Published in final edited form as:

J Cell Physiol. 2018 February ; 233(2): 1523–1534. doi:10.1002/jcp.26043.

Bryostatin and its synthetic analog, picolog rescue dermal fibroblasts from prolonged stress and contribute to survival and rejuvenation of human skin equivalents

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Abstract

Skin health is associated with the day-to-day activity of fibroblasts. The primary function of fibroblasts is to synthesize structural proteins, such as collagen, extracellular matrix proteins, and other proteins that support the structural integrity of the skin and are associated with younger, firmer, and more elastic skin that is better able to resist and recover from injury. At sub-nanomolar concentrations (0.03–0.3 nM), bryostatin-1 and its synthetic analog, picolog (0.1–10 nM) sustained the survival and activation of human dermal fibroblasts cultured under the stressful condition of prolonged serum deprivation. Bryostatin-1 treatment stabilized human skin equivalents (HSEs), a bioengineered combination of primary human skin cells (keratinocytes and dermal fibroblasts) on an extracellular matrix composed of mainly collagen. Fibroblasts activated by bryostatin-1 protected the structural integrity of HSEs. Bryostatin-1 and picolog prolonged activation of Erk in fibroblasts to promote cell survival. Chronic stress promotes the progression of apoptosis. Dermal fibroblasts constitutively express all components of Fas associated apoptosis, including caspase-8, an initiator enzyme of apoptosis. Prolong bryostatin-1 treatment reduced apoptosis by decreasing caspase-8 and protected dermal fibroblasts. Our data suggest that bryostatin-1 and picolog could be useful in anti-aging skincare, and could have applications in tissue engineering and regenerative medicine.

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Disclosure Statement: We declare that we have no conflicts of interests. Authors indicated that they and their Institutes have no other agreements that could be seen as involvement of financial interest in this work.

Authors' contribution: Conceptualization: TKK. Data curation: TKK. Formal analysis: TKK. Investigation: TKK. Methodology: TKK, PAW. Project administration: TKK. Supervision: TKK. Validation: TKK. Visualization: TKK. Writing - original draft: TKK. Review & editing: TKK, DLA, PAW.

Keywords

Bryostatin-1; caspase-8; Erk; bryologues; dermal fibroblasts; keratinocytes; skin care; human skin equivalent (HSE)

Introduction

The most studied approaches to supporting skin health are cosmetological (e.g., topical skin care products, sun protection agents); medicinal (e.g., topical antioxidants, cell stimulators); invasive procedures (e.g., surgical restoration, laser wrinkle removal, hormone replacement therapy) and systemic (e.g., injectable biostimulators). Typical biostimulators include growth factors, growth hormones, vitamins (retinols), and other antioxidants that are injected into the skin to activate dermal fibroblasts and increase their biosynthetic capacity. Activated fibroblasts synthesize and secrete structural proteins, such as collagen and extracellular matrix proteins that support the structural integrity of the skin and increase skin elasticity. Skin rejuvenation with injectable biostimulators attempts to mimic the optimal physiological environment for supporting cellular activity of fibroblasts.

Fibroblast proliferation, differentiation, and survival are associated with activation of extracellular-signal-regulated kinase (Erk) activity in the MAPK signaling pathway. By contrast, stress-associated c-Jun N-terminal kinase (JNK) and p38 activation are associated with fibroblast growth arrest and apoptosis. In aged skin, the activity of Erk is reduced, whereas JNK and p38 kinase are increased, compared to young skin (Shin et al., 2005). Photodynamic therapy (PDT) has also been used to rejuvenate aged skin by activating dermal fibroblast Erk. With PDT, prolonged activation of Erk is mediated by an increase in intracellular reactive oxygen species (ROS) (Jang et al., 2013). Yet PDT-generated ROS can also mediate other toxic cellular effects such as lipid peroxidation, direct/indirect cytotoxicity, and production of free radicals leading to oxidative damage (Brackett and Gollnick et al., 2011). Methods for stimulating Erk independent of ROS can avoid these toxic effects and improve skin health.

