Cell Transplantation, Vol. 15, pp. 823–834, 2006
Printed in the USA.

0963-6897/06 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Development, Characterization, and Use of a Fetal Skin Cell Bank for Tissue Engineering in Wound Healing

Anthony S. De Buys Roessingh,* Judith Hohlfeld,* Corinne Scaletta,† Nathalie Hirt-Burri,* Stefan Gerber,‡ Patrick Hohlfeld,‡ Jan-Olaf Gebbers,§ and Lee Ann Applegate†

*Department of Pediatric Surgery, University Hospital Lausanne, Lausanne, Switzerland
†Orthopedic Cell Therapy Unit, University Hospital Lausanne, Lausanne, Switzerland
‡Department of Obstetrics, University Hospital Lausanne, Lausanne, Switzerland
§Department of Pathology, Cantonal Hospital, Lucern, Switzerland

Wound healing in fetal skin is characterized by the absence of scar tissue formation, which is not dependent on the intrauterine environment and amniotic fluid. Fetal cells have the capacity of extraordinary expansion and we describe herein the development of a fetal skin cell bank where from one organ donation (2–4 cm^2) it is possible to produce several hundred million fetal skin constructs of 9×12 cm^2. Fetal cells grow three to four times more rapidly than older skin cells cultured in the same manner and these banked fetal cells are very resistant against physical and oxidative stress when compared to adult skin cells under the same culture conditions. They are up to three times more resistant to UVA radiation and two times more resistant towards hydrogen peroxide treatment. This mechanism may be of major importance for fetal cells when they are delivered to hostile wound environments. For fetal cell delivery to patients, cells were associated with a collagen matrix to form a three-dimensional construct in order to analyze the capacity of these cells for treating various wounds. We have seen that fetal cells can modify the repair response of skin wounds by accelerating the repair process and reducing scarring in severe burns and wounds of various nature in children. Hundreds of thousands of patients could potentially be treated for acute and chronic wounds from one standardized and controlled cell bank.

Key words: Fetal cell therapy; Tissue engineering; Oxidative stress

INTRODUCTION

The fields of tissue engineering and material science are rapidly producing novel biomaterials with remarkable biological functions. The mechanical properties of these new materials are extremely interesting as they can be tailored for specific applications and the cells that populate them can form actual tissues for maintenance, restoration, or amelioration of function. Therefore, the origin of the cells and their interaction with a biomaterial is extremely important for eventual therapeutic usage. Bringing safe and effective cell therapies to the clinic has several challenges associated with stability and the consistency of the cells. We show herein that fetal skin cells have an innate stability related to their resistance to oxidative stress and, due to their particularly interesting growth characteristics, consistent and safe cell banks can be produced for rapid delivery for patient care.

A very important advantage for the use of fetal cells for therapeutic reasons is that fetal tissue is preimmunocompetent and associated with a reduced capacity to evoke an immunological response in the recipient of such cells. The decreased immunocompetence is associated with the lack of postthymic T-lymphocytes prior to 14 weeks of gestation within fetal tissues (6,9).

Fundamental differences between fetal and adult skin and the fetal and adult skin wound environment may be important in inducing scar-free tissue repair. Early in gestation, the dermis is thin, relatively acellular, and a low extracellular matrix is present. During further development, dermal collagen is deposited and sulphated glycosaminoglycans (GAGS) replace hyaluronic acid (HA) among other nonsulfated GAGS. The extremely rapid growth and the loose extracellular matrix provide a conducive territory for scarless fetal skin repair (7). Another line of evidence showing that it is indeed fetal skin cells themselves that are responsible for scar-free tissue repair

Received March 20, 2006; final acceptance June 8, 2006.
Address correspondence to Dr. Lee Ann Laurent-Applegate, Orthopedic Cell Therapy Unit, University Hospital, CHUV, PAV 03, CH-1011 Lausanne, Switzerland. Tel: (++41) 21 314 3510; Fax: (++41) 21 887 8414; E-mail: Lee.Laurent-Applegate@chuv.ch

is that the in utero environment does not seem to be essential nor sufficient for scarless fetal repair. Fetal skin outside the warm, sterile, growth factor-rich amniotic environment has been shown to be very efficient in healing scarlessly and rapidly. This has been demonstrated with an opossum model as this marsupial is born fetal-like, both physiologically and anatomically, and remains attached to the mother's nipple for 4–5 weeks (4). Despite their extrauterine location, wounds in early pouch young reepithelialize very quickly, synthesize collagen, and heal scarlessly. In contrast, wounds in older pouch young heal more slowly and with scar formation. In this same line, human fetal skin transplanted subcutaneously to an immunoincompetent mouse retained its developmental characteristics and healed scarlessly with restoration of hair follicles and reticular collagen arrangement (14). As the regenerative capabilities of human fetal skin were not disrupted by an adult extrauterine wound environment or contact with adult mouse blood, the scarless capacities appear to be intrinsic to the fetal tissue itself. Scarless wound healing is clearly related to cellular functions and other notable differences between fetal cells and those from either neonatal, young, or old skin cells include extracellular matrix (ECM) ratios, growth factor and cytokine profiles, adhesion and migrating capacity, and resistance to oxidative and physical stresses (7,15,17).

We show herein that oxidative stress resistance is particularly high in fetal skin cells when compared to skin cells of other aged donors. We believe this mechanism is of major importance for fetal cells when they are delivered to hostile wound environments. To assure maximum consistency of the delivered fetal skin constructs, we developed a cell bank of fetal skin cells for the preparation of tissue constructs on a native horse collagen sponge (TissueFleece®, Baxter) for the treatment of chronic and acute wounds in human skin. Careful selection of a donor and an extensive screening to avoid transmissible viral, fungal, or bacterial disease provide a safe and secure utilization of fetal cells for therapeutic usage. We illustrate the diverse clinical use of fetal cell constructs to avoid autografting and lengthy hospitalization.

MATERIALS AND METHODS

Skin Donation and Cell Culture

Cell lines established in the University Department of Obstetrics in Lausanne from fetal skin were used in these studies (12–16 weeks, obtained after pregnancy termination with written, informed consent and approval from the local Medical School Ethics Committee). Skin samples from adult donors (SW2, 24-year-old male; SW12, 39-year-old female; GT, 27-year-old male) were obtained in the Department of Dermatology in the Lausanne University Hospital from non-sun-exposed skin sites also with written, informed consent and approval from the Medical School Ethics Committee.

Epidermal Keratinocyte Culture

Skin samples were washed three times for 10 min each in PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Tissue was treated for ~15 min with trypsin/EDTA and the epidermal cell layer scraped gently away from the dermal tissue with the aid of a dissecting microscope. The epidermal tissue was fragmented and centrifuged at $2000 \times g$ for 15 min. The pellet was then transferred to small tissue culture flasks that contained irradiated (2500 rads) Swiss mouse 3T3 cells at 70% confluence and keratinocyte complete medium as follows: Dulbecco's minimal essential medium/Hams diluted 3:1 (Flow); 10% FCS; 1% glutamine; 0.4 μg/ml 6 hydrocortisone; 10^{-10} M cholera toxin; 5.0 mg/ml insulin; 1.2 mg/ml adenine; 2.5 mg/ml transferrin; 0.14 mg/ml triiodothyronine; 10 μg/ml epidermal growth factor. Keratinocytes were grown at 37°C in a humidified atmosphere with 90% air/10% CO_2. Cells used for human skin grafts were grown in serum-free medium (Gibco, keratinocyte SFM) and for the first 12 h 5% FCS was added to assure a higher cell attachment.

