Use of synthetic serum-free medium for culture of human dermal fibroblasts to establish an experimental system similar to living dermis

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Received: 7 October 2013 / Accepted: 19 February 2014 / Published online: 1 March 2014
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Abstract In this study, we sought to establish a defined experimental system for fibroblast growth similar to that of the living dermis. To this end, we evaluated the growth and biochemical characteristics of fibroblasts cultured with serum-free HFDM-1, a finely tuned synthetic medium for human fibroblast culture. Three culture conditions were used to grow fibroblasts obtained from primary culture: (1) culture with Dulbecco's modified Eagle medium (DMEM) plus 10 % fetal bovine serum (serum-supplemented DMEM), (2) culture with DMEM (serum-free DMEM), and (3) culture with HFDM-1 (HFDM-1), and fibroblast morphology, growth, collagen type I production, and lipid composition were analyzed. Fibroblasts grown in HFDM-1 maintained cell numbers at nearly 100 % from days 14 to 21 and produced more collagen type I than cells grown in serum-supplemented and serum-free DMEM. Arachidonic acid (20:4) and total polyunsaturated fatty acids were lower in cells grown in serum-free DMEM and HFDM-1 than in serum-supplemented DMEM. These results suggested that HFDM-1 recapitulated growth conditions in the dermis better than traditional, serum-supplemented DMEM. In addition, the controlled chemical composition of HFDM-1

eliminated a potential source of variability in cell culture conditions.

Keywords Fibroblast · HFDM-1 · Lipid analysis · Serum-free medium · Synthetic medium

Introduction

Fibroblasts are a cell type found between collagenous fibers and elastic fibers in the dermis and are critical for basic research of hypertrophic scars, keloids, wound healing, and skin diseases. Currently, Dulbecco's modified Eagle medium (DMEM) plus fetal bovine serum (FBS) is usually used for human fibroblast culture (Rittié and Fisher 2005). An autologous serum can be used in place of FBS (Itoh and Hoshi 2005; Yamada et al. 2006; Nishi et al. 2010; Morimoto et al. 2011). Fibroblasts cultured with serum have a high proliferative capacity, similar to that of keloids; however, human fibroblasts in living dermis rarely proliferate and maintain homeostasis of the dermis through collagen production. In addition, the exact composition of serum is undefined, and different lots of serum may cause variations in experimental results. Thus, ideal culture conditions for human fibroblasts used in basic research on hypertrophic scars, keloids, and wound healing should have the following characteristics: allow fibroblasts to maintain

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cell numbers; produce collagen, elastin, acid mucopolysaccharide, and protease; allow fibroblasts to maintain their morphology during long-term passage; and be specifically defined.

Several studies have described successful serum-free culture methods for human fibroblasts (Itoh and Hoshi 2005; Nishi et al. 2010; Bettger et al. 1981; Kan and Yamane 1982; Koch et al. 1997). HFDM-1 is a finely tuned synthetic medium for human fibroblast culture. The manufacturers of HFDM-1 have demonstrated that short-term (9 day) culture of fibroblasts in serum-free HFDM-1 and long-term (20 day) culture of fibroblasts in HFDM-1 plus 0.5 % human serum allowed cells to proliferate as well as cells cultured in DMEM plus 10 % FBS (Itoh and Hoshi 2005). Additionally, several investigators have used HFDM-1 as a culture medium (Yamada et al. 2006; Nishi et al. 2010; Takami et al. 2008). However, no studies have described the growth and biochemical characteristics of fibroblasts cultured in serum-free HFDM-1 in detail.

Therefore, in this study, we sought to establish a defined experimental system similar to that in the living dermis by elucidating the biochemical characteristics of fibroblasts grown in this synthetic medium through analysis of cell growth, collagen production, and lipid composition.

