Concentrated Growth Factors Can Inhibit Photoaging Damage Induced by Ultraviolet A (UVA) on the Human Dermal Fibroblasts In Vitro

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Background: Photoaging is the main cause of extrinsic skin aging. Daily exposure to ultraviolet A (UVA) accelerates the process of photoaging. The present study aimed to understand the role of concentrated growth factors (CGF) on UVA irradiated human skin cells.

Material/Methods: We isolated and subcultured normal human dermal fibroblasts (NHDFs) from 6 different human dorsal skins and established photoaging models of NHDFs irradiated by UVA to detect the influence of CGF on fibroblasts in vitro. Three groups were examined: normal, cellular photoaging model (total dosages of 18 J·cm⁻²), and cellular photoaging model plus CGF. In our study, we used the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay method to measure the cell viability. We also used reactive oxygen species (ROS) assay and superoxide dismutase (SOD) assay to measure respectively the amount of oxygen free radicals and antioxidative enzymes. We compared the migration rates among the photoaging model groups, the control groups, and the CGF-treated culture medium groups that were irradiated.

Results: Our study results indicated that 5% CGF can reduce UVA-induced human skin fibroblasts damage significantly, improve the viability of NHDFs significantly, and largely decrease the UVA irradiation effect (P<0.05). The migration rates of the normal group and the UVA-irradiated NHDFs in the 5% CGF group had significantly increased migration rates (P<0.05), compared to the control medium group. The migration rates of the UVA-irradiated NHDFs in 5% CGF exceed those of the normal group. These results showed that 5% CGF could greatly promote cellular proliferation, migration, and SOD at the same time that the amounts of ROS were markedly decreased.

Conclusions: These experimental findings offer some important insights into CGF’s capacity for scavenging ROS, improving SOD, and increasing migration rates in NHDFs irradiated by UVA.

MeSH Keywords: Antioxidants • Reactive Oxygen Species • Skin Diseases • Superoxide Dismutase • Ultraviolet Rays

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Background

Ultraviolet (UV) radiation is regarded as the reason of extrinsically aging [1]. Based on wavelength, solar UV has 3 main components, including UVA (320–400 nm), UVB (280–320 nm), and UVC (c280 nm). UVA light can penetrate the skin dermis to bring about skin damage, and is considered to be the main wavelength that leads to skin photoaging. Previous studies have argued that UVA has a significant influence on the pathogenesis of photo-dermatoses such as photoaging [2]. Exposure to UVA radiation can bring about several biological phenomena which damage DNA, including oxidative stress, principally through the generation of reactive oxygen species (ROS) [3]. DNA absorbs the UVA radiation, and then UVA reacts with other non-DNA chromophores, bringing about the formation of reactive oxygen species (ROS) which damage lipids, proteins, and DNA in the skin [4]. The generation of reactive oxygen has a negative influence on cells irradiated by UVA radiation. Skin is equipped with an elaborate system of antioxidants and enzymes that maintain the balance between oxidative stress and antioxidant defense [5]. So numerous antioxidants that have the capacity to quench reactive oxygen, have the ability to inhibit the photo-damage in human skin cells. At present, preparations such as herbal preparations (terrestrial herbs and plants), marine products, and blood extracts (platelet-rich plasma) are widely used to repair the photoaging damage. How to prevent skin photoaging is always an interest of dermatology.

Extracts from third generation plasma, i.e., concentrated growth factors (CGF), was first put forward by Sacco in 2006 and is considered a new type of biological scaffold which contains a lot of fibrin and platelets. CGF fibrin gel liquid plays an important role in that it includes several kinds of growth factors and fibrin used in repairing trauma tissue [6]. Not only have clinical experts applied autologous CGF fibrin gel to guide bone regeneration, repair temporomandibular joints, and reconstruct bone defects, but good clinical effect has also been achieved [7–9]. It is known that exposure to UVA has become a public health concern [10,11]. Therefore, attention must be paid to the value of preventing skin photoaging irradiated by UVA. Based on the beneficial effects of CGF in the healing of hard and soft tissue, we suggest that CGF can be developed to become an autologous material for treating photoaged skin. The primary type of dermis cells are fibroblasts in human skin, which are easily affected by UVA, and accordingly are an ideal laboratory model to detect the influence of CGF on photoaging fibroblast. Nevertheless, there is little known about the influence of CGF fibrin gel (liquid) on UVA damage on fibroblasts in vitro. We assumed that CGF fibrin gel (liquid) could also restore the cellular functions of chronically UVA-irradiated normal human dermal fibroblasts (NHDFs) in terms of cellular proliferation and cellular migration. Our current experiment was designed to test the influence of CGF on photo-damage fibroblasts irradiated by UVA.

