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Vitiligo: Repigmentation with Cultured Melanocytes after Cryostorage

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We have developed a technique through which we can multiply melanocytes in culture from a small specimen of normally pigmented buttock skin and reimplant them into depigmented sites of vitiligo. To date, 90 patients have benefited in our hands from such autologous transplantation of pigment cells. Often we have an excess of cells which we would like to store for later use in the event a patient requires further treatment. We report here on 4 cases in which we have cryostored cultured melanocytes for 6–12 months, reimplanted them into vitiliginous sites of the donor after one week of reculture, and obtained optimal repigmentation. We now routinely freeze melanocytes left over after treatment. ***Key words: pigment cell transplantation; bFGF; ultrastructure.***

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In vitiligo, the melanocytes are destroyed and these areas of the skin turn white. Vitiligo affects ~ 1% of the general population and spares no race, sex or age. Most patients with vitiligo are alarmed at the disfiguring lack of skin colour and are concerned that the depigmented areas easily get burned when exposed to the sun. The patients are also frustrated with treatments that do not have much relief to offer (1, 2).

From a small specimen of normally pigmented buttock skin an increased number of melanocytes was obtained in culture and reimplanted into depigmented sites (3, 4). Encouraging results have been obtained in 90 vitiligo patients by transplantation of such cultured melanocytes.

When we have an excess of pigment cells it is of practical importance to be able to store them for later use. To have cryostored cells is especially valuable when one wants to repigment areas that were incompletely repigmented after the first transplantation; when it is initially preferred to repigment some trial spots before more extensive surgery is performed; or when for some reason an operation has to be interrupted or postponed. Left over cells are also useful in case the disease flares up again.

Here we report on cryostored cultured melanocytes which were implanted into vitiliginous areas of the donors after 6–12 months in a freezer and one week of reculture.

MATERIALS AND METHODS

Patients

The transplantations were performed on 3 men and 1 woman, of Scandinavian descent (ages 19–28 years) with generalised vitiligo of 6–13 years' duration, that had progressed over 10–20% of the body. A normally pigmented area of 2×2 cm^2 in the gluteal region was anaesthetized with 5 mg/ml lidocaine without epinephrine and superficially shave-biopsied as earlier described (3, 4).

Cell culture

The melanocytes were released from the biopsy specimen by incubation in 0.25% trypsin/0.1% EDTA/MEM (ethylene-diamine-tetra-acetic acid in Joklik's modified minimal essential medium, Sigma Chemical Co., St. Louis, MO) and cultured in PC-1 serum-free medium without phorbol ester or pituitary extract. Culture method and medium were as earlier described (3, 4).

The cultures were grown for 4 weeks, with medium renewed every third day. Following the addition of 55 μg/ml geneticin (G418 sulfate, Gibco Laboratories Life Technologies, Inc., Grand Island, NY) in one case from day 10–13, in order to kill fibroblasts (6), they yielded 100% melanocytes. No geneticin was used in the other 3 cases, which gave approximately 5–10% fibroblast incorporation. The overall morphology of the cultures and the ultrastructure of the melanocytes are shown in Figs. 1 and 2.

Electron microscopy of cultured melanocytes

Monolayers were fixed *in situ* in 1% formaldehyde/1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer, 30 min at room temperature, washed, kept over night at 4°C in 0.1 M cacodylate buffer, pH 6.8, containing 0.1% L-DOPA (L-dihydroxyphenylalanine, Sigma), then incubated in fresh DOPA solution for 4 h, 37°C, osmicated in the presence of 1.5% potassium ferrocyanide for 30 min, dehydrated in ethanol and embedded in Epon. Ultrathin sections were cut parallel to the growth surface with a diamond knife and counterstained with lead and uranyl ions.

