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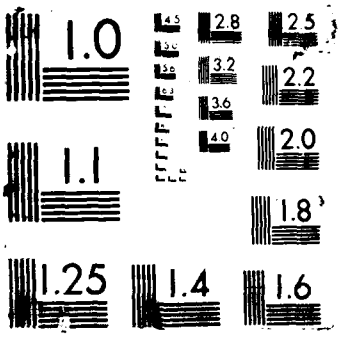
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DEVELOPMENT AND TESTING OF LIVING SKIN EQUIVALENT

ANNUAL AND FINAL REPORT

COVERING THE PERIOD

SEPTEMBER 1, 1980 THROUGH MARCH 15, 1984

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Eugene Bell

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
77 MASSACHUSETTS AVENUE
CAMBRIDGE, MA 02139

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Histology of grafts on recipient rat for up to 24 months showed that the matrix basket-weave pattern established at one month became more pronounced at one year. No hypertrophic scarring was observed in any skin-equivalent graft made although grafts of epidermis alone on a collagen bed without cells did result in scarring. Hypertrophy of the epidermis lessened between one month and one year, and at two years no hypertrophy was observed.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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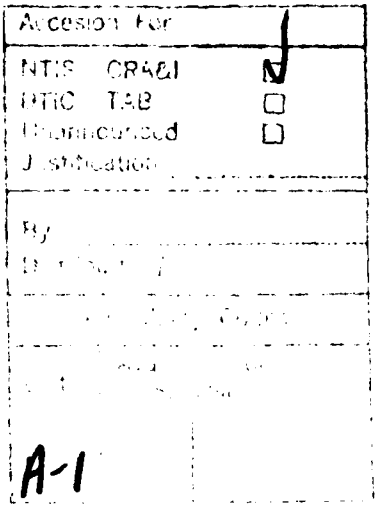

For the Period of September 1, 1980 to February 28, 1981
(6 months)

Survey of Skin Equivalent Grafts Made to Experimental Animals

The extent of wound contraction is measured by the movements of tatoo marks made in the dermis contiguous to the graft at the time of grafting.

1. The Rat Model

a) The use of Isografts in an inbred strain of rats.

In a preliminary series of experiments the potential use of Fischer strain rats has been tested by preparing a series of isografts made by grafting skin equivalents with cells from female donors to male hosts. On the average, wound contraction was blocked about 60%. Inhibition of wound contraction was only 31% in four rats. Two allografts using Sprague-Dawley cells respectively in the dermal and epidermal components of the grafts and Fischer cells in the complementary component were made to Fischer hosts. The first blocked wound contraction 56% and the 2nd, 84%, a 3rd graft made entirely with Sprague-Dawley cells blocked wound contraction only 30%.

b) Tests of Collagens in large grafts

Finding a commercial source of collagen for use in fabrication of skin equivalent grafts has been an important objective, since preparation of collagen in the laboratory is laborious and time consuming. The use of bovine collagen, Telogen, provided by the Collagen Corporation has been compared with our own preparation of rat tail collagen in a pilot experiment.

Two large autografts of skin equivalents were made in Sprague-Dawley rats. One measured 10 cm^2 and was made up with rat tail collagen. The graft healed in excellently and after four months had contracted less than 25%. The second graft measured 23 cm^2 and was made up with Telogen 50% and rat tail collagen 50%. Wound contraction measured 74%.

c) Collagen and Collagen Turnover in Skin Equivalent Grafts and In Vitro.

To measure collagen turnover in grafts in situ C14 acetic anhydride was used to label rat tail collagen at an activity level

of 60,000 cpm/ml. The labeled collagen, at a concentration of 2.1 mg/ml, was used to cast fibroblast lattices (dermal equivalents. The cells were obtained from a pool of Fischer female fibroblasts, maintained in DMEM - 10% FBS. The lattices were allowed to contract and the upper surface covered with epithelial cells, before grafting to male Fischer rats. At designated intervals, the graft and surrounding skin was excised and either prepared for autoradiography or extracted with 0.5 M HAc and counted. Scintilliation counting of the extract showed a loss of 50% of the radioactive label within one week and a loss of over 90% of the labeled collagen within 4 weeks of grafting the lattice. Microscopic examination of the auto-radiograms shows that concentration of grains is reduced first near the dermal-epidermal junction. In time the radioactivity levels are found mainly in the basal region of the graft and are still detectable at 42 days. This suggests that the collagen in the graft lattice is replaced in large part with newly synthesized collagen.