Material and Methods

NHDFs, isolation and culture

Dorsal skin tissues were obtained from 6 adult patients who presented with spine injury and who undertook a corrective procedure at the Department of Spinal Surgery, the Third Hospital, Hebei Medical University. This study was approved by the Ethics Committee of the Hospital of Stomatology, Hebei Medical University.

Fibroblasts were derived from the dermis of human dorsal skin tissue; fibroblasts were isolated and cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum (FBS; Gibco Corporation), streptomycin (100 U/mL), and penicillin (100 U/mL) at 95% relative humidity, 5% CO2, and 37°C. Fibroblasts were identified by immunocytochemistry against mouse anti-human vimentin monoclonal antibodies and mouse anti-CK monoclonal antibody (ZhongShanJinQiao, Beijing, China), and collagen type III polyclonal antibodies (ProteinTech, America). The working dilution of the vimentin and CK antibodies was 1: 100; for collagen III antibodies it was a dilution of 1: 50.

CGF conditioned medium

Intravenous blood was collected in two 10-mL glass-coated plastic tubes with anticoagulant solutions. These tubes were then immediately centrifuged with a CGF centrifuge machine (Medifuge, Silfradent, S. Sofia, Italy) using a program with the following characteristics: 2700 rpm for 2 minutes, 2400 rpm for 4 minutes, 2700 rpm for 4 minutes, and 3000 rpm for 3 minutes. At the end of the centrifugation, there were 4 blood fractions: the upper serum layer, the second buffy coat layer, the third GF and unipotent stem cell layer (CGF), and the lower red blood cell layer (RBC). The CGF liquid was removed from the tube and separated from the RBC and serum layer by using a plastic straw. CGF liquid was kept at 4°C for 14 days in plastic tubes and then frozen at −80°C for 1 hour to separate trapped growth factors and cytokines from the fibrin meshes. After the cycle of freezing-thawing, CGF was filtrated (0.22 um). Then 10% FBS and 90% DMEM were added. The 4 CGF conditioned medium concentrations used were: 5%, 10%, 15%, and 20% conditioned medium [12–15].

UVA treatment

We used a desktop apparatus (Sigma Hightech, Shanghai, China) for UVA irradiation with a spectrum from 320 to 400 nm
as the light source. The intensity of the radiation was measured by an ultraviolet radiation (UVR) radiometer with a UVA sensor prior to each experiment (Photoelectric Instrument Factory of Beijing Normal University, Beijing, China). The incoming dose of UVA absorbed by the cells was 18 J/cm², a dose around equal to approximate 60 minutes of sunshine at the French Riviera (Nice, France) in summer at noon [16]. Fibroblasts were inoculated in 96-well plates and 6-well plates and then irradiated with UVA. Before irradiation, the fibroblasts were rinsed with phosphate-buffered saline (PBS). Then fibroblasts were irradiated in PBS to avoid absorption by culture medium and at the desired intensity without the plastic dish lid. Fibroblasts in the normal groups were treated the same as the cells in the photoaging groups except for the absence of UVA irradiation to guarantee equal treatment conditions. After irradiation, CGF was put into the medium (10% FBS) at the respective concentrations of 5%, 10%, 15%, and 20%. Fibroblasts were directly incubated in culture medium for an additional 24 hours in a humidified atmosphere of 5% CO₂ at 37°C.

Cell growth assay and cell viability assay

Human skin fibroblasts were cultured onto 96-well plate at a density of 6×10⁴ cells/well overnight. The cell numbers in 7-wells of each group were counted at the first, second, and third day after CGF cultivation by MTT assays (Solarbio, China). The absorption was determined in an enzyme-linked immunosorbent assay plate reader (Biotek, America) at λ=490 nm and the background readings were automatically subtracted.

