Brief Communication

Cell Persistence of Allogeneic Keratinocytes and Fibroblasts Applied in a Fibrin Matrix to Acute, Full Thickness Wounds

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HP802-247 is a living cell suspension of cultured allogeneic growth-arrested human male keratinocytes and fibroblasts (1:9 ratio), intended for spray application to chronic wounds. In this study, a small wound was created on the arms of 28 healthy female volunteers (3-mm punch), followed by a single application of HP802-247. At each subsequent week for 8 weeks, a punch excision of the wounds was performed on a cohort of three subjects. Excised specimens were analyzed for allogeneic fibroblast and keratinocyte DNA determined by Y-chromosome short-tandem repeats using PCR amplification followed by capillary electrophoresis, a method with estimated sensitivity of 1 male cell in a background of 8,000 female cells. A complete haplotype attributable to HP802-247 fibroblasts was detected in three of three samples at 1 week, with one partial and one complete fibroblast haplotype detected at 2 weeks, and one partial keratinocyte haplotype detected at 3 weeks postapplication. The findings indicate that HP802-247 can be expected to persist in an acute wound bed for up to 2 weeks postapplication.

Key words: Allogeneic cells; Wound healing; Keratinocytes (KCs); Fibroblasts (FBs); Y-STR; HP802-247

INTRODUCTION

HP802-247 is a novel therapy currently in clinical development for the treatment of ulcers resulting from venous stasis. It is an allogeneic living cell suspension consisting of growth-arrested (80-Gy gamma irradiation) human keratinocytes (KCs) and fibroblasts (FBs) delivered as a spray in a human fibrin matrix. The cells are growth-arrested in order to prevent cell proliferation, focusing cellular metabolism on nonproliferative responses to the wound environment (4). In vitro data show that these cells secrete several growth factors and cytokines of known importance in wound healing, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and granulocyte-macrophage colony stimulating factor (GM-CSF), and that secretion of growth factors is similar before and after

irradiation (2). In two separate phase 2 clinical trials, HP802-247 (5×10^6 cells/ml, the same cell concentration used in the present study) has demonstrated significant efficacy over vehicle control in the healing of chronic, nonhealing, venous leg ulcers (2,3).

Persistence in vivo of the allogeneic HP802-247 cells has both safety and efficacy implications. From a safety standpoint, demonstration that the cells have a limited lifespan when applied to wounds can alleviate concerns regarding neoplastic potential. From an efficacy perspective, establishing the duration of persistence in vivo with some precision allows a better understanding of optimal dosing frequency. Preclinical studies preceding the current work have shown that the HP802-247 growth-arrested cells survive for up to 3 weeks under ideal conditions in culture (unpublished data).

MATERIALS AND METHODS

This was a phase 1 open label study of the persistence of HP802-247 cells in acute excisional wounds of healthy female volunteers. This study was performed in compliance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice (GCP) and was posted on clinicaltrials.gov (NCT01190865). The study was reviewed and approved by the institutional review board (IRB), and all subjects or their legal representatives read, signed, and dated the IRB-approved consent form before taking part in any study activity.

HP802-247

Cells are derived from human neonatal foreskin, obtained under informed consent guidelines and have undergone extensive donor and direct testing for infectious pathogens. HP802-247 was manufactured at DFB Biosciences (Lausanne, Switzerland) according to Good Manufacturing Practice requirements. Details of manufacture and production have been published elsewhere (2). HP802-247 is supplied as two cryopreserved components, C1, a fibrinogen solution, and C2, a suspension of cells (KC/FB = 1:9; 10×10^6 cells/ml, 5×10^6 cells/ml when combined with C1 on the wound) in a thrombin solution. After thawing, these components are sprayed sequentially on the wound bed forming a thin fibrin matrix containing the two living but nonproliferating cell types.

Study Design

Since all of the HP802-247 cells bear a Y-chromosome, a study design in which only female volunteers were enrolled allowed the use of highly sensitive, commercially available, multiplex assays for specific short-tandem repeats unique to the Y-chromosome (Y-STR).

Normal adult females >18 years of age were enrolled. A buccal smear was obtained from all subjects at screening and analyzed for the unlikely presence of Y-chromosome or of translocated Y-STR in the subjects' DNA. Qualified subjects were randomized to one of nine cohorts corresponding to a duration of from 1 to 8 weeks ($n=3$ each) and a "substitute" cohort of four subjects that served as replacements for dropouts from any of the weekly cohorts. A 3-mm punch biopsy wound was created on the inner aspect of the upper arm for each qualified subject at day 1, onto which HP802-247 was applied. HP802-247 applied as directed results in approximately 8,000 cells on the surface of a 3 mm biopsy wound. Subjects returned weekly and a second 3-mm punch biopsy at exactly the same location as the first was taken from the cohort of three subjects scheduled for biopsy that week (according to randomization assignment). These second biopsy specimens were then assayed for the presence of Y-chromosomal material.

