection Rate
0.9% Sodium Chloride Injection, (b)(4) (0.9 % NaCl)	Intravenous	50 ml	0.1 ml/sec
1 in 20 Alcohol in 0.9% Sodium Chloride Injection (b)(4) (EtOH:NaCl)	Intravenous	50 ml	0.1 ml/sec
Polyethylene Glycol 400 (PEG 400)	Intraperitoneal	10 g	- -
Cottonseed Oil (CSO)	Intraperitoneal	50 ml	- -

Intracutaneous test: --b(4)----- White rabbits (1 animal/sex/group) were injected intracutaneously with 0.2 ml of the test article extracts on five sites on one side of the rabbit. At fiver other sites on other side of each rabbit, 0.2 ml of the corresponding control article was injected. The animals were observed for 24, 48 and 72 hours after injection to detect sign of erythema, edema and necrosis. The observations were scored using the Draize scale for scoring skin reaction.

Results:

Systemic Injection test: All animals injected with the test article or control article extracts showed body weight gains. No overt signs of toxicity were observed in any animal.

Intracutaneous test

All animals increased in weight. No signs of erythema or edema observed at any of the test or control article sites. Overt signs of toxicity were not observed in any animals

Conclusion: The test article met the requirements of the test.

Comment:

- The study report did not provide a clear methodology, and did not specify the extraction media or the control articles used. Based on Table 1, the extraction media were 0.9% NaCl and EtOH:NaCl (intravenous route) and PEG 400 and CSO (intraperitoneal route). It is assume that these media were also the control articles.

6.Sub-chronic Toxicity (Subcutaneous Injection Test – Subchronic), Study# 91G-1314; conducted by –b(4)----.; GLP; 1992

The purpose of this test was to evaluate the potential of Apligraf to induce toxic effects following administration via the subcutaneous route in albino rabbits.

Species: -b(4)------ White rabbits (---b(4)----------), 1/sex, 11-12 weeks old, >2.5 kg

Test article: Apligraf (Lot no. 9112.03/9112.24)

Methods: The study was conducted based upon the standards set by the United States Pharmacopeia XXII, National Formulary ----------b(4)----------. Four samples of the test article were surgically implanted into the subcutaneous tissue on one side of the rabbit. On the opposite side of the rabbit, two strips of –b(4)- Negative Control Plastic RS were implanted. Animals were maintained for 90 days and observed daily. After 90 days, animals were sacrificed and the implanted sites were excised for histopathology (slides read by a veterinary pathologist). Per the report, the test is considered negative if, in each rabbit, the reaction to not more than 1 of 4 test article strips is significantly greater than the reaction to that of the negative control strips.

Results: Both animals exhibited body weight gains and neither displayed any clinical signs of toxicity. Significant histopathology differences were noted between the sites implanted with test or control article. In the sites implanted with the test article, slight to moderate infiltrates of polymorphonuclear cells, lymphocytes, macrophages, and giant cells were observed; these findings were generally absent in the sites implanted with the negative control article. The frequency and degree (i.e. scored as slight) of fibroplasia and fibrosis were similar for all implanted sites.

Conclusion: Per the report, cutaneous implantation of Apligraf resulted in a significant tissue response and was considered toxic based on criteria set in the study protocol.

Comment:

- The subcutaneous injection test does not appear to be appropriate for evaluating the safety of a viable cell product. Xenogeneic immune response would be expected with implantation of viable human cell product in immunocompetent animals.

6. **Haemocompatibility (Hemolysis Test – Direct Contact with Rabbit Blood),** Study# 91G-1312; conducted by –b(4)----------; GLP; 1991

The purpose of this test was to determine the hemolytic activity of Apligraf when in direct contact with rabbit blood.

Test article: Apligraf (Lot no. 9112.03/9112.24)
Methods: Fresh rabbit blood, obtained within one hour of testing, was diluted in –b(4)- 0.9% Sodium Chloride Solution. The test article (60 cm^2 of Apligraf) was tested in -b(4)--------- and the positive (Sterile Water) and negative (0.9% Sodium Chloride)

controls were tested in –b(4)-----. A total of 0.2 ml of diluted rabbit blood was added to each vial containing test or control articles, followed by incubation at 37ºC for 60 minutes. Hemolysis was assessed by –b(4)----------; the percent hemolysis of the test article was based on the following formula:

$$\% \text{ Hemolysis} = \frac{\text{(Ave. Abs. of Test Article - Ave. Abs. of Negative Control)}}{\text{(Ave. Abs. of Positive Control - Ave. Abs. of Negative Control)}} \times 100$$

The test article was considered non-hemolytic if the percent hemolysis was ≤5%.