Detection of cellular superoxide dismutase (SOD) assay and the intracellular ROS level

The enzymatic activity of superoxide dismutase (SOD) was measured by the instruction of reagent kits (Nanjing Institute of Jiancheng Biological Engineering, China) [5]. The intracellular ROS level was measured by using ROS reagent kits (Nanjing Institute of Jiancheng Biological Engineering, China) [17]. In brief, fibroblasts were washed with PBS, and then treated with 10 mM DCFH-DA and incubated in the dark for 30 minutes at 37°C. The stained cells were imaged and analyzed by using fluorescence microscopy (Olympus, Japan). The excitation and emission wavelengths were set at 494 nm and 518 nm, respectively. Images were captured randomly using constant time, exposure, and gain.

Wound-healing assay

We used wound-healing tests to measure the migration abilities of fibroblasts. Around 2×10⁵ cells/well reaching 90–100% confluence were plated in a 6-well plate. A wound was generated in the confluent monolayer by scratching the monolayer with a 20-μl pipette tip. Fibroblasts migrating into scratched wound region were photographed and measured on first day to the fourth day.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and Student-Newman-Keuls’ test. All data were processed with SPSS 21.0 statistical software and were expressed as mean ± standard deviation. P<0.05 was considered as significant.

Results

Culture and growth of NHDFs

NHDFs were cultured by the tissue block adherent method. After 5 to 7 days, NHDF cells primitives migrated from the tissue. The primary cultured cells showed microscopically large fusiform fibroblast-like cells. Fibroblast-like cells ultimately fused into forma radial growth around the center of the tissue block after an additional 4 to 6 days (Figure 1A). When the cells fusion rate reached 70–80%, fibroblast-like cells were passaged (Figure 1B, 1C).

Identification of NHDFs

The subcultured fibroblast-like cells were microscopically positive for collagen III and vimentin. (Figure 1D, 1E). CK in fibroblast-like cells reacted negatively by comparison (Figure 1F). These figure testified that the fibroblast-like cells were NHDFs.

The response of CGF on UVA-irradiated NHDFs

To find out the optimal concentration of CGF for the subsequent experiments, we observed the cellular morphology of 5 different CGF concentration gradient by inverted microscope (Figure 2C–2N). We also evaluated the proliferation of the normal group and the proliferative effect of CGF on UVA-irradiated NHDFs at the same time. With CGF ranging from 5% to 20%, we found that 5% CGF could promote cell proliferation when the cells were exposed to UVA irradiation (Figure 2A). In the photoaging model group, however, only the 5% CGF group was observed to increase the cell number as compared to 10% to 20% CGF groups during 1 to 2 days (Table 1). Thus, 5% CGF was used in the following experiments.

Cell viability of CGF on UVA-treated fibroblasts

The influence of CGF on photoaging cell was evaluated in cultured skin fibroblasts. The fibroblasts were separately treated at respective dosage of CGF (0–20%) after UVA exposure. The cell viability of 5% CGF group was markedly increased as evaluated by MTT assay. We found that 5% CGF dramatically increased the cell viability compared with the photoaging group (Figure 2B).
CGF reduced intracellular ROS level in UVA-irradiated cells

Compared with the photoaging group and the 5% CGF group, the amount of ROS was very low in normal cultures (Figure 3A–3C). The amount of ROS formation had comparatively grown by approximate 20-fold upon UVA exposure. The 5% CGF treatment strongly reduced (14–15%) the amount of ROS, indicating that 5% CGF can quench reactive oxygen distinctly. Smaller number of cells was observed in the 5% CGF-treated cells, compared to that in the only UVA-irradiated cells (Figure 3D, Table 2).

Effect of CGF on cellular SOD

The activities of SOD were higher in the normal group than in the photoaging model group. Treatment with 5% CGF increased SOD activities compared with the photoaging group (Figure 3E). In our experimental condition, SOD activity in the normal group was 1.65 times higher than that in the photoaging model group. Treatment with 5% CGF after UVA irradiation increased the activity of SOD to 1.47-fold of photoaging model group (Table 3).