Study Procedures

Subjects meeting criteria for enrollment returned for a baseline visit (day 1). A shallow (into the dermis) 3-mm punch biopsy was taken from the inner aspect of the upper arm following local anesthesia by lidocaine infiltration using clean surgical procedures. Four radial lines centered on the biopsy site were drawn with a gentian violet surgical marker and redrawn weekly, as needed, in order to clearly mark the site for the second biopsy.

HP802-247 was removed from the -80°C freezer and thawed at 37°C for 5 min in a water bath according to the manufacturer's instructions. Immediately following hemostasis and removal of any excess clot, HP802-247 was applied to the wound by sequentially spraying C1 and C2, one actuation ($\approx 130 \mu\text{l}$) each from a distance of ≈ 10 cm. Wounds were dressed with a nonadherent contact bandage (Nexcare™ Tegaderm™, 3M, St. Paul, MN, USA) and covered with gauze.

If a subject left the study for any reason, one of the subjects randomized to the "substitute" group was used as a replacement. The second biopsy was performed identically, as before, at the exact location of the first biopsy. Polysporin ointment was applied to the second biopsy wounds, and subjects were scheduled for a Study Exit visit 2 weeks later for confirmation of healing.

Biopsy Specimens

Biopsied tissue was removed from the biopsy punch using sterile scissors to clip the biopsy tail free of the skin. Using the punch plunger and sterile forceps, the specimen was placed directly into a labeled cryovial on dry ice for transport to the lab. Biopsy specimens were identified by subject number only. Individuals conducting the Y-STR assays were blinded to cohort assignment. Specimens remained frozen pending DNA and Y-STR assays.

DNA Analysis

DNA from volunteer buccal swabs, posttreatment biopsy specimens, and a few of the pretreatment biopsy specimens (for reference purposes) was extracted on the AutoMate Express™ robot using the PrepFiler™ Forensic DNA Extraction Kit. DNA samples were quantified for male human DNA with the Quantifiler® Duo DNA quantification kit on an AB 7500 Real-Time PCR System. Y-STR analysis was performed using the AmpFLSTR YFiler typing kit following manufacturer's guidelines in a GeneAmp® PCR system 9700. Amplified products were electrophoresed and analyzed on an AB 3500xl genetic analyzer using GeneMapper® ID-X fragment analysis software. Following analysis of the volunteer reference samples and clinical samples, DNA was extracted from the two cell lines comprising HP802-247 (KC=donor P112, FB=donor P104) and typed to determine the Y-STR haplotype of the male donors. Analysis of the cell

line DNA followed the same procedure outlined above. All instruments and kits used in the DNA analysis are products of Applied Biosystems, Foster City, CA, USA.

Statistical Analysis

Descriptive statistics of correct chromosome identification were planned for this study as the primary analysis. The primary efficacy variable was the full set of 17 Y-STR loci. If all loci were amplified such that a clear identification of a donor was possible, the test results were categorized as positive. Identification of each cell type (KC, FB) was analyzed as a secondary analysis. Probit analysis was utilized to evaluate the time point in days when 50% of the Y-chromosome loci were correctly detected or identified among the second biopsies. The number of days from the treatment application to the time point when 50% of the genetic loci could be correctly identified (or ID_{50}) was used to quantitatively measure the persistence of growth-arrested keratinocytes and fibroblasts of HP802-247. The probit analysis was performed using SAS® PROC PROBIT (SAS Institute, Inc., Cary, NC, USA). The ID_{50} and its 95% confidence interval (CI) are reported.

RESULTS

Subject Disposition and Demographics

Twenty-eight subjects qualified for the study, received an initial wound, and were randomized into eight cohorts

of three patients for each time point or the “substitute” cohort. Three subjects left the study prematurely, two for noncompliance and one for withdrawn consent. Of the 28 subjects enrolled in the study, 23 (82%) were White and 5 (18%) were African American, with 3 (11%) indicating Hispanic or Latino ethnicity. The median age was 44.4 years (range, 26–68 years), with two subjects >65 years of age.

