J Artif Organs (2001) 4:353–356

ORIGINAL ARTICLE

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Tissue-engineered skin using aggregates of normal human skin fibroblasts and biodegradable material

Abstract Higher-density inoculation of fibroblasts into a three-dimensional scaffold should accelerate wound healing after skin implantation. This study attempted to develop tissue-engineered skin with a higher density of fibroblasts. We first attempted to fabricate three-dimensional high-cell-density aggregates (spheroids) of normal human fibroblasts for application to tissue-engineered skin. Our method consisted of rotational shaking with nontreated dishes, decreasing fibroblast-material interactions, and augmenting cell-cell interaction. To prompt aggregate formation, the medium was supplemented with insulin, dexamethasone, ascorbic acid, and basic fibroblast growth factors that potentiate secretion of extracellular matrices. Under such improved conditions, fibroblasts were able to form spheroidal aggregates within 24 to 36 h of rotational culture. Although the formed aggregates were irregular in shape and were composed of only several cells after 12 h, they became almost spheroidal after 24 h. The aggregates grew even more round after 36 h, and their surfaces became smooth. After 36 h of rotational culture, the fibroblast aggregates were collected and reinoculated onto a biodegradable mesh composed of polyglycolic acid coated with collagen. The aggregates were trapped in the material and became attached after 24 h. Finally, because transforming growth factor-β_3 (TGF-β_3) is known to accelerate wound healing, we conducted a semiquantitative analysis of TGF-β_3 mRNA in both the fibroblast monolayers (two-dimensional culture) and the aggregates (three-dimensional culture). Analysis of TGF-β_3 mRNA expression showed that mRNA expression was greater in the fibroblasts of aggregates than in a monolayer. Therefore, our newly developed dermal graft is expected to accelerate wound healing faster than conventional grafts.

Key words Normal human skin fibroblast · Biodegradable polymer · Tissue-engineered skin

Received: December 4, 2000 / Accepted: March 22, 2001

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Introduction

Dermagraft is a tissue-engineered skin product that is manufactured by growing newborn human dermal fibroblasts on a degradable three-dimensional scaffold. Dermagraft has been designed to replace this dermal layer and potentiate the normal wound-healing process, because the fibroblasts on the degradable scaffold secrete a mixture of growth factors and matrix proteins.[1–5] The fibroblasts accelerate the maturation of the dermal layer and the migration of keratinocytes into the dermal layer. However, when a degradable three-dimensional scaffold with human dermal fibroblasts was transplanted to a mouse, the rate of skin regeneration was not fast. Therefore, the development of an advanced and improved version of Dermagraft was expected.

To improve the regeneration of dermis-like tissue in tissue-engineered skin products, an attempt using growth factors was reported.[6] This attempt clearly demonstrated the efficacy of the material, but there is a drawback in that almost all growth factors, such as the basic fibroblast growth factor, are expensive.

In contrast, as mentioned above, the growth factors released from fibroblasts promote wound-healing processes,

including keratinocyte migration and growth, and neovascularization. Therefore, we theorized that an increase in the density of the fibroblasts in a tissue-engineered skin product would increase the probability of further accelerating skin wound-healing processes when it was implanted in patients.

A cell aggregate in the shape of a spheroid has a three-dimensional structure with a high cell density. If the aggregate can be applied to an artificial skin product, the cell density of the product should be increased. This paper reports on two such attempts. First, we attempted to make aggregates of normal human skin fibroblasts using our original method, which was established to make aggregates of hepatocytes.[7–9] Next, we attempted to make a new type of tissue-engineered skin with an enhanced fibroblast density by inoculating the fibroblast aggregates into a biodegradable polymer mesh.

Materials and methods

Cell culture

Normal human fibroblasts derived from neonatal dermal skin tissue were incubated in DMEM (Dulbecco's modified Eagle medium) supplemented with 2% heat-inactivated (v/v) fetal bovine serum (FBS) in 5% CO_2–95% air. After reaching confluence, the fibroblasts were harvested by treating with 0.05% trypsin-0.01% ethylenediaminetetraacetiz acid (EDTA) solution.

Suspension culture

As a basal medium for a suspension culture, a modified MCDB202 medium was additionally supplemented with 2% FBS, insulin (10^{-6}M), dexamethasone (10^{-5}M), and basic fibroblast growth factor (1ng/ml). These factors, which potentiate the secretion of extracellular matrices, were used so that aggregate formation could be prompt.