Materials and methods

Materials

Triosanoic acid (23:0), used as an internal standard; FBS (from the same lot number for all experiments); trypsin type IX; and trypsin inhibitor type I–S were obtained from Sigma (St. Louis, MO, USA). Porcine pepsin, DMEM, petroleum ether, benzene, methanol, chloroform, and glacial acetic acid were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HFDM-1 was obtained from IFP (Yamagata, Japan). Plastic T-75 flasks and 100-mm dishes were obtained from Asahi Techno Glass Corporation (Funabashi, Japan). Thin-layer silica gel 60 plates were obtained from Merck (Tokyo, Japan).

Normal dermal fibroblasts were obtained from surplus skin samples following surgery at the Department of Plastic Surgery, Kobe University Hospital. All tissue collection procedures were approved by the ethics committee of Kobe University Graduate School

of Medicine in accordance with the Principles of the Declaration of Helsinki.

Methods

Fibroblast culture, morphological analysis, and cell growth analysis

The epidermis of each skin sample was removed with a scalpel, and the remaining tissue was minced into approximately 1×1 mm sections. Nine pieces of the minced dermis were placed on a plastic dish, and a drop of DMEM plus 10 % FBS was placed on each piece. The following day, 10 mL of the medium was added to each dish. Cell cultures were fed every 48 or 72 h until the cells reaches almost 80 % confluence. These procedures were performed using skin samples from nine patients, and nine groups of normal dermal fibroblasts were obtained.

Next, fibroblasts obtained from primary culture were grown under three culture conditions: (1) culture with DMEM plus 10 % FBS (serum-supplemented DMEM), (2) culture with DMEM (serum-free DMEM), and (3) culture with HFDM-1 (HFDM-1). Figure 1 shows the experiment schedule. Between day 0 and day 3, all cells were grown in DMEM only. Media were changed on days 4, 11, and 18. Subculture was carried out on days 7, 14, and 21, and cells were collected on these days for cell growth analyses. On days 7 and 14, medium was collected for analysis of collagen type I production. On day 14, cells were collected for lipid analysis. For morphological analysis, fibroblasts were observed under an Olympus culture microscope (model CK40, Olympus, Tokyo, Japan). For cell growth analysis, the number of cells surviving under different culture conditions was counted using a hemacytometer (Burker-turk line; Erma, Tokyo, Japan). The number of fibroblasts on day 0 was defined as 100 %.

Analysis of collagen type I production in fibroblasts

Enzyme-linked immunosorbent assays (ELISAs) were performed using culture supernatants to detect human collagen type I using a specific ELISA kit (Funakoshi Corporation Ltd, Tokyo, Japan). The concentration of collagen type I was determined by measuring the absorbance of 3,3',5,5'-tetramethylbenzidine (450 nm) using a model 550 microplate reader (Bio-Rad

Fig. 1 Experiment schedule

Laboratories, Hercules, CA, USA). Background levels of collagen type I were measured in each culture medium prior to cell culture. The concentration of collagen type I in each culture supernatant minus that in each medium was normalized to the number of cells; this value was defined as the collagen type I production of a fibroblast.

Lipid analysis

Lipids were extracted by the method published by Folch et al. (1957). In short, cultured fibroblasts were rinsed twice with calcium-free phosphate-buffered saline and scraped into methanol. Cultured fibroblasts were extracted with a 1:2:1.5 ratio of methanol:chloroform:0.1 M KCl in 50 % methanol, and the organic phase was re-extracted with 2.5 volumes of 0.1 M KCl in 50 % methanol. The extracted fraction was evaporated under a nitrogen stream. Samples were divided into two groups: one for whole cell (WC) analysis and one for cell membrane analysis by thin-layer chromatography (TLC). Since the fatty acid composition of each fraction reflects the percent fatty acid amount of all fatty acid-containing lipids in the fraction (Terashi et al. 2000), we then compared the percent fatty acid