Cell Persistence

Table 1 presents the Y-STR haplotype for both fibroblasts and keratinocytes making up HP802-247 as well as the Y-STR loci detected for each wound excision with at least a partial haplotype. A full haplotype characteristic of the fibroblast cell line was detected for all three subjects (1 week cohort) at 1 week postapplication. At 2 weeks postapplication, a full haplotype was detected for one of the three subjects (2-week cohort) and a partial haplotype for a second subject indicating that HP802-247 cells applied to wounds could reasonably be expected to persist for up to 2 weeks postapplication. By 3 weeks, DNA originating from HP802-247 was detected in only one of the three samples (3-week cohort). Interestingly, this was a partial haplotype attributable to the HP802-247 keratinocytes. Table 2 presents the ID_{50} in number of days for the partial DNA profile, >50% DNA profile, and complete DNA profile within approximately 8 weeks posttransplant.

Table 1. Y-STR Haplotypes From Second Biopsy Specimens Compared to HP802-247 Cell Line Haplotypes

Loci	FB (Donor P104)	KC (Donor P112)	Subject Number/Cohort					
			004 Wk 1	023 Wk 1	025 Wk 1	020 Wk 2	024 Wk 2	008 Wk 3
DYS19	14	15	14	14	14	14	14	–
DYS385	12,14	11,13	12,14	12,14	12,14	12,14	12,14	–
DYS389I	13	13	13	13	13	13	13	13
DYS389II	30	29	30	30	30	30	30	29
DYS390	24	25	24	24	24	24	24	–
DYS391	11	11	11	11	11	11	11	–
DYS392	13	14	13	13	13	13	13	–
DYS393	12	13	12	12	12	12	12	13
DYS438	12	12	12	12	12	12	–	–
DYS439	12	12	12	12	12	12	12	–
DYS437	15	15	15	15	15	15	15	–
DYS448	19	19	19	19	19	19	19	–
DYS456	15	16	15	15	15	15	15	–
DYS458	19	17	19	19	19	19	–	–
DYS635	23	23	23	23	23	23	23	–
GATA-H4	12	12	12	12	12	12	12	–
Profile	Full	Full	Full	Full	Full	Full	Partial	Partial
Attribution:			FB	FB	FB	FB	FB	KC

Numbers in the table refer to the number of times the STR sequence is repeated at the given loci. FB, Fibroblast; KC, Keratinocyte; Wk 1, Week 1; Wk 2, Week 2; Wk 3, Week 3 (1, 2, and 3 weeks postapplication, respectively).

Table 2. Posttransplant Time and Probability of Correctly Identifying DNA

Probability	Type of Profile Identification	Estimate (Day)	(95% CI)
ID ₅₀	Partial DNA profile	19	(0, 30)
	≥50% of the 17 loci	17	(0, 28)
	Complete DNA profile	13	(0, 24)

CI, confidence interval; ID₅₀, time when 50% of genetic loci could be correctly identified.

HP802-247, given as a single application at 5×10^6 cells (final concentration)/ml, was found to be safe and well tolerated in the subject population.

DISCUSSION

The persistence of HP802-247 cells was demonstrated for up to 2 weeks (FBs), following a single application to acute excisional wounds in healthy female volunteers. Since a partial haplotype attributable to KCs was detected at 3 weeks in one sample, it is not unreasonable to conclude that KCs also persist for 2 weeks but are simply more difficult to detect due to their nearly 10-fold lower concentration. The results of this study support a biweekly dosing regimen given the persistence of the cells. Interestingly, in a recently completed dose-ranging study in subjects with venous leg ulcers, the dose demonstrating the best overall efficacy was applied biweekly (3). It may be that a biweekly frequency strikes the optimal balance between survival of the allogeneic cells and the additional burden of removal of debris resulting from dying cells.

A limitation of this study is that the detection of DNA originating from the HP802-247 cells does not necessarily equate with the presence of living cells (1). However, the use of a multiplex assay consisting of probes for 17 distinct Y-STR loci does allow a very clear distinction to

be made between the presence of a complete chromosome indicative of an intact cell and a partial Y-chromosome, a distinction not possible using in situ hybridization probes. A second limitation is that the measurement of cell persistence was performed using a weekly wound sampling interval. Nevertheless, the statistical treatment of the data allows for the estimation of an ID₅₀ for the cells with 95% confidence intervals. HP802-247 consists of keratinocytes and fibroblasts in a 1:9 ratio, and so there is a 9× higher probability of detection of the fibroblast DNA over keratinocyte DNA, assuming all other factors being equal. Indeed of the six positive results, five were attributable to the fibroblast component of HP802-247.

ACKNOWLEDGMENTS: This study was supported by Healthpoint Biotherapeutics. We are grateful to Drs. Innes Cargill, Darrell Lange, Vincent Ronfard, and Mr. Tommy Lee for their critique and suggestions. Drs. Dickerson, Slade, and Ms. Weedon are employees of Healthpoint Biotherapeutics, the manufacturer of HP802-247. Dr. Planz, Mr. Reece, and Ms. Kirkpatrick have no financial interest.

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