GRANT NO: DAMD17-91-Z-1007

TITLE: EFFICACY OF ALLOGENIC CULTURED KERATINOCYTE GRAFTS FOR BURN WOUNDS

PRINCIPAL INVESTIGATOR: Anthony A. Meyer, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
300 Bynum Hall, CB #4100
Chapel Hill, North Carolina 27599

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PREPARED FOR: U.S. Army Medical Research and Development Command, Fort Detrick
Frederick, Maryland 21702-5012

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.
Our initial proposal reviewed the previous work on cultured keratinocyte grafts, specifically allografts, done over the last 15 years. The principal hypothesis of this proposal was that although keratinocytes do not express Class II histocompatibility antigens in vitro, once animals are grafted with allogeneic cultured keratinocytes, these keratinocytes will sensitize the animals and lead to subsequent rejection. If this hypothesis is not true, however, keratinocyte allografts could be used as a major source of wound coverage for patients with large burns or other open wounds. This Midterm Report will review the progress on this grant as it relates to the initial proposal.
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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June 1, 1993

Commander, U.S. Army Institute of Surgical Research
ATTN: SGRD-USX (Col. Basil A. Pruitt, Jr., M.D.)
Fort Sam Houston, Texas 78234-6200

RE: Mid-Term Report of Grant DAMD 17-91-Z-1007

Name of Grantee: University of North Carolina at Chapel Hill

Name of Principal Investigator: Anthony A. Meyer, M.D., Ph.D.

Period of Report: 3 May 1991 to 2 May 1993

I. Background

Our initial proposal reviewed the previous work on cultured keratinocyte grafts, specifically allografts, done over the last 15 years. The principal hypothesis of this proposal was that although keratinocytes do not express Class II histocompatibility antigens in vitro, once animals are grafted with allogeneic cultured keratinocytes, these keratinocytes will sensitize the animals and lead to subsequent rejection. If this hypothesis is not true, however, keratinocyte allografts could be used as a major source of wound coverage for
patients with large burns or other open wounds. This mid-term report will review the progress on this grant as it relates to the initial proposal.

II. Summary of Progress

In the "Statement of Work", our initial proposal predicted the completion of steps 1, 2, and 3 by the end of the first 24 months. This has generally been achieved and, in some areas, been surpassed. The following list delineates our achievements in these studies.

a) Cultured keratinocytes have been successfully grown for C57BL/6 and CBA mice. These two mouse strains were chosen as replacements for the B10 and B10.A animals described in the initial proposal. The H-2 genotypes of these two strains represent a major histocompatibility difference rather than a single haplotype difference, resulting in easier determination of sensitization.

b) Cultured keratinocyte allografts have proven to survive for up to three weeks in our present model. Although allograft success is only 30-35% compared to approximately 50% in keratinocyte autografts, this does demonstrate the presence of graft and graft survival. A summary of this data is shown in Table 1.

c) Allograft has been identified specifically from excised grafts on CBA animals by using monoclonal antibody specific for the Class II H-2\(^b\) haplotype of C57BL/6 animals. This has been demonstrated repeatedly on western blots as being identical to the Class II antigen expression induced by interferon gamma on C57BL/6 cultured keratinocytes. Notably, this antigen appears in vivo whether or not the animal has been exposed to interferon gamma. This is extremely important because there is spontaneous generation of this antigen after grafting. Examples of this from 5 animals are shown in Figure 1, which demonstrates Class II H-2\(^b\) antigen expression from cultured keratinocytes excised 3 days after grafting onto H-2\(^k\) recipients.

d) Animals grafted with allogeneic cultured keratinocytes fail to demonstrate sensitization when assessed by mixed lymphocyte response or presence of serum cytotoxic antibody. This data was reported at the Society of University Surgeons and is shown in Figures 2 and 3. It has been accepted for publication.
in Surgery. This data would suggest that there is no immune response to cultured keratinocyte allografts and that these allografts may not induce graft injection.

e) Subsequent studies have been performed using cultured keratinocyte allografts followed by full-thickness tail allografts to determine if animals can be sensitized as assessed by accelerated second set rejection. This study, shown in Table 2, demonstrates that these animals are in fact primed by keratinocyte allografts. Furthermore, when excised and assayed by western blot, these wounds demonstrate Class II histocompatibility antigen expression. An abstract with this data has been submitted to the Association of Academic Surgeons.