Dermal Fibroblast Culture

Dermal tissue was dissected into <0.5-mm^3 fragments and grown in DMEM supplemented with 10% FCS and glutamine and the cells were used for experimentation between passages 0 and 3. They were grown to confluence before splitting and rinsed twice with PBS and counted.

Cell Bank Synthesis

From one original 4-cm^2 biopsy (skin sample from 14 weeks gestation), 100 6-cm plates were seeded with whole tissue fragments ~4 per plate (<0.5 mm^3). These fragments were grown in DMEM supplemented with only 10% fetal bovine serum (Hyclone). When cell growth advanced after approximately 1 week, dishes of tissue and cells were trypsinized [0.25% trypsin/0.1% ethylene diaminetetraacetic acid (EDTA)]. At this point 90 plates were frozen into individual units in liquid nitrogen. Cells were centrifuged at $2000 \times g$ for 15 min and resuspended in a freezing solution of DMEM (5 ml) + FCS (4 ml) + DMSO (1 ml, Fluka) and frozen in 1-ml aliquots (1×10^7 cells) at −80°C in Nalgene Cryo 1°C Freezing Containers (Nalgene) to achieve a −1°C/min rate of cooling and freezing curve. After 24 h, cells were transferred to liquid nitrogen for longer storage. We have seen that cells frozen in this manner are capable of being stored for at least 10 years in our laboratory. Ten plates were amplified to 200 plates of which 190 units

were for the secondary frozen stock. This was repeated once again with a third frozen stock composed of 198 vials. The remaining 2 units were amplified to 40 units and these were frozen as the usable "construct" bank at 5×10^5 cells/vial, which can be directly seeded into two collagen sponges (9×12 TissueFleece®, Baxter). Cell cultures were grown at 37°C in a humidified atmosphere of 95% air/10% CO_2.

Radiation Sources and Exposure Conditions

The UVASUN 3000 lamp (Mutzhas, Munich, Germany) emits wavelengths between 330 and 450 nm at a dose rate of 300 W/m^2 at a convenient irradiation position. The spectral output of the lamp was analyzed with a calibrated Optronic model 742 spectroradiometer (Optronics Laboratories, PA, USA) and shows a broad peak between 360 and 410 nm. The UVASUN 3000 lamp is equipped with an infrared filter and a filter that cuts off sharply all wavelengths below 335 nm. In addition, cells were irradiated with plastic tissue culture lids that permit no transmission of UVB or UVC radiations. For simplicity, we will refer to this radiation as UVA, although the small component of near-visible radiation may contribute to the biologic effects. Radiation fluences were monitored by an International Light Radiometer, IL 1700 with UVA detector head (No. 566 with filter W1327), calibrated against the spectroradiometer.

Radiation and Chemical Treatments

We have previously shown that epidermal keratinocytes are extremely resistant to oxidative and physical stresses (2,3). Fetal cells are also equally resistant (data not shown) and therefore for the cellular sensitivity studies we compare adult and fetal fibroblasts. Cells were plated in 60- or 100-mm-diameter Falcon culture dishes and grown to 75% confluence. Just prior to irradiation or chemical treatment of cells, the growth medium was removed and the cell monolayer was rinsed twice with phosphate-buffered saline (PBS, 0.14 M NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). For UVA irradiation, cells were covered with PBS and irradiated at 25°C. Irradiation periods were on the average from 13 to 55 min maximum. For hydrogen peroxide treatment, cells were covered with PBS containing the appropriate concentration of H_2O_2 (0.1–2.4 mM) and treated 30 min at 37°C in a 5% CO_2 incubator.

Survival Assays

Dishes of cells (60 mm, ~75% confluent) that had received either hydrogen peroxide or UVA radiation treatments were trypsinized, diluted, and plated at 200–5000 cells per dish (60 mm, three dishes per treatment). The dishes were incubated at 37°C for 12–14 days, after which they were stained with methylene blue and the colonies (>20 cells) were counted with a microscope. All experiments were carried out with both the laminar flow hood illumination system and room fluorescent lights extinguished.

Cell Growth Characteristics and Stability

Cell growth curves were established for fetal and adult human skin fibroblasts. Cells from flasks at 75% confluence were trypsinized and counted. Plates of 1000 cells were established in triplicate and counted at various time points between 5 and 20 days. Cells (fetal and adult skin fibroblasts) were also frozen under several conditions as cell pellets at −20°C, −80°C, and liquid nitrogen and also in association with different concentrations of DMSO. The stability of cells was also determined by refrigerating cells either in pellets or associated in a collagen matrix.

Fetal Skin Construct Preparation

Preliminary experiments investigating cell seeding density (from 10^2 to 10^5 cells cm^2) and growth periods (from 1 to 7 days) on a 9×12-cm equine collagen sheet of 2 mm thickness dry weight (Baxter, Switzerland) were performed in order to determine optimal conditions for fetal skin construct preparation. Fetal cells at passages 3 or 4 were placed in 20 ml medium (DMEM containing 10% FBS) and seeded on the collagen sheet by making small incisions at 2-cm intervals into the collagen matrix with a sterile, small-bored Pasteur pipette. The sheet containing the fetal cells (denominated later as fetal skin construct) was placed into a 37°C incubator at 95% relative humidity and 10% CO_2. An additional 30 ml of medium was added 1 h later, whereas changes of medium occurred every second day. Fetal skin cell constructs remained at the same 2-mm thickness as the original dry collagen sheets. A seeding density of 2.5×10^3 cells/cm^2 with cells in passages 3–4 and a growth period of 2 days were used for the patients.

Immunohistochemistry

Fixed tissue sections of 5 μm thickness were used for the immunohistochemistry. All incubations were done in a humidified chamber in the dark unless otherwise specified. For p63 detection, nonspecific binding was blocked by an incubation for 2 h at 25°C with a solution of PBS containing 5% fetal calf serum (FCS), 7% normal goat serum (NGS), and 0.1% Triton X-100. Tissue sections were then incubated with p63-specific antibodies [p63 (a p53 homolog at 3q27-29) Ab-1, Clone 4A4] at a 1:2000 dilution in PBS containing 5% FCS, 5% NGS, and 0.1% Triton X-100 for 30 min (Neomarkers, Fremont, CA, USA). Immediately following this incubation, tissue sections were washed three times for 10 min each in PBS and the sections treated with biotinylated

goat anti-rabbit at 1:200 in a solution of PBS with 5% FCS, 1% NGS, and 0.1% Triton X-100 for 3 h at 25°C. Tissue sections were washed four times for 5 min each in PBS and then treated with Vectastain ABC (Vector, Burlingame, CA, USA) as indicated by the company for 3 h at 25°C. After this incubation, tissue sections were washed three times for 10 min each in PBS and treated with 0.5 mg/ml 3,3′-diaminobenzidine with 0.32 μl 30% H_20_2 added just before an incubation of 1–2 min. All samples were treated at the same time. The antibody staining for p63 is represented by brown coloration. The samples were washed for 5 min under running water. They were counterstained with (Harris' hematoxylin), dehydrated, and mounted with Merckoglas. (Merck, Switzerland).

Clinical Study Design

Only patients who were candidates for autografts (mesh or full thickness) after 7–10 days of traditional treatment were considered in this study. Once included in the study, 1-cm^2 skin biopsies were taken from the patients to prepare cultures with the intention of possibly using autologous grafts after wound bed preparation with the fetal skin constructs if healing was not progressing rapidly. The study protocol was approved by the local ethics committee. Both the children (if at an appropriate age) and their parents gave written, informed consent.