Single prepared cells were finally resuspended at 1.66×10^6 cells/ml with the medium for a rotational culture. Five milliliters of the suspension was conducted into a dish (diameter, 35mm) fabricated for nonadhesive cell culture. The shaking speed was set at 80rpm, so that the cells would be distributed in the center of the dish. The dishes were placed in a humidified CO_2 incubator. After the aggregates formed, they were reinoculated into a culture dish and a scaffold composed of polyglycolic acid (Vycryl, Ethicon, NJ, USA; diameter 15mm) coated with type I collagen (1.0mg/ml–500μl) and placed in a six-well plate (diameter, 35mm).

Analysis of gene expression

Fibroblasts composed of monolayers (control) and of aggregates were harvested in isogen. These were left at room temperature for 5min to dissolve the cells into the isogen. After 0.2ml of chloroform was added, the samples were vigorously shaken for 15s and allowed to stand for 2min at room temperature. Centrifugation at 12000×*g* for 15min then induced an aqueous phase, which included RNA. After 0.5ml of isopropanol was added, the samples were stored for 5 to 10min at room temperature, then centrifuged for 10min at 4°C (12000×*g*). The precipitates were resuspended in at least 1ml 70% ethanol and centrifuged for 5min at 4°C (7500×*g*). The precipitates were briefly dried and then dissolved in water. The total RNA obtained was reverse-transcribed into single-stranded complementary DNA (cDNA) using superscript reverse transcriptase according to the manufacturer's instructions. The cDNA was used as template DNA for amplification by polymerase chain reaction (PCR). PCR primers for transforming growth factor-β_3 (TGF-β_3) were purchased from Ambion (TX, USA). We designed primers for human G3PDH. The PCR conditions for both sets of primers consisted of an initial denaturation step of 94°C for 5.0min, followed by 34 cycles of denaturation at 94°C for 0.5min, annealing at 58°C for 1.5min, and extension at 72°C for 1.0min. In the final step, the extension time of the DNA step was 10min. The PCR products were separated on a 2% Tris-borate/EDTA buffered agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Results

Formation of aggregates of normal human skin fibroblasts

We first attempted to make spheroids of normal human dermal fibroblasts using our original culture system. When we used dishes with low levels of protein retention and cell adhesion, normal human skin fibroblasts began to aggregate within a few hours. The general shape of the aggregates was irregular, rather than smooth. The diameters of the aggregates increased over time, and the surrounding shapes became smoother. After 24 to 36h of rotational culture, smooth-shaped aggregates were formed, as shown in Fig. 1. On the other hand, when we used normal culture dishes for tissue culture, aggregates of fibroblasts could not form. Because the adhesive force of the fibroblasts was strong, almost all fibroblasts adhered to the surface of the dishes (data not shown).

Inoculation of aggregates into the scaffold

When the fibroblast aggregates were completely formed in the suspension culture system, we inoculated the aggregates into a scaffold composed of polyglycolic acid coated with type I collagen. To increase the frequency of contact between the aggregates and the scaffold, we used medium volumes of 1.5 to 2.0ml in a six-well dish. The dish with the fibroblast aggregates and the scaffold was put on the same shaker that was used to form the aggregates. This action trapped the aggregates in the mesh of the polyglycolic acid, and the aggregates attached to the mesh within 24h. Al-

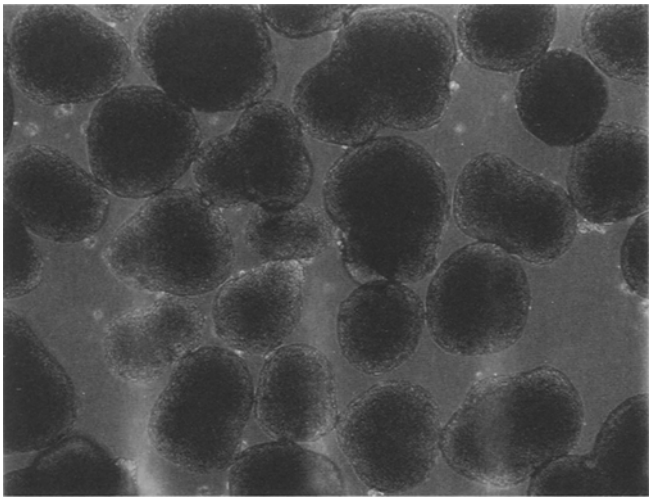

Fig. 1. Formation of normal human skin fibroblasts by suspension culture

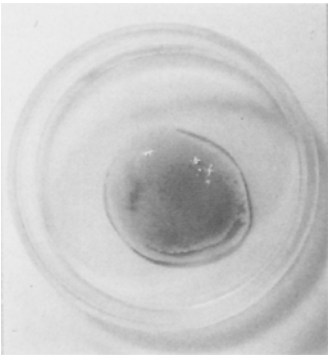

Fig. 2. Tissue-engineered skin composed of aggregates and biodegradable material

though the aggregates of 10 wells were inoculated into one scaffold, almost all of the aggregates were adhered within 24 h, as shown in Fig. 2.