These data take us to the conclusion of step 3 and 24 months into the grant period outlined in the "Statement of Work" in our initial proposal. We have demonstrated the confirmation of hypotheses 2A, 2B, and 2C as listed on page 21 of the initial proposal.

III. One area in which we hoped to be successful in our technical objectives was not reached. This was the demonstration of Class I and Class II histocompatibility antigens on biopsies of cultured skin using immunohistochemistry or immunofluorescent techniques. We continue to work on these techniques, but have not found specific and consistent results with them. The use of western immunoblotting with monoclonal antibody specific for the donor allotype, however, has provided us not only with a means for identification of donor-specific graft, but also with a method for detection of antigen expression capable of sensitization of the recipient.

IV. In addition to our present findings as outlined in the "Technical Objectives and Goals", we have found important information relating to the persistence of feeder layer fibroblasts in mature cultured keratinocyte grafts. Preliminary data suggests that these fibroblast feeder layers persist even into the third passage of human keratinocytes. Fibroblast persistence could potentially lead to sensitization of the graft recipient to the fibroblast feeder layer resulting in rejection of subsequent keratinocyte autografts. This would be consistent with the findings of delayed graft loss of keratinocyte autografts in current human studies. Preliminary findings from these studies were presented at the 1993 American Burn Association Meeting.
V. Future Continued Research

We will continue along our "Statement of Work" for the next two years. Our initial plans are to elucidate the response to keratinocyte grafts in animals after burn injury. We will begin these experiments within the next two months. We will also continue to clarify the mechanisms by which keratinocyte allografts sensitize the host and lead to graft loss. We are presently working on the identification of cytotoxic T lymphocytes which could lead to graft rejection but would not generate an increased mixed lymphocyte response or serum cytotoxic antibody.

Future goals include a better means of following keratinocyte allografts and development of molecularly-altered keratinocyte grafts. Specific manipulation of keratinocytes by "knock-out genes" or other molecular biology alterations could produce an immunologically inert graft allowing for universal coverage of patients needing skin grafts.

Sincerely,

Anthony A. Meyer, M.D., Ph.D.
Professor of Surgery
University of North Carolina
Department of Surgery
164 Burnett Womack
Campus Box 7210
Chapel Hill, NC 27599-7210

cc: U.S. Army Medical Research and Development Command
    U. S. Army Medical Research Acquisition Activity
<table>
<thead>
<tr>
<th>Graft Take</th>
<th>Gross Exam</th>
<th>Histology</th>
<th>Gross Exam or Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUTO CK</td>
<td>55.2% (21/38)</td>
<td>50.0% (19/38)</td>
<td>73.7% (28/38)</td>
</tr>
<tr>
<td>ALLO CK</td>
<td>30.0% (12/40)</td>
<td>35.0% (14/40)</td>
<td>50.0% (20/40)</td>
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<tr>
<td><strong>Days 2-10</strong></td>
<td></td>
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</tr>
<tr>
<td>AUTO CK</td>
<td>60.0% (18/30)</td>
<td>56.7% (17/30)</td>
<td>83.3% (25/30)</td>
</tr>
<tr>
<td>ALLO CK</td>
<td>33.3% (10/30)</td>
<td>43.3% (13/30)</td>
<td>60.0% (18/30)</td>
</tr>
</tbody>
</table>

§ Two AUTO CK animals that died two weeks postgrafting were not included in the analysis.
Table 2

<table>
<thead>
<tr>
<th></th>
<th>ALLO CK (n=10)</th>
<th>AUTO CK (n=8)</th>
<th>ALLO FT(^1) (n=11)</th>
<th>Control (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>median graft survival</td>
<td>9 days*</td>
<td>13 days</td>
<td>9 days</td>
<td>13 days</td>
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ALLO CK reject earlier than control, *p<0.001 compared by Wilcoxon rank order and Chi squared.
**FIGURE 1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>CNA</th>
<th>N/6</th>
<th>Animal #</th>
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<th>Positive Control</th>
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<tbody>
<tr>
<td>Skin</td>
<td>Skin</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
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<tr>
<td>Standards</td>
<td></td>
<td></td>
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</tbody>
</table>

- **Histocompatibility Antigen Band**
- Class II

| Kl275 con | 1/6 | 5 | 4 | 3 | 2 | 1 | P | D2 | SP |