Therapeutic Application of Fetal Skin Constructs

Two patients with wounds and one patient with second degree deep burn are shown for this application (Table 1). Explanation of the study was given 24 h before the first planned treatment in order to allow time for the patients and parents to study the information before giving written, informed consent. Fetal skin constructs were placed directly on the lesions and overlayered with petroleum jelly-coated gauze. Cotton gauze bandages protected the construct and the gauze. No stitches, staples, or biological glue were needed. The procedures were performed under sterile conditions. The surgeon's evaluation of healing determined the number of procedures. Bandages were changed every 3 or 4 days to ensure stability of the dressing and also as a function of availability of the anesthesia teams. General anesthesia or analgesia with nitrous oxide (N_2O) was used depending on the aspect of the wound and the need of debridement at the beginning of the treatment when necrotic tissue had to be removed. After closure of the skin, regular cream application (Bepanthen®, Roche and hospital preparation) was begun. Pressure garments such as used for traditional burn care were individually made to order and were worn day and night, removed for bathing and massage only.

RESULTS

Cultured Cell Populations From Fetal Skin

Indeed, both epidermal keratinocytes and dermal fibroblasts can be cultured separately from fetal skin (Fig. 1a, b). As with young and adult skin, epidermal fetal keratinocytes need many additional growth factors and a feeder layer for proper culture and do not have a capacity for extensive passage numbers (limited to 4–5 passages). Epidermal cells were all p63 positive (Fig. 1a) and pure cell cultures were obtained from the epithelium (Fig. 1b) and from the dermal tissues (Fig. 1c). Thus, p63 provides a traceable marker for epidermal cell presence. As we wished to produce a consistent culture condition for fetal skin for the cell bank development, whole fetal skin was used as starting material with no enzymatic treatment and minimal medium requirements (DMEM + 10% Hyclone serum). The media requirements were efficient for cell type selection and consistency. No p63 cells were assessed from passage 0 to passage 8, indicating that only dermal fetal cells were included in the final culture. As there is no specific marker available for fibroblasts, exclusion of other cell types was routinely accomplished with specific antibodies (i.e., melanocytes, myoblasts, osteoblasts, etc.). We have previously shown that keratinocytes are much more resistant to oxidative stress when compared to the underlying dermal fibroblasts. As this was seen to be also the case for the fetal cells, we used the fibroblasts from fetal

Table 1. Summary of Patients

No.	Age	Type of Injury	Body Site	Delay Before Treatment (Days)	No. of Constructs	Time to Closure (Days)	Follow-up (Months)
1	12 years	Impalement, 7 × 6 cm	thigh	15	7	50	8
2	10 days	Pressure ulcers to muscle layer, 3 × 2 cm and 4 × 5 cm	arm	7	3	15	16
3	15 months	Second and third degree burn—scalding water, 8% body surface	feet and legs	21	8	10	14

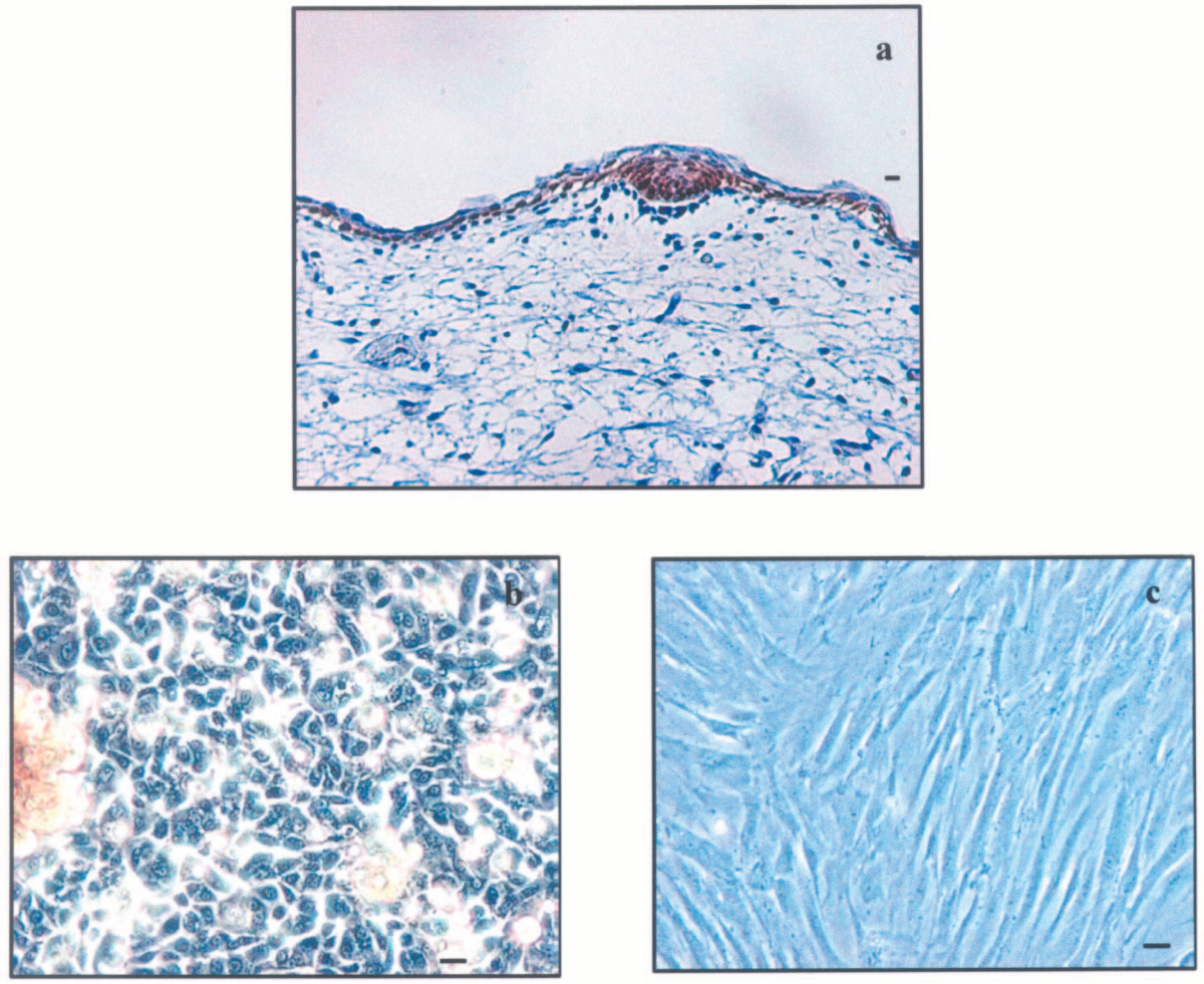

Figure 1. Histological section of fetal skin (12 weeks gestation) stained with p63 antibodies (brown coloration, A4A, Neomarker) and the p63-positive epidermal keratinocyte population of cells in culture along with the negative population of dermal fibroblast cells. Scale bar: 50 μm.

and old skin for characterizing the differences in resistance to physical and oxidative type stresses.