Expression of mRNA for TGF-β_3 and G3PDH

The expression of TGF-β_3 mRNA in normal human fibroblasts of aggregates was compared with that in monolayers, by employing reverse transcription (RT)-PCR. The expression of G3PDH mRNA as a housekeeping gene in both fibroblasts was also analyzed for a standard. As shown in Fig. 3, the mRNA expression of TGF-β_3, one of the major growth factors related to wound healing,[10,11] increased in fibroblasts of aggregates in contrast to that of a monolayer in the three independent experiments.

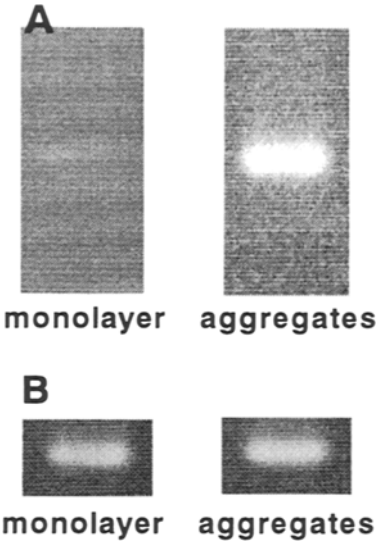

Fig. 3. Expression of mRNA in human fibroblasts in aggregate and monolayer by RT-PCR methods (*left lane*, control; *right lane*, aggregates). **A** TGF-β_3, **B** G3PDH

Discussion

It has been reported that it is not easy to organize normal fibroblasts into spheroidal aggregates. In this paper, we report our formation of aggregates of normal human skin fibroblasts using a rotational culture. When the same conditions as those of hepatocytes were used, however, it was difficult to form aggregates of human fibroblasts, because the adhesion of normal human skin fibroblasts to the dish is strong. Therefore, we changed the material of the vessel used to form the aggregates from a glass dish coated with silicone to a plastic dish fabricated for nonadhesive cell cultures. In addition, to increase cell–cell interaction (and at the same time, to decrease cell–substrate interaction), the shaking speed was set at 80 rpm, so that the cells were distributed in the center of the dish. Although Dai and Saltzman[12] attempted to form aggregates of a mouse fibroblast cell line in a suspension culture, they were not successful under the conditions they employed. Subsequently, they reported that the addition of water-soluble conjugates of cell adhesion peptides containing a sequence of three amino acids, Arg-Gly-Asp (RGD), and poly(ethyleneglycol) (PEG) did result in the formation of aggregates. In our experiments, however, we were able to form aggregates of normal fibroblasts in a suspension culture without the addition of water-soluble conjugates of cell adhesion peptides. As compared with the experimental conditions of Dai and Saltzman, our research differed in many points, including cell density, rotation speed, substrate for formation of the aggregates, method used to load shear to the cells (rotational culture vs. spinal flask), and medium (to promote cell–cell interaction, we supplemented the medium with insulin, dexamethasone, and basic fibroblast growth factor).

Takezawa et al.[13,14] made an aggregate of normal adult human dermal fibroblasts by using a thermoresponsive polymer composed of poly-*N*-isopropyl acrylamide (PNIPAAm) and type I collagen. On the basis of characteristics of the human normal skin fibroblasts in the aggregates, they suggested that aggregates of normal human skin

fibroblasts are good in vitro experimental models reflecting the in vivo status of cells in living tissues or organs. Therefore, it was thought that the aggregates of normal human skin fibroblasts that we formed by rotational culture would not only become tissue-engineered skin, but also might be a good in vitro experimental model as an alternative to animal experiments.

Because fibroblasts release many kinds of cytokines, such as growth factor, a high-density inoculation of fibroblasts might accelerate wound healing. In our experiment, when aggregates of fibroblasts were inoculated into a polyglycolic mesh, a large number of fibroblasts (with a minimum density of 4.0×10^6 cells/cm^2) adhered to the material. In addition, the expression of TGF-β_3 mRNA, a cytokine related to wound healing, increased in fibroblast aggregates compared to that in a monolayer. Therefore, when aggregates of fibroblasts were used as tissue-engineered skin, the final amount of TGF-β_3 existing and accumulating in the skin would become much greater than that in a monolayer. Thus, our tissue-engineered skin with aggregates of fibroblasts is expected to accelerate the healing of wounds involved in skin transplantation.

Acknowledgments This work was partly supported by a Grant for the Research for the Future Program (JSPS-RFTF96I00202) from the Japan Society for the Promotion of Science (JSPS) and by a grant from the Japanese Ministry of Education, Science, and Culture.

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