Freezing of melanocytes

The melanocytes were lifted from the culture flask with 3 ml of the above trypsin solution per 150-cm^2 culture flask. Immediately after cell detachment, the trypsin was neutralised with 3 ml undiluted newborn-calf serum (Gibco) per flask, the contents of the flask were transferred to a 15-ml test tube, and the cells centrifuged into a pellet. The pellet was resuspended in 1 ml cryoprotectant for each 10 million cells. The cryoprotectant solution consisted of 8% dimethyl sulphoxide (DMSO, Mallinckrodt, Inc., Paris KY) in undiluted newborn-calf serum. Cells and protectant were mixed by gentle pipetting and transferred to a 1.8-ml cryotube. The tube was put on ice for 10 min to allow the DMSO

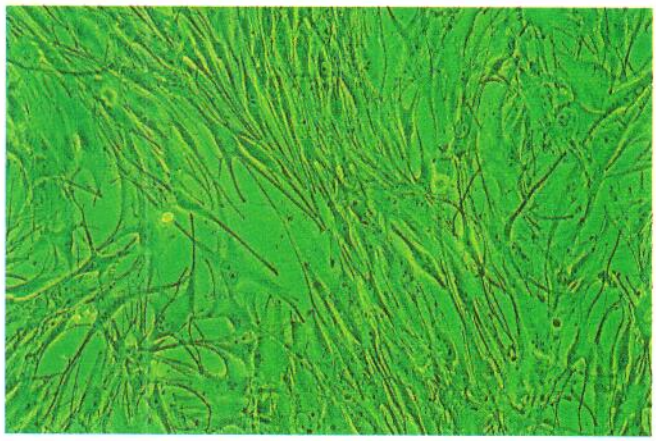

Fig. 1. Melanocytes *in vitro*. Photomicrograph (phase) of culture *in situ* at 3 weeks of growth in culture.

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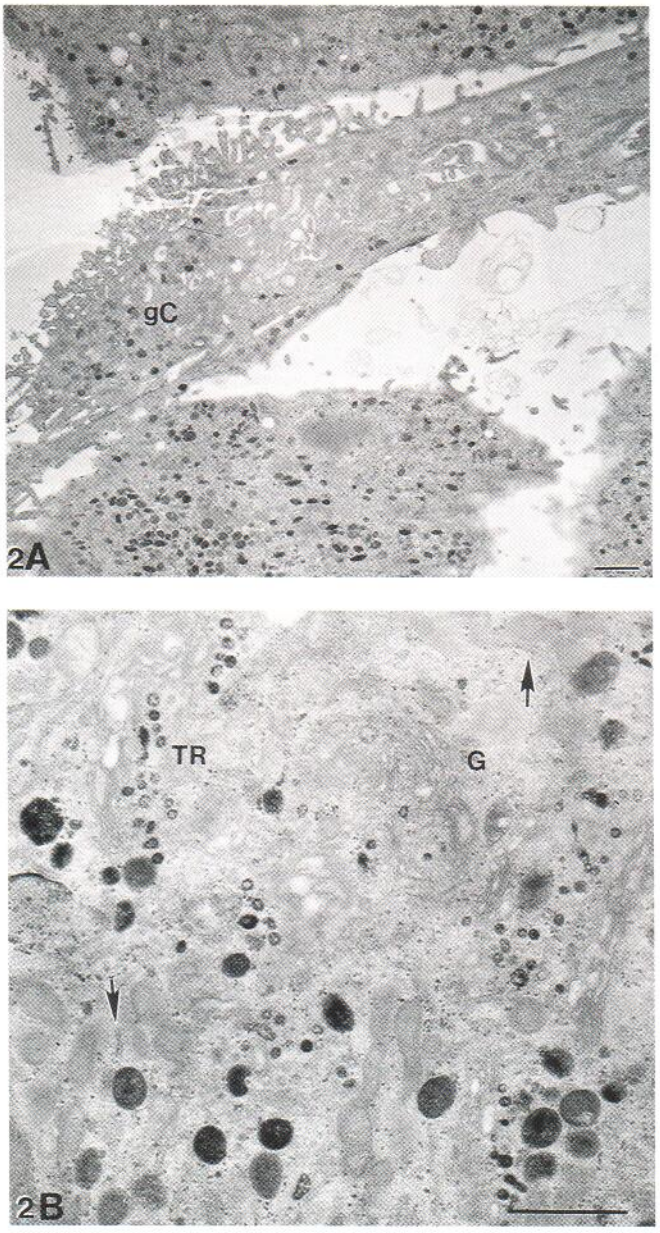

Fig. 2. Electron micrographs of cells *in situ* after DOPA reaction, at 4 weeks of growth in culture. (A) melanocytes show differentiated cytoplasm with melanosomes (electron-dense, *black dots*) and a growth cone (*gC*); (B) cell body with morphologically normal endoplasmic reticulum (*arrowheads*) and Golgi apparatus (*G*), DOPA-reactive trans-Golgi reticulum (*TR*) and melanosomes. Scale bars: 1 μm.

to penetrate the cells and then placed in a −70°C freezer for freezing and storage.