Cell Growth and Stability of Fetal Versus Adult Skin Fibroblasts

The cell growth of fetal skin fibroblasts has been shown to be much greater than of skin fibroblasts of adult donors. Fibroblast outgrowth can be observed the first day when fetal skin fragments are placed into culture dishes. The same is usually observed after 5–6 days for adult skin fragments treated under the same conditions. Once cultures were established, the fetal cells continued to grow at a much faster rate than cells from adults (Fig. 2A). By analyzing the cell number as a function of days in culture when starting with a low number of cells (100 or 1000), there existed a sharp difference at 12 days of culture. This is perhaps due to the difference in cloning efficiency, which was approximately 90–98% for fetal cells and 20–50% for adult cells. In the same manner, fetal cells frozen as simple cell pellets even at −20°C for up to 3 months were able to show similar outgrowth as under normal freezing conditions with DMSO in liquid nitrogen. Adult skin fibroblasts (two of the three cell lines) showed a limited amount of cell growth at −80°C freezing conditions with DMSO as a conserving agent but not at −20°C. Fetal skin fibroblast were even able to show considerable cell growth following refrigeration for 2 weeks (cloning efficiency of 25%).

Cell Survival of Fetal Versus Adult Skin Fibroblasts Following UVA Radiation

We have previously observed that neonatal fibroblasts from foreskin tissue were more resistant to oxidative stress (1,2). It was of interest to see how cells from different ages of gestation reacted also to oxidative

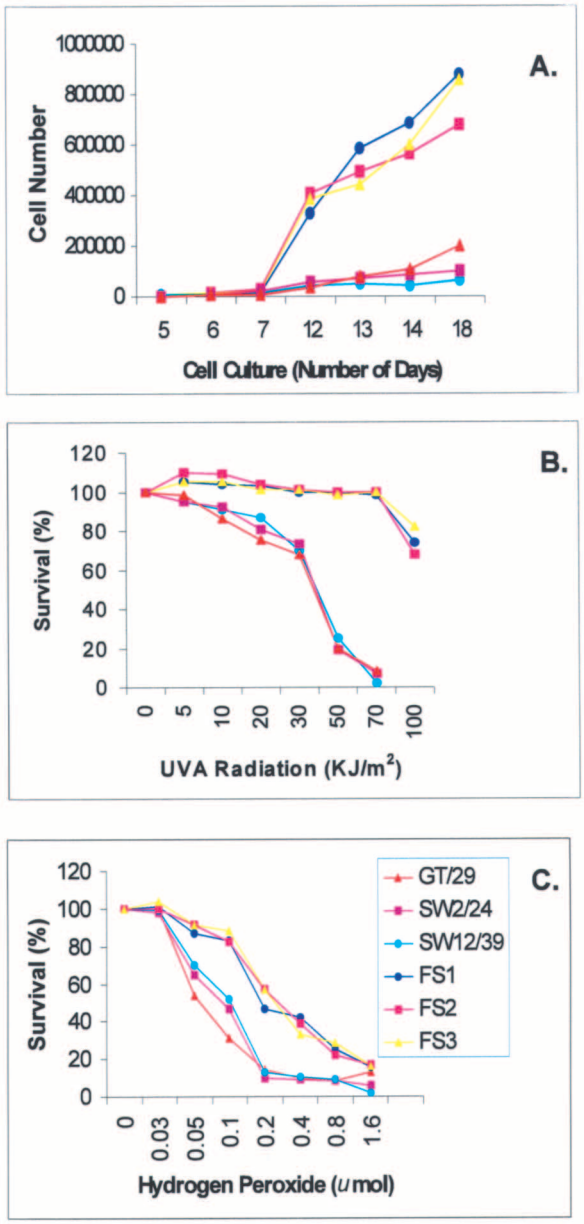

Figure 2. (A) Cell growth of dermal skin fibroblasts as a function of time for three individual fetal skin cell lines and three adult skin cell lines starting with 1×10^3 cells for each sample. Each data point is represented by the average cell number from three culture plates. (B) Percentage survival of dermal skin fibroblasts as a function of exposure to UVA radiation for three individual fetal skin cell lines and three adult skin cell lines. Each data point is represented by the average number of clones from three culture plates. (C) Percentage survival of dermal skin fibroblasts as a function of treatment with hydrogen peroxide for three individual fetal skin cell lines and three adult skin cell lines. Each data point is represented by the average number of clones from three culture plates.

stress and compare their resistance to adult skin cells. Even though there is a great difference between fetal cells and adult cells regarding their resistance to UVA radiation as the oxidative stress, no differences were seen between ages 12–16 weeks in gestation. By looking at the percentage of survival as a function of UVA radiation fluence, there was an extreme resistance marked in the three fetal skin cell lines tested in early passage number when compared to adult skin cell lines in the same passages (Fig. 2B). Even following the highest fluence of UVA radiation (taking approximately 50 min), only approximately 20% of the fetal cells were killed. However, a dose of 30–50 kJ/m^2 (a dose that gives a perceptible erythema to human skin) was capable of killing 50% of adult skin cells.

Cell Survival of Fetal Versus Adult Skin Fibroblasts Following H_2O_2 Treatment

The inactivation curves for the adult skin fibroblast were biphasic in nature, which is characteristic of hydrogen peroxide as the oxidizing agent. This ws not seen for the fetal skin cells and there was a gradual decrease in cell survival with an increasing concentration of hydrogen peroxide. The fetal skin cells were 1.5 times more resistant to this type of oxidizing stress when compared to adult skin cells under the same culture conditions and passages (Fig. 2C).

Cell Bank Synthesis

With one organ donation of 2–4 cm^2 of skin, we were able to develop a skin cell bank capable of producing >270 million skin constructs (9 × 12 cm) for therapeutic usage (Fig. 3). This is the quantity of cells that can be reasonably managed by one individual for the development of the bank in our hospital laboratory conditions. The cell bank consisted of dermal fetal cells even though whole fetal skin was used as starting material. We did not want to use enzymatic treatment of the original tissue to simplify and standardize culture conditions. Only minimal medium requirements, DMEM + 10% Hyclone serum, similar to those used for cell banking for vaccine development, were implemented. The media requirements were efficient for cell type selection and consistency throughout passaging (0 to 8). No p63 staining was found, indicating that only dermal fetal cells were included in the final culture. As there is no specific marker available for fibroblasts, exclusion of other cell types was routinely accomplished with specific antibodies (i.e., melanocytes, myoblasts, osteoblasts, etc.). These cells were routinely tested for Mycoplasma along with all bacterial and fungal infections. For the original tissue donation, the person was tested at 0 and 3–6 months for antibodies as described in Table 2. The fetal tissue was also examined for genetic or pathological abnormalities.