d. Use of skin equivalent grafts on burned grafts

In conjunction with Dr. Paul Ehrlich at the Shriners Burns Institute, eight large skin equivalent grafts were made to rats; they measured about 6 x 8 cm^2. Eight control autografts were also made. All grafts were applied 10 days after making scalding water or hot metal burns, at which time the eschar was removed and the grafts laid on the underlying granulation tissue. All the autografts failed mainly due to infection but one of the eight tissue equivalent grafts was successful. It inhibited wound contraction and persisted with no infection. The animal was sacrificed at 98 days. Since autograft controls were lost because of infection, the techniques of grafting need to be addressed.

2. The Rabbit Model

The rabbit ear was chosen as a model because no panniculus muscle underlies the skin of the ear. A first graft to the ear was made with a skin equivalent lattice. The epithelium was good. After 5 months the graft had changed from 2.3 x 1.4 to 2 x 1.5. The quality of the healed graft was excellent; there was some fur growth on the edges of the graft. Other grafts are in progress.

For The Period of March 1, 1981 to July 31, 1982

1. Structural Integration of Grafted Skin Equivalents

Bilayered skin equivalents, composed of a sheet of epidermal cells overlying a collagen lattice populated with fibroblasts, quickly become structurally integrated with the surrounding host skin after grafting to Wistar/Lewis rates. Three days after transplantation, the skin equivalent lies on a bed of host granulation tissue and is loosely attached to the adjoining host dermis. Blood vessels begin to invade the collagen lattice by five days after grafting. By the seventh day a fully keratinized, hypertrophic epidermis covers the surface of the graft and blood vessels penetrate the lattice to the base of the epidermis. Vascularization of the graft is accompanied by activation and proliferation of the fibroblasts and by a condensation of the collagen matrix. During the second week after grafting, the collagen fibrils become organized into thin fibers that show a basketweave pattern of birefringence when examined using polarized light. By one month the structure of the skin equivalent has become stabilized. The fibroblasts now resemble the quiescent fibrocytes of normal, resting dermis and the epidermis remains moderately hypertrophic. The graft lacks secondary derivatives such as hair follicles and sweat glands, presumably because the stem cells are lost during the isolation of the epidermal cells.

2. Differentiation of a Skin Equivalent after Grafting to Experimental Animals.

The skin equivalent (SE) that we have developed for use in skin grafting is a bilayered structure consisting of a multi-layered epidermal sheet overlying a collagen lattice populated with fibroblasts. In our previous light miceoscopic studies of grafts applied to Fischer or Wistar/Lewis rats we found that the vascularization of the graft between five and seven days after implantation is accompanied by an activation of the fibroblasts and a condensation of the collagen matrix. To follow this reorganization of the SE at the electron microscopic level, we have examined thin sections of grafts biopsied at intervals ranging from zero to nine days after implantation. At the time of grafting, the basal epidermal cells lack hemidesmosomes and have not yet formed a basement membrane (BM). Five days after grafting blood vessels from the host granulation tissue have begun to invade the collagen lattice of the SE which is populated by fibroblasts resembling the quiescent fibrocytes of normal dermis. At the base of the epidermis, the cytoplasmic plaques of the hemidesmosomes have developed, and short stgretches of BM appear in the adjoining extracellular space. At seven days the fully keratinized epidermis lies on a nearly continuous BM and capillaries extend to the base of the epidermis. Fibroblasts encircle the capillaries and now appear activated, with an

abundant, dilated RER and an extensive GA. By nine days after grafting, the BM is continuous and well-organized. Although most of the collagen fibrils in the dermis are randomly oriented, small arrays of fibrils lie adjacent to the fibroblasts. Implantation of the SE and its subsequent exposure to humoral factors thus triggers the differentiation of the epidermis and the activation of the fibroblasts which reorganize the collagen matrix to form the basketweave pattern characteristic of the mature graft.