Defrosting

After 6–12 months' storage at −70°C, the cells were defrosted by placing the cryotube directly in a 37°C-bath of sterile distilled water. Immediately after the content had thawed (~1–2 min), it was transferred to a test tube containing PC-1 medium and centrifuged. The supernatant was removed by pipette and the cell pellet carefully resuspended in culture medium, and transferred to a culture flask. Special care in handling is required while the cells are in cryoprotectant. The melanocytes were cultured for one week with two medium renewals in order to give them a chance to recover from the damage of freeze/thawing and to wash away residual DMSO. The survival rate of the defrosted cells was around 70%.

Preparing cells for transplantation

Immediately prior to the transplant procedure, the cultures were lifted with trypsin/EDTA as above; the trypsin was neutralized with soybean extract (Sigma); the cells were centrifuged and resuspended and washed twice with MEM. Finally they were resuspended in a small volume of PC-1 medium, 0.1 ml/1 × 10^6 cells, and taken to the patient's bedside.

Preparing transplant sites

In one patient two recipient areas extended over 20 cm^2 in the right inguinal region (Fig. 3A) and 10 cm^2 on the medial aspect of the right arm (Fig. 3B). The areas were cleaned with 70% ethanol, anaesthetized with Fluor-Ethyl spray (Gebauer Pharmaceutical Preparations, Cleveland, OH). In the other 3 patients the transplant sites were anesthetized with EMLA-cream (Astra, Sweden), under plastic occlusion for 2–3 h. The areas covered (4–200 cm^2) were situated on the face, back, legs, feet, hands and fingers. The epidermis was removed by superficial abrasion with a high-speed dermabrader (25,000 rpm) fitted with a regular diamond fraise wheel. The goal was to remove the epidermis to the dermal-epidermal junction, the level to which melanocytes home during skin development and wound healing, but not deeper to prevent scarring.

Transplantation and care of transplant site

The melanocyte suspension was applied to the denuded areas from a small syringe to an estimated density of 1,200 cells/mm^2, as described before. The transplant was secured with dacron netting (Millipore, Bedford, MA) or Delnet (Frastec, Bronx, NY), covered with gauze moistened with PC-1 medium, followed by Tegaderm dressing (3 M Medical Surgical Division, St. Paul, MN). The patients remained in bed for ~10 h. After one week the moist dressing was replaced by a dry one, which was worn for another week. Three days after the removal of the second dressing, the patients began to expose the treated areas to natural sunlight, without using sun screen but restricting the exposure during the first 2 weeks to 15 min every other day.

RESULTS

The areas transplanted with previously frozen and recultured cells repigmented, as did the areas treated with non-frozen cells. Pigmentation was seen after 2 weeks, and after another 2–3 weeks the entire transplanted areas were pigmented, as illustrated in Figs. 3C, D. During the first year some areas were hyperpigmented, but the colour more and more matched the patient's natural skin colour and after a year it was almost perfect.

DISCUSSION

The success of this procedure is due in large part to the healthy state of the cells in primary culture, before freezing. None of the abnormal features described for human and murine vitiligo melanocytes *in vitro* (7, 8), such as melanophagosomes and dilated endoplasmic reticulum, are evident in our vigorously proliferating cultures (Fig. 1).

The survival ratio of ~70% after cryostorage can probably be increased by finding a more optimal cryoprotective, more optimal speed of freezing for this cell type, and immersion storage in liquid nitrogen. We now save all left-over cells with an eye to defrosting and transplanting them, if and when the patient advises us of newly developed leukodermic spots.