composition of each sample in the total lipids extracted from WC and the paired TLC-extracted fraction, both of which reflect fatty acids in cells. In the TLC group, the fraction was suspended in 100 μ L of 1:1 chloroform:methanol after evaporation and was applied to a TLC plate. After TLC of the cellular lipids, the phospholipid-containing area of the plate was scraped, and the material was eluted from the silica during transmethylation with 6 % methanolic HCl. In the WC group, the procedure of applying a TLC plate was omitted. One hundred microliters of triosanoic acid was added at this time as an internal standard, and the sample was heated for 3.5 h at 80 °C to induce formation of fatty acid methyl esters (FAMES). FAMES were resuspended in 5 mL petroleum-ether, evaporated, and resuspended in 100 μ L chloroform for analysis. A total of 0.5 μ L chloroform was used for each analysis.

Twenty-three biological FAMES plus 23:0 were analyzed using a Shimadzu gas chromatograph (GC) model GC-14B (Shimadzu Corporation, Kyoto, Japan). FAMES were eluted with scrubbed helium at a flow rate of 2.79 mL/min at 210 °C for 16 min, at a gradient of 4 °C/min until 220 °C, and then held at 220 °C until 18.5 min. The flame ionization detector output of the GC and data were evaluated with a C-R8A Shimadzu Chromatopac (Shimadzu Corporation).

Statistical analysis

All data were analyzed using Student's *t* tests in case of two groups comparison and ANOVA with Bonferroni correction in case of multiple groups. All the categorical data are presented as mean $\pm$ standard deviation (SD). *p* values <0.05 were considered statistically significant (after applying for Bonferroni correction).

Results

Morphological changes and cell growth of fibroblasts in each medium

Analyses of morphology and cell growth are basic and critical for studies of cultured cells. To determine morphological changes in fibroblasts cultured in each medium, we observed cells under an Olympus culture microscope. As shown in Fig. 2a, b, fibroblasts grown in

Fig. 2 **a** Morphology of the fibroblasts in serum-supplemented DMEM, serum-free DMEM, and HFDM-1 on day 7, 10, and 14. **b** Morphology of the fibroblasts in serum-supplemented DMEM, serum-free DMEM, and HFDM-1 on day 17 and 21

serum-supplemented DMEM did not undergo morphological changes, while fibroblasts grown in serum-free DMEM became atrophic on day 14. Moreover, fibroblasts grown in HFDM-1 maintained a fibroblast-like morphology and seemed to become slightly hypertrophic. Examination of the proliferation of fibroblasts grown in each medium demonstrated that serum-supplemented DMEM allowed cells to maintain cell numbers throughout the experiment (Fig. 3). In contrast, fibroblasts grown in serum-free DMEM exhibited a sharp decrease in cell numbers on day 7, and the majority of these cells died by day 21. Fibroblasts grown

in HFDM-1 exhibited an increase (116.2 %) in cell numbers on day 7, followed by a slight reduction on day 14 to 86.6 % of the cell number on day 0. This cell number was then maintained through day 21 of the experiment. These results indicated that culturing fibroblasts in serum-supplemented DMEM allowed fibroblasts to proliferate in a manner similar to that of tumor cells, while culture in serum-free DMEM led to rapid cell death. In contrast, culture in HFDM-1 allowed fibroblast numbers to be maintained nearly 100 % between days 14 and 21 and therefore may be most similar to conditions in the living dermis.