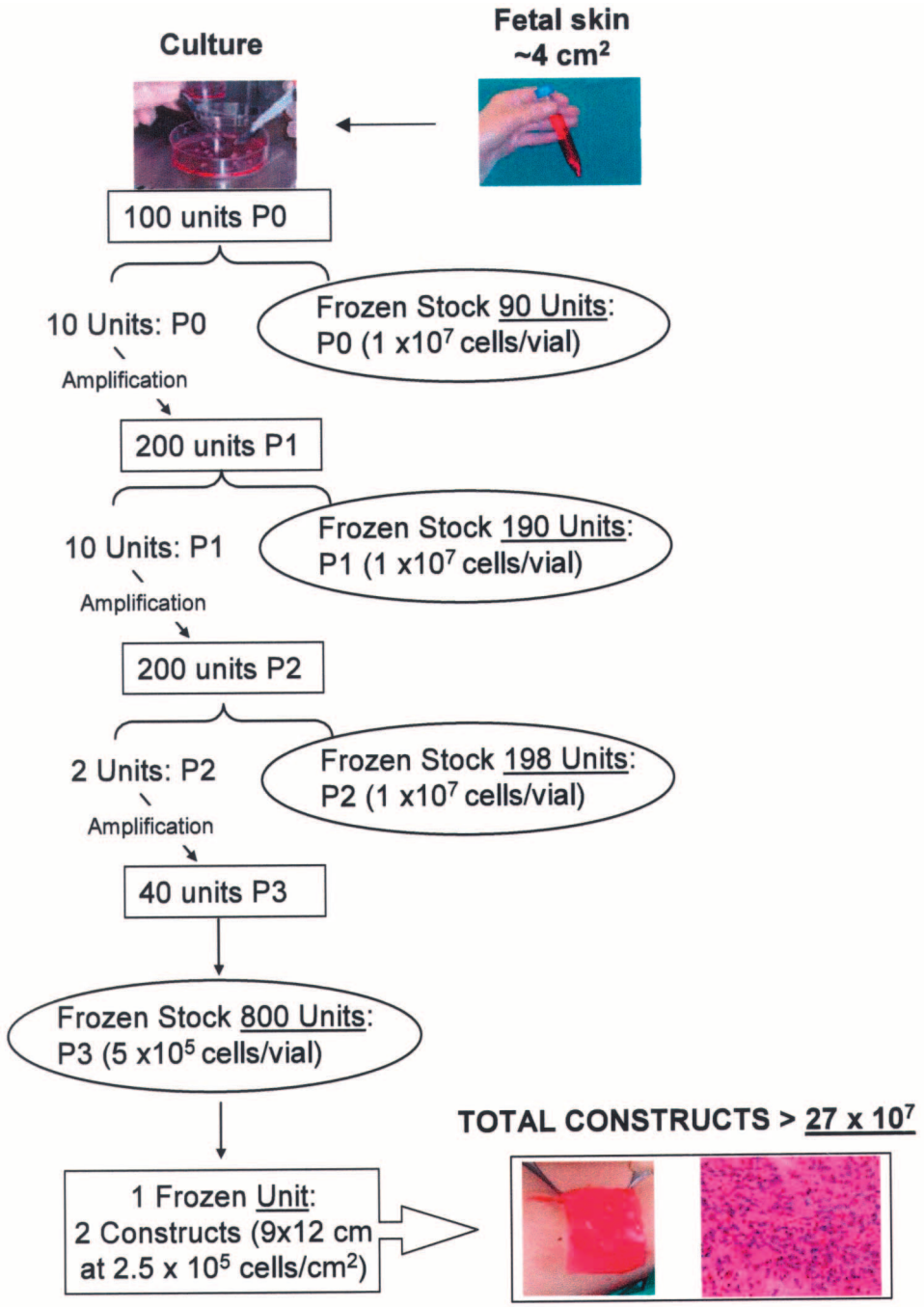

Figure 3. With one organ donation of 2–4 cm^2 of skin, it is possible to establish a fetal skin cell bank that is capable of producing >270 million skin constructs (9×12 cm) for therapeutic usage.

Three-dimensional fetal skin constructs associated with a collagen matrix (equine collagen, Baxter, Switzerland) were produced from the cell bank developed in a process to assure consistency for delivery to the patient. Vials of frozen cells were stocked to provide rapid processing and minimal culture times of the biological constructs (only 48 h necessary). Cells were used for construct development from passages 3–4 similar to what is accomplished for skin autografting techniques.

Clinical Application

Wounds. The first case concerns a 12-year-old boy with a wound on the inside of his left thigh inflicted while running in the forest where his left leg violently

Table 2. Description of Viral Testing for Donor Patients at 0 and 3 Months

Antibodies Tested	Company
HBsAg-Cobas Core HbsAgII EIA	Hoffmann-La Roche AG
Anti-HIV-1/HIV-2	Hoffmann-La Roche AG
Cobas Core Anti-HIV-1/HIV-2 EIA DAGS II	Hoffmann-La Roche AG
Anti HCV-Cobas Core Anti-HCV EIA	Hoffmann-La Roche AG
AXSym Anti-HCV EIA, Version 3.0	Abbott Diagnostika
PCR-HCV—Cobas Amplicor, Version 2.0	Hoffmann-La Roche AG
Treponema pallidum: Serodia—TP.PA	Fujirebio, Almedica AG
Anti-CMV—Vidas CMV IgG	BioMérieux SA
ETI-CYTOK-M reverse	Sorin Diagnostics S.r.L
Toxoplasma gondii—Toxo-Screen DA (IgG)	BioMérieux SA
Toxo-ISAGA (IgM)	BioMérieux SA

struck a tree branch, causing a wound of 7×6 cm in diameter and deep enough to expose the fat (Fig. 4). This child did not want hospitalization and insisted on outpatient care. The wound was incised and stitched and a drain was inserted. Ten days later the edges of the skin had turned a deep purple and the stitches had given way. At 12 days the wound was opened and on the 15th day a fetal skin construct was applied (Fig. 4).

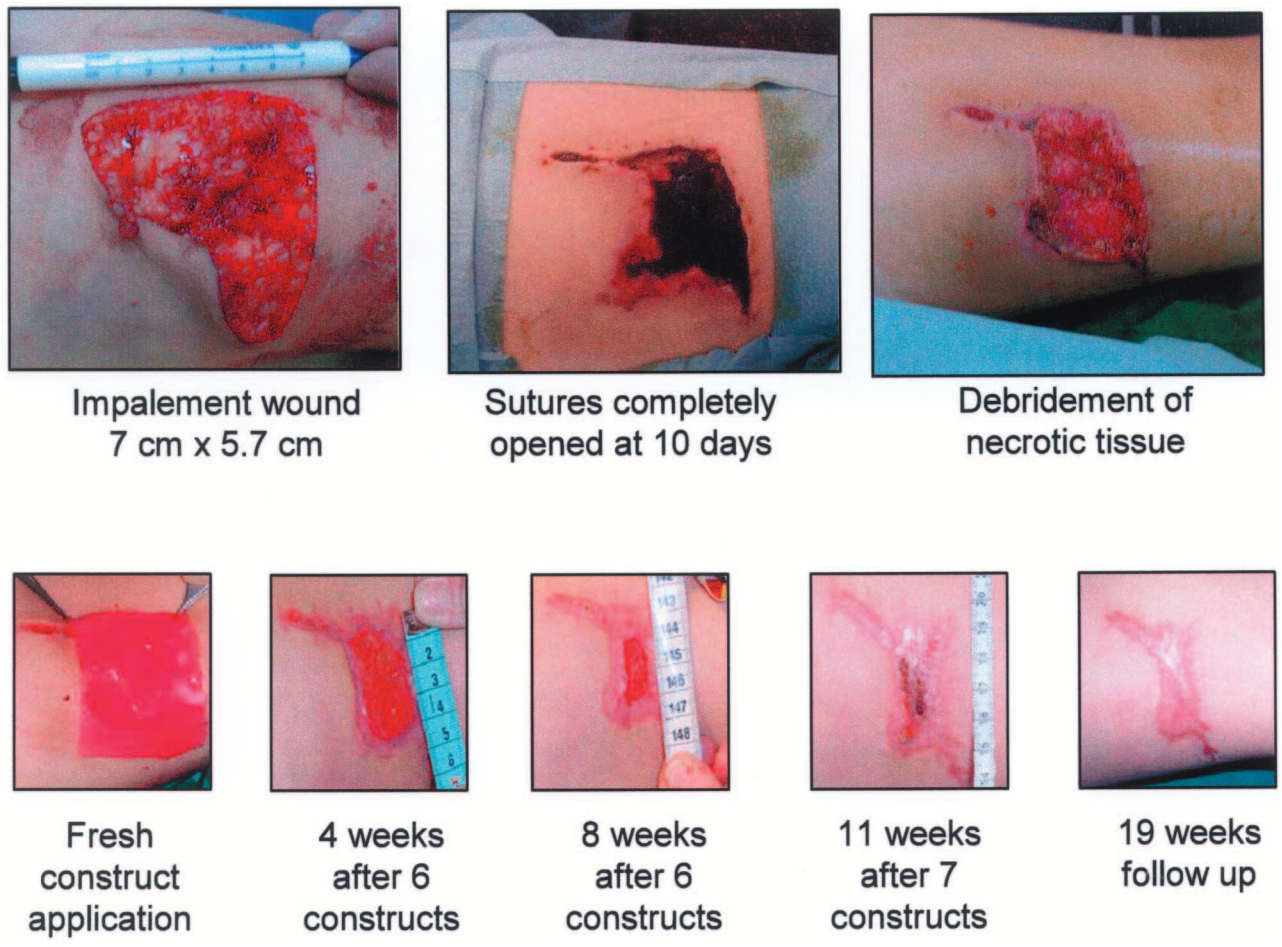

Figure 4. Male 12-year-old boy that had impalement injury to right thigh. Fetal skin constructs were applied seven times during 11 weeks showing progressive closure of this large physical wound.