Agents such as hesperetin glucuronide from flavonoid metabolism (Proteggente et al., 2003), pentoxifylline and α -tocopherol (Laurent et al., 2005) have been found to modulate dermal fibroblasts to improve skin health. Bryostatin-1 is a macrolide lactone that was first isolated from the bryozoan, *Bugula neritina* (Pettit et al., 1982). Bryostatin-1 has been shown to have a wide range of biological effects, including anticancer effects (Zhang et al., 1996), synaptogenic effects that enhance memory (Sun and Alkon, 2005; Kuzirian et al., 2006), and anti-HIV effects (Pérez et al., 2010). The effect of bryostatin-1 is related to the induction of PKC-activated pathways involved in some of the most important cellular functions such as cell survival, proliferation, protein synthesis, and apoptosis. In stressed conditions prolonged bryostatin-1 treatment reduced apoptosis by decreasing caspase-8 and protected dermal fibroblasts. We have shown that bryostatin-1 activates Erk (Khan and Alkon, 2006) leading us to hypothesize that bryostatin-1 and its synthetic analogs (Wender et al., 2004) can be used to activate dermal fibroblasts to improve skin health. We investigated the effect of bryostatin-1 and its synthetic analog, picolog, on Erk activation and the survival rate of *in*

vitro cultured fibroblasts on the structural integrity of a human skin equivalent (HSE) composed of primary human fibroblasts and keratinocytes.

Materials and Methods

Reagents

Bryostatin-1 (Cat# 2383) was purchased from Tocris Bioscience (Minneapolis, MN). The Erk inhibitor PD98059 (Cat# P215) was purchased from Sigma-Aldrich (St. Louis, MO). P44/42 (Erk1/2) rabbit monoclonal antibody (Cat# 4695) and phosphoP44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit polyclonal antibody (Cat# 9101) were purchased from Cell Signaling Technology (Danvers, MA). Human/mouse caspase-8 antibody (Cat# AF1650) was purchased from R&D Systems (Minneapolis, MN). β -actin (AC-15) antibody (Cat#sc-69879) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). β -tubulin antibody (Cat# 04–1049) was purchased from Millipore (Temecula, CA). The design and synthesis of the synthetic analog of bryostatin-1, picolog, were conducted according to published methods (Wender et al., 2014). The chemical formula of bryostatin-1 and picolog are presented in Fig.1. Picolog was synthesized by function-oriented synthesis using bryostatin-1 as therapeutic lead in a step-economical fashion. Picolog was found to be the most potent new class of 'B-Ring' analog of bryostatin-1 in terms of PKC activity (Wender et al., 2015). Bryostatin-1 and picolog were dissolved in DMSO (50 μ M) and aliquots were stored at -20°C . Stock solutions were dissolved in culture medium at different concentrations just before each experiment.

In-depth description of dermal fibroblast cell lines

In-depth description of skin biopsy samples are presented in Table 1. Skin fibroblast samples AG09555 (isolated and banked from a 53-year-old healthy female), AG11358 (isolated and banked from a 73-year-old healthy male) and AG04560 (isolated and banked from a 59-year-old healthy male) (all from the Aging Cell Culture Repository of the National Institute on Aging) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Fresh fibroblasts were isolated from a skin punch biopsy (2–3 mm) from a 45-year-old healthy female subject (0078F45). The skin biopsy was conducted by qualified personnel under the supervision of Dr. Shirley Neitch with IRB approval of the Marshall University (Huntington, WV). All subjects signed informed consent forms under the Marshall University rules and regulations. The method of isolating fibroblasts from skin biopsies was followed as described elsewhere (Khan and Alkon 2006). We selected skin fibroblast stains 1:1 ratio of male and female (healthy age range 45–73 years) and both from Caucasian and Black race. All biopsies were taken in ante-mortem state. For this study, we used dermal fibroblasts from passage 6 to 14.