Figure 1. Characterization of human dermal fibroblasts (HDFs). Primary culture of fibroblasts (inverted microscope, 40×) (A); Subculture culture of fibroblasts (inverted microscope, 100×) (B); Subculture culture of fibroblasts (inverted microscope, 200×) (C); Identification of NHDFs. Optical microscope showing positive for collagen III (Polymer, 200×) (D); Identification of NHDFs. Optical microscope showing positive for vimentin (Polymer, 200×) (E); Optical microscope showing negative for CK (polymer, 100×) (F).
Concentrated growth factors can inhibit photoaging damage induced...

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Wound-healing assay

The wound-healing migration test indicated that 5% CGF might accelerate the migration ability of fibroblasts in the photoaging model group. The ability of the fibroblast cells to repopulate and move the wounded area was enhanced. The migration rates of the photoaging group, 5% CGF group, and the control group were 2.35±0.84%, 74.9±3.17%, and 62.07±4.23%, respectively, after culturing for 1 day (Figure 4A–4L, Table 4).

Discussion

Skin aging is a complex biological phenomenon. Long-term UVR irradiation causes skin damage, leading to skin photoaging, and the main damage is in the dermis, which is also the main area for repairing skin trauma [18]. UVA can penetrate the dermal layer of the skin [19]. So, it is worth mentioning that UVA mainly influences the human papillary dermis fibroblasts. Fibroblasts are the major target site of UVA radiation, and they play an important role in modulating the changes in aging-specific biological characteristics of skin. We detected the influence of CGF on photo-damage in the most abundant fibroblasts in human skin dermis. Thus, NHDFs are a great target for UVA-irradiated damage. In the present study, we used skin tissue from the back of adults as a source of cells in order to simulate the effect of UVA on adult skin.

Unlike intrinsic aging, photoaging can be prevented and alleviated. CGF can provide a good microenvironment for cell proliferation and migration of fibroblasts.
proliferation, as it contains a number of growth factors, including TGF-β1, VEGF, IGF, and PDGF-BB [20,21]. However, cell proliferation is a complex process. It is not clear what the mechanism of the various active factors is on fibroblasts proliferation. There are several active factors in self-rich CGF (liquid), so the biological efficacy of CGF (liquid) cannot be determined for individual factors. Thus, we wanted to find the most appropriate CGF concentration. For instance, the equivalent ratios of angiostimulating factors suggest quite contrasting effects such as VEGF, basic fibroblast growth factor, and angiostatic factors (e.g., endostatin and thrombospondin-1). Similarity, the effects of the TGF-β1 and PDGF-BB vary according to the initial state of the cells, with TGF-β1 acting on differentiation and PDGF-BB acting on proliferation [15]. In our current experiment, the distinct photoaging phenomenon was detected in cultured dermal fibroblasts after they were UVA-irradiated at a dose of 18 J/cm². According to our MTT assay results, this UVA-induced photoaging was characterized by decreased cell viability of 55%, in comparison with normal cells, in the first day UVA irradiation. At the same time, the cell viability in the 5% CGF group was reduced by 29.5% compared to that in the normal cells, indicating that 5% CGF slowed the UVA-induced cell photoaging process and inhibited damage against UVA-treated cells.

Our previous experiments showed that CGF can facilitate tissue repair and healing [9]. However, it is still unknown what

### Table 1. The results of MTT assay (mean ±SD).

| Groups            | 1 day later (OD) | 2 days later (OD) |
|-------------------|------------------|-------------------|
| Photoaging model  | 0.28±0.05        | 0.41±0.08         |
| 5% CGF+UVA        | 0.42±0.06*       | 0.62±0.07*        |
| 10% CGF+UVA       | 0.24±0.09        | 0.36±0.08         |
| 15% CGF+UVA       | 0.27±0.08        | 0.34±0.07         |
| 20% CGF+UVA       | 0.25±0.05        | 0.34±0.06         |
| Normal            | 0.60±0.06        | 0.73±0.09         |

The 5% CGF group compared with the photoaging model group and the normal group respectively during 1 to 2 days, *P<0.05.