Fig. 3 Cell growth of the fibroblasts in serum-supplemented DMEM, serum-free DMEM, and HFDM-1. Number of the fibroblasts on day 0 was defined as 100 %, and Y axis was defined as log2 of % of day 0. Error bar means SD (N = 9)

Fig. 4 Collagen type I production per fibroblast cell of fibroblasts in serum-supplemented DMEM, serum-free DMEM, and HFDM-1. The concentration of collagen type I in each sampled culture supernatant minus that in each medium itself divided by the cell number. This value was defined as the collagen type I production of one fibroblast. * $0.01 < p < 0.05$, ** $p < 0.01$; no asterisk means no significant difference (after applying for Bonferroni correction). Error bar means SD (N = 9)

Collagen type I production of fibroblasts grown in each medium

Collagen type I is a classic molecular marker of normal fibroblasts in the dermis. Thus, we next determined the levels of collagen type I production during different culture conditions using ELISA. Fibroblasts grown in serum-supplemented DMEM and serum-free DMEM produced little collagen type I

(Fig. 4). However, fibroblasts grown in HFDM-1 produced significantly more collagen type I than fibroblasts grown in serum-supplemented DMEM and serum-free DMEM. These results indicated that fibroblasts grown in HFDM-1 may have a collagen-producing capacity similar to that of normal dermis fibroblasts.

Fatty acid composition of fibroblasts in each medium

Fatty acids are the main constituents of lipids, which act as sources of energy, components of the cell membrane, and physiologically active signaling molecules (Terashi et al. 2000; Graber et al. 1994). Moreover, lipids are involved in scar formation and wound healing (Louw 2000; Shakespeare and Strange 1981; Balazs et al. 2001; Tachi 1998; Cardoso et al. 2004), and arachidonic acid has been shown to play a role in scar formation (Nomura et al. 2007). Therefore, since lipid analysis can provide insight into changes in cell morphology, proliferation, and differentiation, we compared the fatty acid composition of total lipids from each sample extracted by WC and the paired TLC-extracted fraction. A total of 10 of the 23 fatty acids in WC- and TLC-extracted groups were identified (Fig. 5). From both extractions, arachidonic acid (20:4) and total polyunsaturated fatty acids (PUFAs) tended to be lower in cells grown in serum-free DMEM and HFDM-1 than in cells grown in serum-supplemented DMEM (Fig. 5). These results indicated that culturing cells in serum-supplemented conditions promoted the synthesis of arachidonic acid (20:4) and total PUFAs.

Discussion

Dermal fibroblasts maintain homeostasis of the dermis by producing collagen, elastin, acid mucopolysaccharide, and protease (Ross 1975). In contrast, in serum-supplemented DMEM, the traditional fibroblast growth medium, fibroblasts proliferate at a high rate, similar to that observed in keloid tissue, whereas fibroblasts grown in serum-free DMEM stop proliferating, senesce, and die. Therefore, in vitro studies in fibroblasts may not accurately reflect the phenotypic characteristics of fibroblasts due to nonoptimal culture conditions. HFDM-1 is a finely tuned synthetic medium for human fibroblast culture, made by adding

Fig. 5 Percent compositions of fatty acids of the fibroblasts in serum-supplemented DMEM, serum-free DMEM, and HFDM-1. * $0.01 < p < 0.05$, ** $p < 0.01$; no asterisk means no significant difference (after applying for Bonferroni correction). Error bar means SD. EFA essential fatty acid, TLC thin layer chromatography, WC whole cell (N = 9)

to Eagle's minimal essential medium: HEPES, L-glutamine, lipids, sodium bicarbonate, sodium selenite, gentamicin sulfate, non-essential amino acids, recombinant human insulin, recombinant human epidermal growth factor, dexamethasone, and synthetic polymer (Itoh and Hoshi 2005; Yamada et al. 2006; Morimoto et al. 2011; Takami et al. 2008). It contains no animal-derived components and is thought to promote a normal fibroblast phenotype. However, until now, extensive characterization of primary fibroblasts grown in HFDM-1 had not been performed. In the current study, we compared the growth characteristics and phenotypes of primary fibroblasts cultured in traditional serum-supplemented DMEM, serum-free DMEM, and HFDM-1. Our results suggested that, while serum-supplemented DMEM promoted overgrowth of fibroblasts and serum-free DMEM caused cell death, HFDM-1 promoted fibroblast growth most similar to that observed in the normal dermis.