Dermal fibroblast culture and treatment

Primary skin fibroblasts isolated from skin biopsies and maintained in Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Invitrogen, Grand Island, NY) supplemented with 10% FBS in 6-well culture plates (37 $^{\circ}\text{C}$, 5% CO_2 , 90% humidity) until reaching 90–100% confluence. Cells were cultured in completely serum-free medium to simulate stress conditions. After culture in serum-free media for 24 hours, cells were treated with either

vehicle; 0.03, 0.3, or 30 nM bryostatin-1; or 0.1, 1, 5, 10, or 100 nM picolog. We previously found that sub-nanomolar concentrations of bryostatin-1 are effective for cellular activation (Khan et al. 2009) and we used the same 0.3 nM dose for most experiments. Nanomolar concentrations of picolog were previously found to be effective for cellular activation (Khan et al. 2009). The condition of the cultured skin fibroblasts was monitored with an inverted cell culture microscope (Westover Digital AMID Model 2000, Westover Scientific, Bothell, WA), controlled by a computer and images were captured with image acquisition software (Micron 2.0.0, Westover Scientific).

Epidermal keratinocyte culture

The Clonetics™ keratinocyte system containing normal adult human epidermal keratinocytes (NHEK) was used as source of epidermal keratinocytes (Lonza, Walkersville, MD). Human adult epidermal keratinocyte cells were cultured in keratinocyte basal medium supplemented with appropriate growth factors (Clonetics KGM-Gold™ BulletKit™ [cat# 00192060] contains one 500 ml bottle of keratinocyte basal medium-gold supplemented with bovine pituitary extract (BPE), 2 ml; hEGF, 0.5 ml; insulin, 0.5 ml; hydrocortisone, 0.5 ml; transferrin, 0.5 ml; epinephrine, 0.25 ml; GA-1000, 0.5 ml) in T25 culture flasks in an incubator at 37°C, 5% CO₂, and 90% humidity. The growth medium was changed the day after seeding and every other day thereafter. Keratinocytes were used up to 18 population doublings. All cell preparations tested negative for mycoplasma, bacteria, yeast, and fungi. The condition of the skin keratinocytes was monitored with an inverted cell culture microscope (Westover Digital AMID Model 2000, Westover Scientific), controlled by a computer and images were captured using image acquisition software (Micron 2.0.0, Westover Scientific).

Immunoblot analysis

Protein lysates (20 µg of protein each) were boiled in 2× Laemmli buffer for 10 min and separated using a 10% gradient Tris-Glycine gel (or 4–20% gradient Tris-Glycine gel for caspase-8). Separated proteins were transferred to nitrocellulose membranes and the membranes were blocked in 2% BSA dissolved in (1× PBS) at room temperature (RT) for 15 min. Membranes were then incubated with P44/42 (Erk1/2) rabbit monoclonal antibody (1:1000), PhosphoP44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit polyclonal antibody (1:1000), rabbit polyclonal caspase-8 antibody (0.5µg/mL), anti-β-tubulin antibody (1:10000), and anti-β-actin antibody (1:1000), respectively for 1 hour at RT. Membranes were washed 3 times with standard immunoblot washing buffer and further incubated with alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:10000 dilution for 45 min. Membrane fractions were washed 3 times with standard immunoblot washing buffer and developed using the 1-step NBT-BCIP substrate (Thermo Scientific, Rockford, IL). Signal intensities of the images were recorded in the ImageQuant RT-ECL (GE Life Sciences, Piscataway, NJ) and densitometric quantification was performed using the IMAL software (Blanchette Rockefeller Neurosciences Institute at West Virginia University, Morgantown, WV). Intensities of Erk1/2 and p-Erk1/2 signals were normalized against β-actin for each lane. Intensities of caspase-8 signals were normalized against β-tubulin for each lane.

Cell viability test by MTT assay

Cell viability assay was conducted by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide dye absorbance. After treatment, cells were labeled with MTT and dissolved by adding SDS-HCl solution to each well and mixing thoroughly with a pipette tip. Absorbance was read at 570 nm using a microplate reader. Data were presented in terms of relative cell viability normalized with normal fibroblasts culture.