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD – standard deviation; OD – optical density; CGF – concentrated growth factors; UVA – ultraviolet A.

### Figure 3. Effect of 5% CGF on UVA-induced ROS generation in NHDFs. NHDFs were treated with different concentrations of CGF after exposure to 18 J/cm² of UVA. Then, ROS were detected with ROS assay kits. Treated cells were imaged by using fluorescence microscopy. The ROS of the photoaging model group, the 5% CGF group, and the normal group in fibroblast cells respectively observed by inverted microscope, 200× (A–C). Fibroblast cells were treated with 5% CGF after UVA irradiation. ROS clearly decreased in the fibroblast cells treated with 5% CGF after UVA irradiation (B). ROS was largely distributed in the cytoplasm of normal cells after UVA irradiation (A). The number of cells in ROS results is shown in (D). Data are expressed mean values ± standard deviation (SD). Columns with different superscript letters are significantly different (n=9, P<0.05) (D). Effect of 5% CGF on changes of SOD activities. The enzymatic activity of SOD was assayed. Data are expressed mean values ± standard deviation (SD). Columns with different superscript letters are significantly different (n=9, P<0.05) (E).
Table 2. The results of ROS assay (mean ±SD).

| Groups  | Photoaging model | 5% CGF+UVA | Normal    |
|---------|------------------|------------|-----------|
| ROS (cells) | 65.556±3.3582*  | 22.778±2.4381* | 3.333±1.8028* |

The 5% CGF group compared with the photoaging model group and the normal group respectively, * P<0.05. ROS – reactive oxygen species; SD – standard deviation; CGF – concentrated growth factors; UVA – ultraviolet A.

Table 3. The results of SOD assay (mean ±SD).

| Groups  | Photoaging model | 5% CGF+UVA | Normal    |
|---------|------------------|------------|-----------|
| SOD (U/mL) | 22.472±1.2656*  | 31.8462±2.3332* | 37.183±2.5315* |

The 5% CGF group compared with the photoaging model group and the normal group respectively, * P<0.05. SOD – superoxide dismutase; SD – standard deviation; CGF – concentrated growth factors; UVA – ultraviolet A.
Table 4. The results of wound-healing assay (mean ±SD).

| Groups              | First day unclosed wound region (pixel)* | Fist day migration rates* |
|---------------------|-----------------------------------------|---------------------------|
| Photoaging model    | 9.33±3.06                               | 2.35±0.84%                |
| 5% CGF+UVA          | 408.33±14.23                            | 74.9±3.17%                |
| Control             | 300.33±9.45                             | 62.07±4.23%               |

The relative migration abilities of each group cells were observed from the difference area of unclosed wound region and measured in the first day, * P<0.05. SD – standard deviation.

Figure 4. Analysis of the migration of fibroblasts treated with UVA and concentrated growth factor (CGF). Wound-healing test for fibroblasts of the photoaging model group from the first to the fourth days (inverted microscope, 40×) (A–D); wound-healing test for fibroblasts of the 5% CGF group from 1 day to 4 days (inverted microscope, 40×) (E–H); wound-healing test for fibroblasts of the control group from 1 day to 4 days (inverted microscope, 40×) (I–L).

The effect of CGF (liquid) is on UVA-treated fibroblasts. It’s the first time that a study has demonstrated the protective effect of CGF (liquid) on UVA irradiation fibroblasts. Oxidative stress induced by ROS is also a primary cause of photoaging and also promotes cell death [22,23]. Cells exhibit oxidative stress from exposure of ROS, such as nitric oxide, hydroxyl radicals, and superoxide. ROS can cause premature aging and skin cancer, and are also chemically reactive molecules containing oxygen, such as peroxides and oxygen ions [24]. For this reason, the measurement of ROS production might be a valuable method to estimate UV-induced oxidative damage. A possible method to protect skin against photo-damage is to take advantage of...
powerful antioxidants to quench ROS [25]. Antioxidants can prohibit cell death by means of scavenging ROS or by suppression. Our study has proven that the mechanism of photoaging in fibroblast cultures by UVA irradiation contains ROS generation. The amount of ROS in the photoaging group was increased by UVA irradiation, but the amount of ROS in the 5% CGF fibroblast group was considerably decreased. The present experiment showed that 5% CGF might scavenge ROS to protect fibroblasts against oxidant damage and might be providing protective effect on fibroblast by acting as an antioxidant.