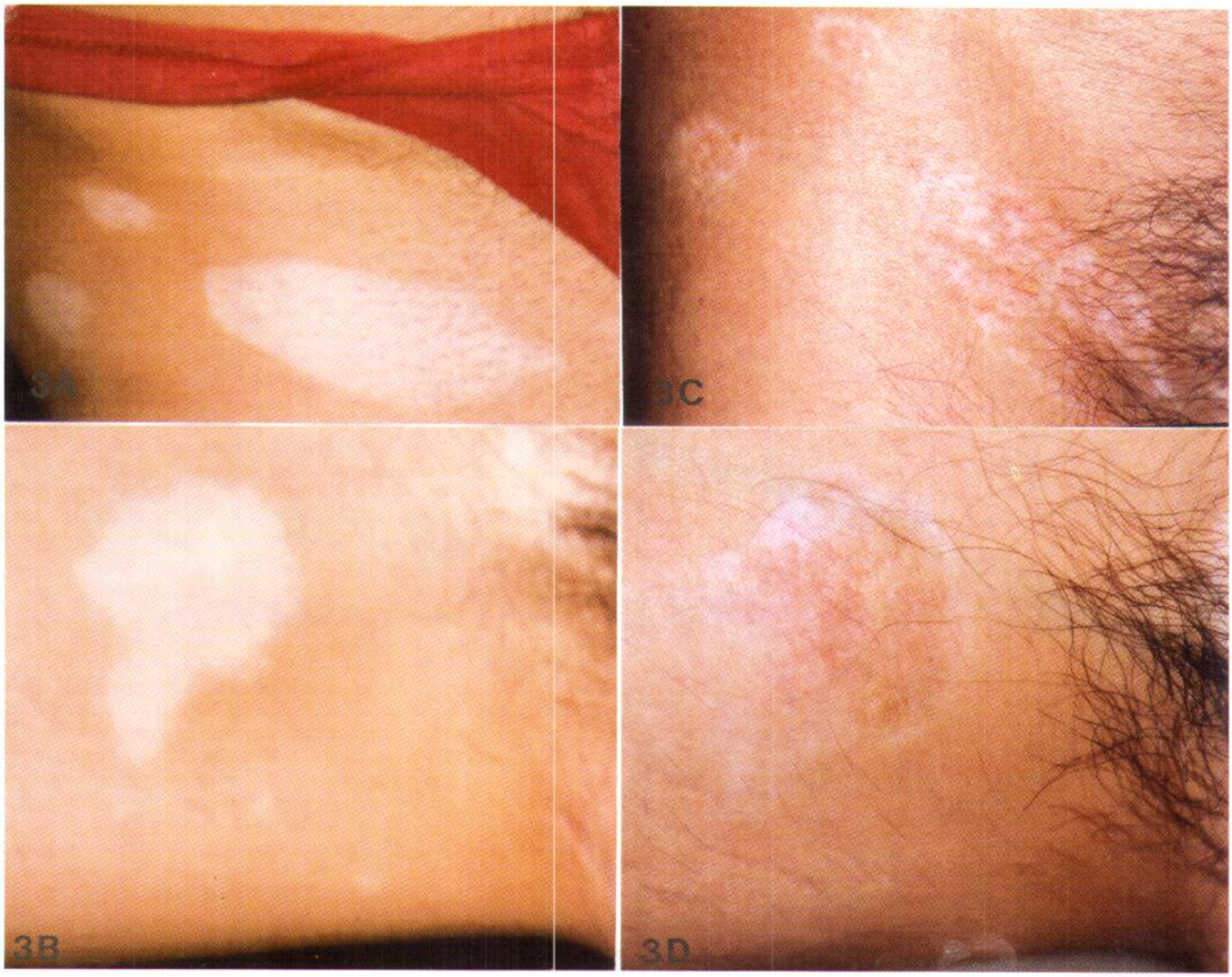

Fig. 3. Recipient sites before and one year after melanocyte transplantation. Right inguinal region (A) and right arm (B) before treatment; (C, D) the same sites 12 months after transplantation of melanocytes that had been multiplied in culture for 4 weeks, cryopreserved for one year, and recultured for one week.

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