Cell viability test by Trypan blue assay

Trypan blue exclusion test was conducted to determine the percentage of viable cells present in a cell suspension after specific treatment. After each treatment cells were removed from the 6-well cell culture plates by trypsinization and washed twice with PBS (1X). Trypan blue solution (0.4%) was added with 1:1 ratio into cell suspensions. A drop of the trypan blue/cell mixture was kept on a hemacytometer and number of viable/non-viable cells were count under an inverted cell culture microscope (Westover Digital AMID Model 2000, Westover Scientific), controlled by a computer and images were captured using image acquisition software (Micron 2.0.0, Westover Scientific). The percentage of viable cells was calculated as follows: viable cells (%) = (total number of viable cells per ml of aliquot)/(total number of cells per ml of aliquot) × 100. Significant number of cells was lost in case of prolong serum free cell cultures without bryostatin-1/picolog treatment.

Preparation of human skin equivalents (HSEs)

Human skin equivalents (HSEs) has been prepared in laboratory by the method described below. We engineered an HSE composed of both epidermal and dermal layers; keratinocytes and fibroblasts were utilized to prepare the bilayer structures (Shevchenko et al., 2010). Dermal fibroblasts were cultured on a 6-well collagen-coated surface (Biocoat Cell Environment, Becton Dickinson, Bedford, UK) or BD BIOCOAT™ plates (BD Biosciences, San Jose, CA). Fibroblast cells were maintained in DMEM with low glucose (Invitrogen) supplemented with 10% FBS, and were grown to 100% confluence. Culture medium was removed and the cells were washed three times with DMEM (without serum) to remove all growth factors. Approximately 1 million cultured dermal fibroblasts cells were plated into each well of a 6-well plate coated with collagen (Biocoat Cell Environment, Becton Dickinson, Bedford, UK) or BD BIOCOAT™ plates (BD Biosciences, San Jose, CA). Fibroblast cells were allowed to form a layer on the collagen matrix for 24 hours (in DMEM with low glucose (Invitrogen) supplemented with 10% FBS). After 24 hours fibroblasts culture medium was removed from the cellular matrix and washed twice with sterile 1XPBS. Previously cultured epidermal keratinocytes were added to the fibroblasts as the second layer (Fig. 2). The dermal fibroblast layer and epidermal keratinocyte layer were allowed to form HSE in keratinocyte basal medium supplemented with appropriate growth factors (keratinocyte basal medium-gold supplemented with BPE-, 2 ml; hEGF, 0.5 ml; insulin, 0.5 ml; hydrocortisone, 0.5 ml; transferrin, 0.5 ml; epinephrine, 0.25 ml; GA-1000, 0.5 ml) in an incubator at 37°C, 5% CO₂, and 90% humidity. After 24 hours, the HSEs were treated with vehicle or increasing doses of bryostatin-1. The condition of the HSEs was monitored with an inverted cell culture microscope (Westover Digital AMID Model 2000, Westover Scientific), controlled by a computer and images were captured via image acquisition

software (Micron 2.0.0, Westover Scientific). HSEs have been used commercially as clinical skin substitutes (Orcel[®] from FortiCell Bioscience, Englewood Cliffs, NJ; FDA approved in 2001/2008; Apligraf[®] from Genzyme Tissue Repair Corporation, Cambridge, MA, FDA approved in 2007) and as *in vitro* models for skin biochemical tests and toxicity tests (Mertsching et al., 2008; Zhang and Michniak-Kohn, 2012).