Cellular oxidative stress irradiated by UVA is expressively reinforced so that antioxidative capacity is accordingly elongated [17]. It is the production of high levels of ROS induced by UVA in skin cells that causes oxidative damage to numerous biological macromolecules such as those in proteins, nucleic acids, and cell membranes. ROS have strong effects on the initiation and progression of skin aging. Normal cells have a defense system against ROS, including antioxidant enzymes like SOD and GSH-Px [5]. Oxidative stress can lead to an imbalance of the cellular antioxidant system. It is a concern that SOD is a major antioxidant enzyme that provides a defense against the damage of cells by oxidative stress in potentially cytotoxic reactivity [26]. SOD plays an important role in scavenging ROS in the antioxidant system. It is important for maintaining the dynamic balance of ROS in the body that SOD viability directly reflects radical scavenging ability in an organ. SOD can protect cells irradiated by UVA from oxidative damage [5]. On the basis of measuring activities of SOD, we have examined the influence of CGF on oxidative stress induced by UVA irradiation. In our present experiment, we found that 5% CGF meaningfully upregulated SOD concentrations and downregulated ROS productions in fibroblasts after treatment with 18J/cm² of UVA. This demonstrated that UVA irradiation dramatically and effectively suppressed the activities of SOD significantly in fibroblasts, indicating that UVA decreased the capacity of human dermal tissues to scavenge ROS and cause oxidative damage. However, 5% CGF effectively inhibited the changes in the activities of SOD and ROS levels induced by UVA radiation. Compared with the photoaging group, 5% CGF inhibited SOD activities and reduced ROS levels in fibroblasts treated by UVA. It was shown in our study that 5% CGF can protect fibroblasts from UVA induced oxidative damage, and that it can increase effectively the antioxidant activity of fibroblasts enabling the cellular systems to scavenge oxygen free radicals, and provide protection to UVA-irradiated fibroblast by promoting antioxidant enzymes in vitro.

There are prominent parallels between the pathways involved in photoaging of the skin and those concerned with wound healing. It has been hypothesized that skin aging is analogous to a wound that is sufficiently extensive to overwhelm the skin’s inherent repair mechanisms [27]. Therefore, we used the scratch test to simulate the process of the wound healing after UVA irradiation. CGF promotes wound healing and reduces the possible mechanism of scar formation: CGF contains a large number of growth factors [6]. Fibroblasts can play a leading role in the recovery process of cutaneous connective tissue [28]. Growth factors stimulate fibroblasts to rapidly migrate to the wound, and promote the proliferation of fibroblasts [29,30]. The migration rate of UVA-irradiated fibroblasts was decreased, the migration rate of UVA-irradiated fibroblasts was significantly increased in the presence 5% CGF, and the migration rate of the 5% CGF group was higher than that of the normal group. It was thus concluded that CGF displayed a positive protection effect on UVA-treated skin damage.

Conclusions

It was concluded that 5% CGF located in the extracellular fluid has the capability of free radical-scavenging and can weaken UVA-treated oxidative stress in fibroblasts in human skin dermis. The present study primitively elucidated that the supplementation of 5% CGF fibrin gel (liquid) to culture medium efficiently reduced UVA-treated fibroblast damage in vitro and promoted wound healing. It showed several advantages of 5% CGF, making it a potential candidate for treating prematurely aged skin. The clinical use of CGF doesn’t elicit an inflammatory response or elicit immune-rejection and is safe, as its source is from autologous venous blood. Additionally, the preparation of CGF has many excellent characteristics such as it is an economical, convenient, simple, and rapid process. The CGF characteristics of safety without the risk associated with allogeneic products, easy preparation, autologous nature, antiphotoaging, and the sustained release of growth factors, makes CGF a promising biomaterial for clinical application for skin lesions caused by UVA and was shown to slow the photoaging process. However, more clinical trials are needed to establish an appropriate application method of this material.

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