For this case, seven constructs were applied on the wound at intervals of 3–4 days. The first three constructs were applied under general anesthesia and the last four with N_2O. Eleven weeks later the wound had healed completely with residual scarring. A compressive garment was worn and the scar massaged twice daily with cream (Bepanthen®, Roche and hospital preparation).

The second case concerns a baby born at 37.5 weeks of gestation and weighing 3500 g (Fig. 5). The 33-year-old mother had a bicornuate uterus. At birth the baby presented two pressure lesions on the left forearm. One of these lesions, anterior to the left elbow, measured 3×2 cm. The second lesion was posterior to the left forearm and measured 4×5 cm. The skin and subcutaneous tissue were absent and the lesions extended to the muscle layer. On examination, the baby was unable to move her left forearm and left hand spontaneously. The forearm was in a prone position, the fingers were flexed, and the hand hung limply. The baby could not extend her fingers or straighten her wrist. The mobility of the left arm and left shoulder was normal. For the first few days, the lesions were disinfected and protected, but after 1 week they showed no sign of closing and were filled with granulation tissue. On the eighth day, the baby was anesthetized to allow cleaning of the wounds. Excision of necrotic skin and fat tissue down to the muscle aponeurosis was done. A second debridement with aponeurectomy showing necrosis that had penetrated through to the muscle layer was performed on the 13th day. The application of tissue-engineered fetal skin was started 14 days after birth.

For this case (Fig. 5), constructs were placed directly on the wounds and overlayered with petroleum jelly-coated gauze and the entire forearm was then covered with gauze. The process was repeated three times during a 1.5-week period. The outer bandage was changed every day and the petroleum jelly-coated gauze only every third day. Complete closure was seen on both deep wounds on this patient and no autografting was necessary after three application of fetal skin constructs. As we had previously assessed in children with burns (10), the wound healing began from the border with the fetal skin constructs stimulating rapid healing of the patient's own

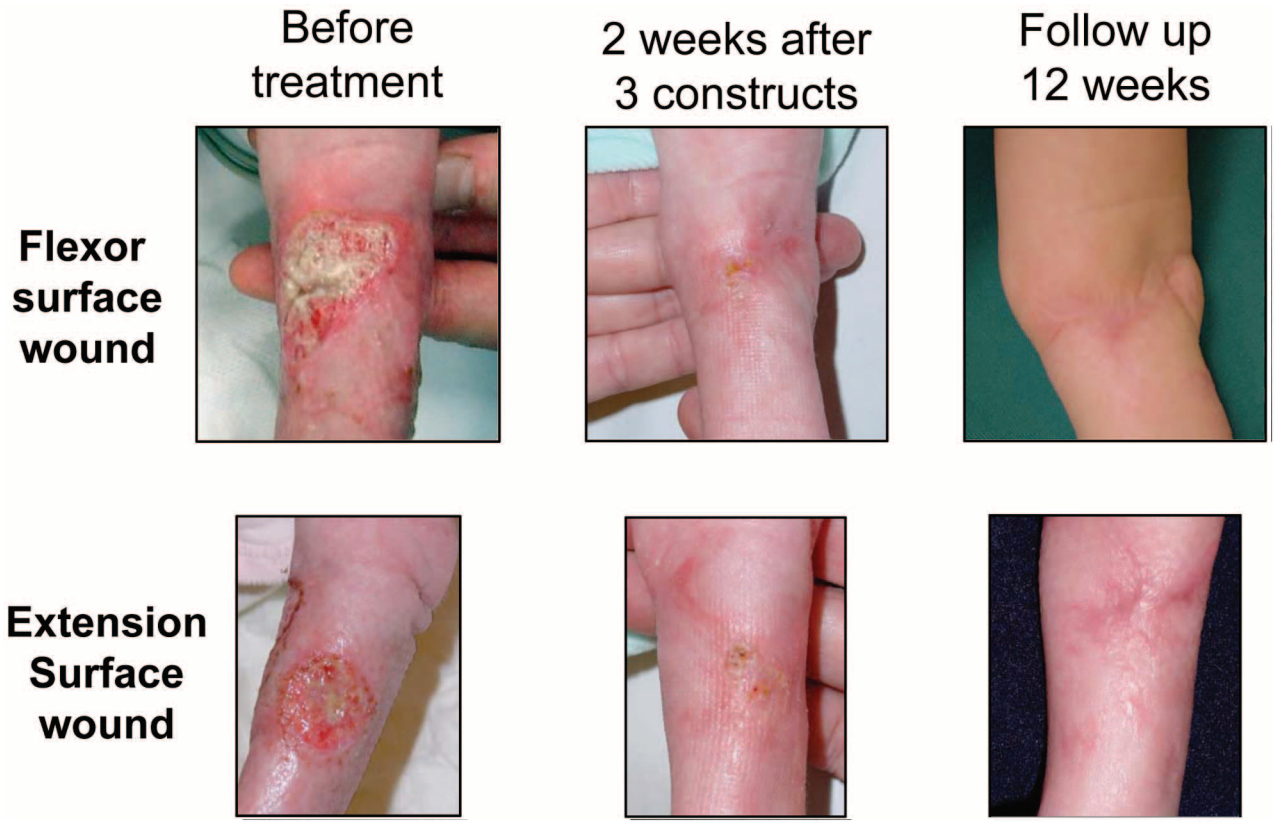

Figure 5. Female 10-day-old girl with ulcerations through to the muscle layer. Both interior and exterior wounds, which were resistant to closure in the first week, were treated three times in 15 days, showing complete closure at this time and eliminating the necessity to perform an autograft.

skin, both dermal and epidermal. This is also supported from the biopsy data showing no presence of fetal cells in healed burn sites as assessed by FISH technique (male donor fetal cells Y chromosome not present in female burn patient) (10). The fetal skin constructs were completely biodegraded at each bandage change. The lesions healed within 7 days and a cast was then applied to maintain the forearm and the hand in a neutral position. Physiotherapy was started a few days after birth.

Burns. The third child arrived at the emergency room after having burned his left hand and his legs with steaming water. Eight percent of his skin was burned (Fig. 6) and all the burned skin was blistered. For the first few days, the burn was disinfected and protected by dressings. After 4 days, the boy was anesthetized to allow cleaning of the wounds and the phlyctena were incised. On the 7th, 9th and 12th days, debridement with removal of the necrotic tissue was performed and was found to be a deep second degree burn. Tissue-engineered fetal skin therapy was started 21 days after the accident.