3. Fibroblasts in Isogeneic Skin Equivalents Persist after Grafting.

We have fabricated skin equivalents by combining fibroblasts from female Fischer rats with collagen to form a lattice and overlaying the lattice with a suspension of epidermal cells. The epidermal cells attach and form a sheet which differentiates. These skin equivalents were then grafted to male Fischer rats in order to follow the fate of the fibroblasts after implantation. Biopsies of the skin equivalent were taken between five days and 13 months after grafting and examined histologically or placed in tissue culture to permit karyotyping of the resident fibroblasts. At least twenty-two chromosome spreads were prepared from each population of fibroblasts that grew out of the biopsies taken nine days, one month, seven months, and 13 months after grafting. Ninety-one percent of the fibroblasts from the graft biopsied at nine days were female, with this proportion decreasing to 64% at one month. This initial sharp drop is followed by a slow, linear decline which continues thgrough the 13th month when 42% of the fibroblasts are female. We conclude that fibroblasts of the grafted skin equivalent become permanent residents of the skin of the host rat.

4. Acceptance of Allogeneic Fibroblasts and Rejection of Xenogeneic Fibroblasts in Skin Equivalent Transplants.

Living skin equivalents (SE) were prepared using cultured fibroblasts in a collagen matrix overlaid with isogeneic keratinocytes. SEs prepared from allogeneic rat fibroblasts or xenogeneic rabbit or human fibroblasts were transplanted to recipient rats. Biopsies of SE grafts were examined histologically. At intervals, karyotyping was performed on biopsied grafts to determine the percentage of donor fibroblasts remaining in the graft. Results showed that allografted fibroblasts in SEs were accepted by recipient rats after a transient mononuclear response. A secondary stimulus in the form of a second SE allograft did not provoke an immune response in either the original graft or the challenge graft. Xenografts were rejected.

5. Differentiation and Morphogenesis of Keratinocytes Grown on Contracted Collagen Lattices.

Keratinocytes cultured on a contracted collagen lattice containing fibroblasts form a tissue sheet which differentiates into a multilayered epidermis. Human keratinocytes obtained from

a biopsy were passaged twice on plastic before application as a suspension onto the contracted lattice. In addition to covering the lattice, or dermal equivalent, mixed epidermal cells separated from both the dermal and epidermal components of skin form crypts or follicular structures containing some keratin. These crypts are are better developed if the keratinocytes are cultured in contact with air. On the outer surface of the epidermis a corneum is observed. Desomsomes are seen and tonofilaments can be identified in electron micrographs. Keratinocytes plated on collagen lattices containing fibroblasts are shown to synthesize a range of keratin polypeptides including the 65K Dalton protein. If the keratinocytes alone or with fibroblasts are incorporated into a collagen lattice at the time of casting, the keratinocytes within the lattice form pearls. Each pearl arises from a single cell which undergoes divisions. The pearls contain keratin after 11 days in cullture. In these preparations the keratinocytes on the surface of the lattice multiply to form an epidermal sheet but differentiation depends on culture conditions. The epidermal sheet becomes multilayered only if the lattice contains both keratinocytes and fibroblasts and if it is exposed to air. If the lattice is cast only with keratinocytes and exposed to air, no keratinocytes multiply on the surface. If the lattices are immersed in the medium with or without fibroblasts only a monolayer of keratinocytes is obtained.

For the Period of August 1, 1982 to July 31, 1983

Our laboratory has continued on the bilayered skin equivalent (SE), working toward optimization of the material to be grafted. In this report we summarize the fate of 169 grafts. Wound contraction in 80% of the grafts was of the order of 20%. We have frozen and thawed SE lattices with good survival of the fibroblasts in the dermis. Keratinocytes were healthy following freezing and thawing, but some shearing at the dermal-epidermal interface was observed. An unsuccessful attempt was made to improve attachment of epidermis to the dermal portion of the SE by casting the SE with the equivalent of dermal papillae. Splitting of the lattice along the plane of the base of the papillae was observed after grafting. Further experiments are underway on improving the epidermal attachment by casting ridges in the dermis and by increasing time in vitro before freezing or grafting.