Results

Protection of human skin fibroblasts by bryostatin-1 and its synthetic analog, picolog cultured under long-term serum starvation

Skin fibroblast cells isolated from a skin punch biopsy of a 45-year-old healthy male (AG11358) were cultured in 6-well plates. After 24 hours under serum starvation, cells were treated with different concentrations (0.03, 0.3, 30nM) of bryostatin-1 and picolog (0.1, 1, 5, 10, 100 nM) or vehicle. After an additional 15 days in serum-free conditions, cells in bryostatin-1 (Fig. 3A)/picolog (Fig. 3B) were healthy and growing compared to cells cultured without bryostatin-1/picolog (Fig 3a, b). Bryostatin-1/picolog treatment protected skin fibroblasts against complete serum starvation for the prolonged time period of 35 days, with total loss of cellular integrity in the untreated condition (Fig 3A, B). The cell viabilities after different treatment were assayed after keeping all cultures for additional 1 day in 10% serum. There was huge difference in cell viabilities between treatments (bryostatin-1/picolog) with untreated groups (Fig. 3).

This experiment was repeated in three additional primary skin biopsy derived fibroblasts cells lines (banked skin fibroblast cells from a 59-year-old healthy male (AG04560), banked cells from a 53-year-old healthy female (AG04560) and fresh fibroblasts isolated from a skin punch biopsy from a 45-year-old healthy female subject (0078F45)) and provided similar results (data not shown).

Protection of human skin fibroblasts cultured on collagen matrix by bryostatin-1 (Bry) under long-term serum starvation conditions was studied next (see supporting documents). To better mimic human skin in culture, we cultured human skin fibroblasts from three different healthy subjects (0078F45, AG09555, and AG04560) on a collagen matrix. Serum starvation and treatment were done as before. Bryostatin-1 protected skin fibroblasts from serum starvation for a prolonged time period of 25 days (Bry+) (Supporting documents), whereas cellular integrity was lost in the untreated cultures (Bry-) (Supporting documents Fig. 1).

Concentration dependence of protection of human skin fibroblasts by bryostatin-1 and its synthetic analog, picolog cultured under long-term serum starvation

Concentration dependence of protective effects of bryostatin-1 and its synthetic analog, picolog with respect to long-term serum starvation were studied (Fig. 4). At 15 days of serum starvation bryostatin-1 was effective to cell survival from 0.03 nM to 30 nM concentration and picolog was effective to cell survival from 0.1 nM to 100 nM (Fig. 4A). For long term serum starvation (35 days) both bryostatin-1 (30 nM) and picolog (100 nM) were less effective at higher concentrations (Fig. 4B). When cells treated with 30 nM bryostatin-1/100nM picolog and kept 35 days of serum starvation cell viabilities were very

low (Fig. 4C). Effective doses of bryostatin-1/picolog were necessary for cell survival in long term stress induced by serum free condition.

Protection of human skin fibroblasts by picolog cultured under long-term serum starvation conditions by MTT assay

Beside cell viability assay by Trypan blue we tested MTT assay. Picolog (5nM), a synthetic analog of bryostatin-1 also protected human skin fibroblasts after 15 days of culture in serum free condition (Fig. 5A, B). Cell viability tested by MTT assay after 15 days of culture showed that picolog treatment increased cell viability (Fig. 5C).

Bryostatin-1 prolonged (several hours) Erk1/2 activation in human dermal fibroblasts

We also examined the effect of the Erk inhibitor PD98059 on fibroblast survival after bryostatin-1 treatment under serum-starved conditions (Fig. 6A). Cells were treated with vehicle or bryostatin-1 in the presence or absence of PD98059 (30 μ M concentration) (Inhi). Cells treated with bryostatin-1 alone showed best cellular integrity (Fig. 6A; panel: Bry+ Inhi-). Untreated cells (Fig. 6A; panel Bry-Inhi-) and cells exposed to the Erk inhibitor showed less cellular integrity and greater evidence of cell death (Fig. 6A, panel Bry-Inhi+, Bry+ Inhi+).

To examine how bryostatin-1 sustained fibroblast survival after exposure to long-term serum-free culture conditions, we conducted an immunoblot analysis of Erk in fibroblast cells cultured under serum-starved conditions and treated with bryostatin-1 for various lengths of time (Fig. 6B). Bryostatin-1 induced activation of Erk1/2 in fibroblasts cultured under serum-free conditions (Fig. 6B, C), an effect that could be blocked by addition of the Erk inhibitor (30 μ M concentration) (Fig. 6D).