For this case (Fig. 6), the constructs were placed directly on the burn and overlayered with petroleum jelly-coated gauze and the entire hand and the legs were covered with gauze. The process was repeated seven times during a 3-week period. The outer bandage and the petroleum jelly-coated gauze were changed every 2–3 days. This patient presented with a staphylococcus infection during the third week of treatment, which was traced to a transmission from a family member. We therefore stopped fetal skin construct applications. Both feet and the upper left leg of the patient were already completely healed, so the infection was treated with local and systemic antibiotics. It was assessed that a portion of the right leg extending to the ankle and a 2-cm region of the calf on the left leg had not yet healed sufficiently and required partial thickness grafts thereafter to complete closure. The lesions healed within 2 months (Fig. 6). Intensive cream massages (Bepanthen®, Roche and hospital preparation) started after wound closure. Esthetical and functional results were excellent in this child where fetal skin constructs were applied; the skin was supple and thin with a good mobility. Moreover, a recovery of skin pigmentation was observed in all of the sites that were not autografted. It illustrates for the first time on one of our patients the autograft technique (which is considered at the golden standard) and fetal skin constructs at a parallel site.

DISCUSSION

The skin, like many other tissues, is constantly replenished with new cells produced by stem cells. One of the genes in skin cells that is vital for maintaining epithelial stem cells is p63 and thus provides an excellent marker for locating these cells in the epidermal layer of skin. It has been shown, at least with a mouse model, that in the absence of p63, regenerative proliferation for limbs, craniofacial, and epithelial development is not efficient (18). In fetal skin, the epidermis is only 1–2 layers thick at 12–14 weeks of gestation and ~2–4 cell layers thick at about 16 weeks (8). In all of the layers of skin during development, it is possible to detect strong p63 nuclear marking in all epidermal cells and in developing hair follicles. These cells (stem cells, p63-positive epidermal keratinocytes) have gained much attention because of their ability to produce different cell types but little is known on how they function and why they are able to constantly proliferate, although it seems that p63 plays a role in maintaining the population of epithelial stem cells. The dermal tissue does not contain individual p63-positive cells and there is not yet a marker identified that characterized the dermal fibroblasts that are capable of rapid regeneration. Recently, we have seen that by comparing fetal skin fibroblasts to adult that there are 900 different genes that are altered between these populations when statistically restricted at 0.001 (5). Perhaps by looking at the more extreme differences between fetal skin fibroblasts and adult at old ages we will be able to isolate a possible candidate marker similar to that found in the epidermis. Indeed, we have identified a significant difference between fetal and older cells with nine genes of the TGF-β superfamily (manuscript in preparation). Genes were either up- or downregulated depending on the biological function, such as TGF-β1, which is highly implicated in scar formation in wound healing (13) and for which the gene expression is approximately 7 times higher in young and old skin cells compared to fetal skin cells. This difference in TGF-β1 gene expression may participate in improved wound healing. It is unknown why the fetal cells are much more resistant to oxidative stress. They may be more efficient in scavenging potential damaging free radical intermediates or perhaps they are more efficient in processing and repairing oxidative damage to critical cellular targets. For certain antioxidants that we have seen to be very important for protection in human skin cells (heme oxygenase and ferritin) (2,3), we do not see significant differences constitutively or inducible between fetal, young, and old skin cells. Interestingly, in preliminary studies looking at proteins by 2D gel analysis in fetal cells in low and high passages, we have been able to identify several proteins that change dramatically and they are implicated in oxidative stress functions (16). Fetal skin cells have proven to be resistant to physical and oxidative type stresses, which seems to afford a great stability to these cells. Their proliferation capacity has been optimized such that very large numbers of skin cells can be frozen in processes to assure their consistency for therapeutic usage.

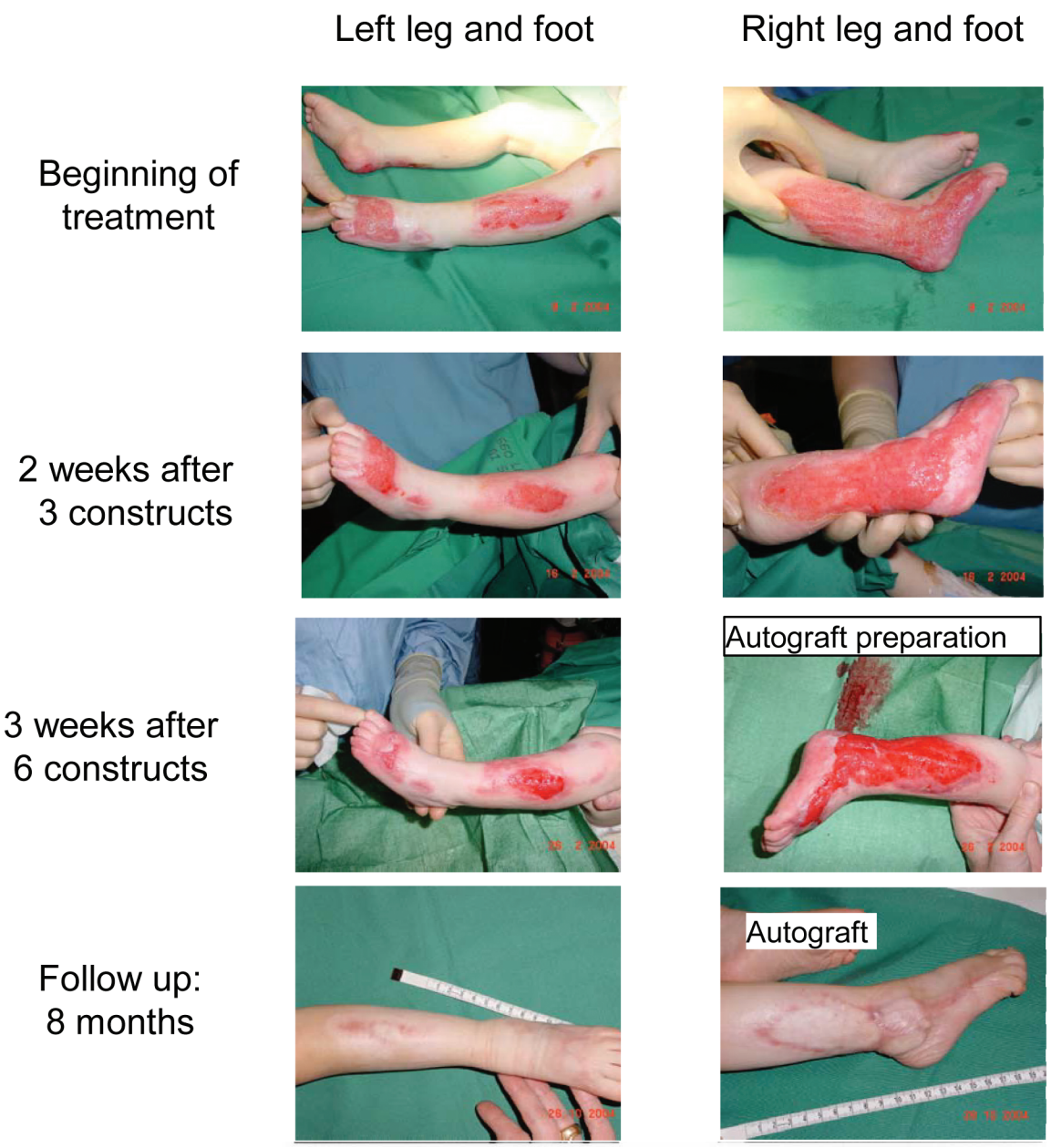

Figure 6. Male 18-month-old boy burned with water on both feet and legs producing a deep second degree burn. Right and left legs and feet were treated with fetal skin constructs at every bandage change (every 48 h) during a 20-day period when, at this time, the child presented with a staphylococcus infection. For unclosed surface, a partial thickness autograft taken from the abdomen was utilized. There are distinct divisions on the right foot and lower leg and in the middle of the wound on the upper left leg of this patient where fetal constructs were used for closure compared to the autograft sites.