Results: The percent (%) hemolysis of the test article was 0.23%.

Conclusion: Apligraf was considered to be non-hemolytic

Comments:

- Hemolysis testing with fresh human blood would be more appropriate to evaluate for hemocompatibility.

CONCLUSION

Apligraf®, which is identical to Apligraf® (oral), is approved under a PMA filing (reviewed by CDRH) for the treatment of venous leg and diabetic foot ulcers; therefore, limited additional preclinical studies were conducted in support of this BLA. The preclinical studies conducted to support the clinical trials for Apligraf® (oral) were performed under an IDE application (reviewed by CDRH). It should be noted that Apligraf® was regulated as a medical device during the clinical development of both the cutaneous and oral mucosal wound indications. Although this product was converted to a BLA regulatory pathway, additional preclinical studies were not conducted because clinical data existed by that time.

Pharmacology studies conducted by the sponsor demonstrated that Apligraf® exhibits characteristics similar to a skin graft. *In vitro* studies showed that following physical injury (via a –b(4)-------- the keratinocytes within Apligraf® were able to migrate, re-epithelialize and keratinize. In addition, Apligraf® secreted growth factors into the ----------b(4)----------. Apligraf® was also shown to act as a barrier for the penetration of microorganisms. *In vivo* transplantation of Apligraf® onto a full-thickness cutaneous wound in nude mice resulted in graft integration with the host tissue. The transplanted Apligraf® remained durable, as demonstrated by the persistence of human keratinocytes and fibroblasts, for at least one year in the nude mice. Apligraf® transplants on cutaneous wounds in nude mice were shown to be compatible with periodontal dressings (i.e., Barricaid and Coe-Pak™) and at least one anti-microbial rinse solution (0.12% chlorhexidine solution).

Although the sponsor's hypothesis is that Apligraf® functions to enhance wound healing by secondary intention, the results from the *in vivo* studies in the murine wound models did not provide direct evidence to support this position. Apligraf® appeared to function as a skin graft (i.e., healing by primary intention) in the nude mouse full-thickness wound model. In contrast, Apligraf® did not integrate or show long-term persistence following application to cutaneous wounds in humans; the mechanism for this species difference was not explored.

In vitro analysis of the cellular components of Apligraf®, allogeneic keratinocytes and dermal fibroblasts, did not show induction of an alloimmune response when evaluated by the –b(4)---------- assay. The potential for Apligraf® to induce an alloimmune response by indirect allorecognition was not evaluated.

The potential toxicity of Apligraf® was evaluated via studies that reflect the initial medical device framework (i.e., biocompatibility testing in conformance to ISO-10993 standards). Tests performed included: 1) General Safety Test, 2) Cytotoxicity, 3) Sensitization, 4) Intracutaneous Reactivity/Irritation, 5) Systemic Toxicity (Acute + Subacute), 6) Subchronic Toxicity, and 7) Hemocompatibility. This biocompatibility testing paradigm did not reveal any findings of significant biological concern. The subcutaneous injection of Apligraf® into rabbits resulted in tissue reaction at the implanted site due to the xenogeneic immune response.

The toxicology study designs as discussed in the International Conference on Harmonisation (ICH) Safety ('S') guidelines, consisting of pharmacokinetics, acute toxicology, chronic toxicology, genotoxicity, carcinogenicity, reproductive and developmental toxicity, safety pharmacology, and immunotoxicity (http://www.ich.org/cache/compo/276-254-1.html) were not conducted due to the nature of Apligraf® and the extensive clinical experience in the cutaneous indications. In addition, some aspects of these studies were captured in the biocompatibility testing.

Key Words/Terms: Apligraf® (oral); pharmacology; oral mucosal wound; keratinocyte; fibroblast; skin graft; toxicology; pharmacology