Prolonged bryostatin-1 treatment protected dermal fibroblasts by reducing apoptosis

Cysteine-aspartic acid protease (caspase) family of proteins plays a central role of cellular apoptosis. Here we examined the role of caspase-8, the initiator enzyme in apoptosis in prolong treatment of bryostatin-1 in dermal skin fibroblasts from AG09555. Prolonged bryostatin-1 treatment reduced the expression levels of caspase-8 (Fig. 7). Caspase-8 expression was significantly lower after 3 days of bryostatin-1 treatment. This study was repeated with culture skin fibroblasts from AG04560 cell line and similar results were found.

Protection of human skin equivalents (HSEs) by bryostatin-1 treatment

HSEs were created by layering skin fibroblasts cultured on collagen matrix to form a dermal matrix with human keratinocytes seeded on top to form an epidermal layer (Fig. 2). HSEs were treated with vehicle or bryostatin-1 and monitored under microscope. After 9 days of culture, HSE treated with bryostatin-1 showed only a few dead cells (Fig. 8A, see Bry+ column), whereas untreated HSE cultures showed a significant number of dead cells (Fig. 8A, Bry-column). After 12 days in culture, the number of dead cells in the HSE cultures treated with bryostatin-1 had increased, but there was almost a complete breakdown of HSE without bryostatin-1 treatment. We also saw better cell survival within HSEs after two treatments with a dose of bryostatin-1 (0.3 nM) compared HSEs that received only one 0.3

nM dose or untreated HSEs (Fig. 8B; panel: Bry-). This study was repeated with culture skin fibroblasts from AG09555 cell line and similar results were found.

Discussion

In this study, we showed that primary cultures of human fibroblasts can be sustained long-term under serum deprivation conditions after treatment with bryostatin-1 or its synthetic analog, picolog. A greater number of treated fibroblasts than untreated fibroblasts survived in serum-free culture for up to 3 weeks. The same protective effect of bryostatin-1 was seen with cultures of HSEs, with fewer dead cells after 9–12 days in culture than untreated HSEs. Bryostatin-1 treatment activated the Erk signaling pathway in human fibroblasts, and the protective effect of bryostatin-1 was lost with the addition of a specific Erk inhibitor. Together, these findings suggest that bryostatin-1 activates the Erk pathway to support fibroblast survival under stress conditions, consistent with bryostatin-1's known role in modulating protein kinase C.

Fibroblasts are the primary cell type responsible for synthesizing collagen fibers that form a structural scaffold for the skin. Dermal fibroblasts also produce structural proteins such as elastin and glycosaminoglycans (GAGs). Elastin protein gives the skin its elasticity. Activation of Erk in fibroblasts increases matrix metalloproteinase (MMP)-3 and collagen type Ia messenger RNA and protein (Jang et al., 2013). Bryostatin-1 has been shown to stimulate protein synthesis (Alkon et al., 2005), and synthesis of extracellular matrix proteins. The present study showed that bryostatin-1 induced Erk cellular signaling, which was necessary for its observed effect on fibroblast survival. Thus, bryostatin-1 and its synthetic analogs could help maintain or improve skin health by enhancing Erk-mediated biosynthesis of structural proteins like collagen in dermal fibroblasts.

At sub-nano molar to nano molar concentrations bryostatin-1 has been shown to activate PKC α /e (Khan et al., 2009; Yi et al., 2012). This leads downstream of PKC to ERK activation. Growth factors and mitogens use the Ras/Raf/MEK/Erk signaling cascade to transmit signals from cell surface receptors on the cell membrane to regulate gene expression and normal cell proliferation, differentiation, and survival and to prevent normal apoptosis. Growth factor signaling, particularly via the EGFP-induced Erk- MAPK pathway, has been the subject of intense research for development of pharmacologic inhibitors for the treatment of cancer (Roberts and Der, 2007). Therefore, bryostatin-1 and its synthetic analogs could provide an anti-aging strategy that achieves fibroblast activation without stimulating oncogenic pathways.