For skin-equivalent tissues to be useful they must be available for grafting on call. To make this possible allografting and cold storage are necessary. We have shown that allografted fibroblasts in SEs are accepted by the recipient rat without the use of immunosuppressants. Rejection of SE allografts was not provoked by a second SE allograft or by a second graft of allogenic skin, although the skin was rejected. Grafting the recipient first with allogenic skin and then with the SE allograft led to rejection of the skin but not of the SE graft.

Large grafts (25-50 sq cm) were made with partial success. All provided adequate wound coverage during healing so that no recipients died, but the inhibition of wound contraction ranged from 20%-80%. Small grafts (6-10 sq cm) contract about 20%. We attribute the increased wound contraction in some animals to mechanics of grafting, to incomplete keratinization, and to incomplete contraction of the SE before grafting because of the method used for casting large lattices.

Histology of grafts on recipient rats for up to 24 months showed that the matrix basket-weave pattern established at 1 month became more pronounced at 1 year. No hypertrophic scarring was observed in any skin-equivalent graft made although grafts of epidermis alone on a collagen bed without cells did result in scarring. Hypertrophy of the epidermis lessened between 1 month and 1 year, and at 2 years no hypertrophy was observed.

Karotyping of fibroblasts grown from graft centers and from successive rings of skin surrounding the graft showed that in the first 2 weeks after grafting, donor and host fibroblasts moved freely into and out of the SE graft. By 1 month 30% of the

fibroblasts in a ring of skin 8-13 mm from the graft edge were fibroblasts which had migrated out of the graft.

The SE was capable of normal wound repair. When 2 mm strips were cut from the center of a healed SE graft, granulation tissue was formed at the base of the wound and the wound closed to form a raised linear scar.

TABLE I

SUMMARY OF SKIN EQUIVALENTS GRAFTED

	Months on Recipient before Biopsy					
	<1	1-3	6-9	12-18	24	Total
Autograft--rat	4	1	4	3	5	17
Autograft--rabbit	6	3	1	1		11
Isograft--rat	37	13	13	1		64
Allo fib, iso ker--rat	15	12	3			30
Allo fib, iso ker--rab	8	6				14
Iso fib, allo ker--rat	7	2	1			10
Allo fib, allo ker--rat	3	5				8
Xeno fib, iso ker--rat	14	3				17
						169

Key: allo = allogenic
iso = isogenic
xeno = xenogenic
fib = fibroblasts
ker = keratinocytes
rab = rabbit

For the Period August 1, 1983 to March 1, 1984

1. Large grafts to the rat

We continued to test different techniques for replacing large areas of skin with skin equivalent grafts. Rats were prepared for grafting by removing about 20% (3 x 6 cm) of the skin from the barrell. The skin adjacent to the graft bed was secured to the underlying muscle by deep sutures. Histologic examination of the graft biopsied at 14 days showed excellent healing with no change in graft bed area. Sequential sections of the graft showed that the two skin equivalent lattices were completely integrated. It was possible to distinguish between pairs of grafted skin equivalents only at one level, near the graft center where they overlapped. There a small tongue of epidermal cells extended into the graft about 1/4 of the graft thickness and the organization of the collagen on either side of the epidermal tongue was slightly different, with one side having larger collagen bundles. This presumably represents the remnant of the epidermal cells on the surface of the inferior lattice where the two lattices overlapped.

Other recipients were maintained for two months so that the grafts could be monitored over a longer interval. At the time of unbandaging at 14 days, the grafts had contracted between 20% to 40% of original area, possibly because the bandage was loose in some cases. No further wound contraction occurred during the next six weeks. The centers of a few grafts became scabbed within one week after unbandaging, and this scab was maintained. Histologic examination of a large graft with scabbed center shows that the epidermis is damaged under the scab but the dermis is intact. In long-term studies the scabbed areas healed, but were reinjured throughout the lifetime of the graft. The damage occurs over the spine of the rat and may be due to abrasion since the graft area is not protected by fur. Alternatively, since the dermal equivalent does not now contain retae, the attachment of the epidermis to dermis in the skin equivalent may not be as strong as in normal skin.

We conclude that immobilization of the edges of the graft bed allows rapid and complete healing of the SE graft. On the basis of our experiments, the use of overlapping grafts for repair of large defects seems to be feasible.

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