The dermal fibroblast is mandatory for tissue reconstruction in deep wounds. It is known from clinical experience that when skin grafts are performed on wound sites such as burns it is preferable that the epidermal keratinocytes have associated dermal fibroblasts to provide better wound healing (12). As we have previously shown for burns (10), we have shown herein that only fetal dermal fibroblasts are necessary to repair other types of wounds in a one-step, nonsurgical procedure without the need for additional autografting techniques. These results clearly mark the difference between our developed technique and other industrial products already on the market using neonatal dermal fibroblasts where a two-step surgical procedure is mandatory and

autografting is still necessary (Dermagraft™, Allodermm™, Hyalograft 3D™, etc.) (11). Additionally, our fetal skin constructs have an advantage for their rapid production (48-h culture period). For reconstructed skin products (fibroblasts and kerotinocytes) such as Apligraft™, Orcel™, and Polyactive™, 6–8 weeks of cell culture is necessary to prepare the final product (11). Short culture spans are of high interest not only for cost but also for safety and contamination risks. Fetal skin cells clearly provide a cell type of interest for industrialized processing and clinical use.

Clinical application of fetal skin constructs to date has shown that these tissue-engineered products can be applied in very diverse situations where normally the autograft technique is employed because it is the golden standard. Using fetal skin constructs for burns and wounds has shown that autografting can be completely avoided and the quality of skin obtained following repair is of very high quality. A major improvement in the speed and quality of skin repair was seen as opposed to techniques of traditional mesh or split grafting or autografting with cell cultures used to date. New skin showed rapid repair with high elasticity, improved color, and no hypertrophic granulation tissue or cheloid formation. This was clearly illustrated in our one patient that was subjected to the two techniques in parallel due to his unfortunate staphylococcus infection occurring in the middle of the treatment protocol. Also, in the majority of situations, no hospitalization is necessary as would usually be the case for the autografting technique. By using fetal skin cells, we are able to further optimize safe, consistent, and effective cell therapies for our patients. Fetal skin cells have an innate stability and resistance to oxidative stress that prepares them for hostile wound environments. Due to their particularly interesting growth characteristics, consistent and safe cell banks can be produced, providing a simple, rapid, and efficient therapeutic treatment of wounds and burns of all nature.

ACKNOWLEDGMENTS: These studies were funded by the Foundation of Gynecology and Obstetrics of Switzerland and matrix substrates for clinical use (Baxter, TissueFleece) were kindly provided by Baxter, Switzerland and Neocutis, SA (spin-off company of the University Hospital of Lausanne, Switzerland).

REFERENCES

1. Applegate, L. A.; Frenk, E.; Gibbs, N.; Johnson, B.; Ferguson, J.; Tyrrell, R. M. Cellular sensitivity to oxidative stress in the Photosensitivity Dermatitis/Actinic Reticuloid Syndrome. J. Invest. Dermatol. 102:762–767; 1994.
2. Applegate, L. A.; Noël, A.; Vile, G.; Frenk, E.; Tyrrell, R. M. Two genes contribute to different extents to the heme oxygenase enzyme activity measured in cultured human skin fibroblasts and keratinocytes: Implications for protection against oxidative stress. Photochem. Photobiol. 61:285–291; 1995.
3. Applegate, L. A.; Scaletta, C.; Fourtanier, A.; Mascotto, R.; Seité, S.; Frenk, E. Expression of DNA damage and stress proteins by UVA irradiation of human skin in vivo. Eur. J. Dermatol. 7:215–219; 1997.
4. Armstrong, J. R.; Ferguson, M. W. J. Ontogeny of the skin and the transition from scar free to scarring phenotype during wound healing in the pouch young of the marsupial *Monodelphis domestica*. Dev. Biol. 169:242–260; 1995.
5. Burri, N.; Federici, E.; Hohlfeld, P.; Scaletta, C.; Applegate, L. A. Genomic analysis of human fetal and old skin cells. 2nd World Union of Wound Healing Societies Congress, Paris; 2004.
6. Crombleholme, T. M.; Langer, J. C.; Harrison, M. R.; Zanjani, E. D. Transplantation of fetal cells. Am. J. Obstet. Gynecol. 164:218–230; 1991.
7. Dang, C.; Ting, K.; Soo, C.; Longaker, M. T.; Lorenz, H. P. Fetal wound healing. Current perspectives. Clin. Plastic Surg. 30:13–23; 2003.
8. Erch, J.; Stallmach, T. Assessing gestational age from histology of fetal skin: An autopsy study of 379 fetuses. Obstet. Gynecol. 94:753–757; 1999.
9. Gabbianelli, M.; Boccoli, G.; Cianetti, L.; Russo, G.; Testa, U.; Peschle, C. HLA expression in hemopoietic development. Class I and II antigens are induced in the definitive erythroid lineage and differentially modulated by fetal liver cytokines. J. Immunol. 144:3354–3360; 1990.
10. Hohlfeld, J.; de Buys Roessingh, A.; Hirt-Burri, N.; Chaubert, P.; Gerber, S.; Scaletta, C.; Hohlfeld, P.; Applegate, L. A. Tissue engineered foetal skin constructs to avoid autografting for burns. Lancet 366:840–842; 2005.
11. Horch, R. E.; Kopp, J.; Kneser, U.; Beier, J.; Bach, A. D. Tissue engineering of cultured skin substitutes. J. Cell. Mol. Med. 9:592–608; 2005.
12. Jones, I.; Currie, L.; Martin, R. A guide to biological skin substitutes. Br. J. Plastic Surg. 55:185–193; 2002.
13. Lin, R. Y.; Sullivan, K. M.; Argenta, P. A.; Meuli, M.; Lorenz, H. P.; Adzick, N. S. Exogenous transforming growth factor-beta amplifies its own expression and induces scar formation in a model of human fetal skin repair. Ann. Surg. 222:146–154; 1995.
14. Lorenz, H. P.; Longaker, M. T.; Perkocha, L. A.; Jennings, R. W.; Harrison, M. R.; Adzick, N. S. Scarless wound repair: A human fetal skin model. Development 114:253–259; 1992.
15. Sandulache, V. C.; Dohar, J. E.; Hebda, P. A. Adult–fetal fibroblast interactions. Effects on cell migration and implications for cell transplantation. Cell Transplant. 14:331–337; 2005.
16. Vuadens, F.; Crettaz, D.; Scaletta, C.; Servi, E.; Quadroni, M.; Schneider, P.; Hohlfeld, P.; Applegate, L. A.; Tissot, J-D. Plasticity of protein expression during culture of fetal skin cells. Electrophoresis 24:1281–1291; 2003.
17. Wong, L. Medical Research Council Tissue Bank. In: Report of the human fetal tissue transplantation research panal, Vol. II. Bethesda, MD: National Institutes of Health; 1988:D267–D282.
18. Yang, A.; Schweitzer, R.; Sun, D.; Kaghad, M.; Walker, N.; Bronson, R. T.; Tabin, C.; Sharpe, A.; Caput, D.; Crum, C.; McKeon, F. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398:714–718; 1999.