In this study, we analyzed the production of collagen type I, the main collagen produced by the dermis, as a molecular marker of fibroblasts grown in each medium. DMEM-grown fibroblasts produce no collagen type I, regardless of the presence of FBS.

Interestingly, fibroblasts cultured in HFDM-1 produced much more collagen type I than fibroblasts grown in serum-supplemented DMEM and serum-free DMEM. These results supported that the environment in HFDM-1 may be more similar to those in the living dermis than those in DMEM for cultured fibroblasts. However, it is possible that HFDM-1 may simply increase the capacity for production of collagen type I in cultured fibroblasts due to induction of other phenotypic changes.

Fatty acids are classified into three types: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs. In the human body, SFAs, such as palmitic acid (16:0), are synthesized from glucose and acetate-carbon resources. MUFAs are also produced from precursor SFAs by certain enzymes (Marcelo and Dunham 1993). On the other hand, PUFAs, such as arachidonic acid (20:4), cannot be synthesized by the human body and must be obtained from dietary sources. These are called essential fatty acids (EFAs; Ziboh and Chapkin 1988). Arachidonic acid is an essential source of leukotrienes and prostaglandins (Marcelo and Voorhees 1983; Ziboh 1973) and has a key role in inflammation, but is not detected in normal dermis

(Nomura et al. 2007). Slow-proliferating fibroblasts in serum-free DMEM and HFDM-1 contained less 20:4 and total PUFAs than those grown in serum-supplemented DMEM. Therefore, we hypothesized that the growth and proliferation of fibroblasts is linked to the level of PUFAs, such as arachidonic acid. In a previous report, we demonstrated that normal dermis contained less arachidonic acid and total PUFAs than hypertrophic scars in vivo (Nomura et al. 2007). Taken together, our results suggested that HFDM-1 recapitulated growth conditions in the dermis better than the more traditional medium, serum-supplemented DMEM. In addition, the controlled chemical composition of HFDM-1 eliminates a potential source of variability in cell culture conditions, suggesting that use of HFDM-1 may allow for more consistent, standardized results in primary fibroblast cultures.

In the body under normal conditions, fibroblasts are in a static condition. They grow during wound healing. Really rapid growth is not necessarily the hallmark of a physiological cell maintained in vitro. On the other hand, fibroblasts in matured hypertrophic scar and matured keloid seem to rarely proliferate and produce collagen. Therefore, fibroblasts grown in HFDM-1 may be similar to not only those in the living normal dermis but also those in matured hypertrophic scar and matured keloid. But at least, our results supported that fibroblasts grown in HFDM-1 may be more similar to fibroblasts in the living dermis than fibroblasts grown in serum-supplemented DMEM and serum-free DMEM.

In conclusion, while our data did support that HFDM-1 may facilitate maintenance of the primary fibroblast morphology in cultured primary fibroblasts, fibroblasts cultured in HFDM-1 began to die after day 22, eventually leading to complete cell death (data not shown). Furthermore, some of the fibroblasts grown in HFDM-1 seemed to become hypertrophic by day 21, a characteristic not observed in normal living dermis. This suggested that further improvements to HFDM-1 could provide even more optimal growth conditions for primary fibroblasts. Therefore, since some reports have suggested that addition of specific fatty acids to serum can control cell function in epidermal keratinocyte culture (Garner et al. 1995; Marcelo et al. 1994), the use of HFDM-1 plus fatty acids should be a key to establishing an experimental system more similar to that found in vivo. We already experimented with the use of HFDM-1 plus linoleic acid (18:2),

palmitic acid (16:0), and eicosapentaenoic acid (20:5 [n-3]). Fibroblasts grown in this medium were similar to those in only HFDM-1 in terms of morphology, cell growth, and collagen type I production, but there were some differences in fatty acid composition (data not shown). Further consideration on added materials is needed.

Conflict of interest The authors have no conflict of interest to declare.

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