Caspase-8 is an initiator enzyme in the programmed apoptosis processes. The programming of cell death occurs through intrinsic and extrinsic pathways. In the intrinsic pathway, mitochondria release cytochrome C that activates caspase-9 while in extrinsic pathway of apoptosis, cell surface death receptors such as Fas activate caspase-8 or -10 and initiate the process of apoptosis. Caspase-8 activates caspase-3 that ultimately leads to cell destruction. In this study we found at very low concentration (sub nM) bryostatin-1 reduced the scope of cell death by apoptosis by reducing expression levels of caspase-8 (Fig. 5 A , B). Inhibition of caspase-8 was found to reduce brain infarction and promote neuronal survival following

ischemic stroke (Shabanzadeh et al., 2015). In another study from our Institute, bryostatin-1 treatment prevented synaptic loss (reduced neuronal apoptosis) and induced synaptogenesis after cerebral ischemia in an animal model (Sun et al., 2008).

HSEs have been used to explore the fundamental processes of skin cells, including wound healing (Xie et al., 2010), formation of epidermis (Johnson et al., 1992), and stem cell maintenance (Boehnke et al., 2007). They have also been used for *in vitro* testing of skin models (Poniec et al., 2002) and toxicity testing (Oliver et al., 1988; Lelièvre et al., 2007). Human epidermal keratinocytes and dermal fibroblasts cultured in separate layers on a Type I bovine collagen sponge by FortiCell Bioscience has been approved by FDA for use as an HSE for clinical skin replacement and grafts (Orcel®). Cell Systems Biotechnologie GmbH has introduced a product called “Advanced Skin Test 2000 (AST2000)” consisting of a dermal equivalent with embedded fibroblasts as a base and an epidermal layer of keratinocytes on top. AST2000 can be used for testing in *in vitro* skin permeation and toxicology screening models. We developed a similar model to test the effect of bryostatin-1 on fibroblast survival and viability. Our study demonstrated the protective effect of bryostatin-1 on HSE cell survival in culture. An earlier study found that skin fibroblasts can activate keratinocyte outgrowth in the presence of collagen (Tuan et al., 1994). Further studies are needed to determine whether bryostatin-1 treatment of HSEs stimulates fibroblast biosynthesis of structural proteins to enhance keratinocyte outgrowth and skin structural integrity.

One major application of HSEs is as clinical skin replacements. The market for clinical skin replacement and grafting procedures in the US in 2009 was \$18.9 billion, and it is estimated to increase to \$24.3 billion in 2019 (Tissue Engineering and Cell Transplantation: US Markets for Skin Replacements and Substitutes; Report #A426; Medtech Insight: Bridgeport, PA, USA, August 2010). According to the annual plastic surgery procedural statistics, there were 15.9 million surgical and minimally-invasive cosmetic procedures performed in the United States in 2015, a 2 percent increase over 2014 (<https://www.plasticsurgery.org/news/press-releases/new-statistics-reflect-the-changing-face-of-plastic-surgery>). Fibroblast-targeted therapies have been approved by the FDA and used to improve outcomes of fibroblast transplants, split-thickness-skin grafts, and partial-thickness-skin grafts. Addition of bryostatin-1 to the cocktail of growth factors and hormones used with HSEs or autologous fibroblasts prior to grafting could further improve graft survival through fibroblast activation. Slowing epidermal and dermal turnover rate and a prolonged cell cycle lead to gradual loss of skin elasticity and slower wound healing. Bryostatin-1-induced activation of fibroblasts could have applications in surgical wound healing and treatment of cutaneous wounds. In skin care or anti-aging applications, bryostatin-1 could be used topical route or injected with other ingredients of systemic therapies (HA, vitamins, hormones, growth factors, amino acids) into the dermis to promote fibroblast activation and survival and improve skin integrity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are deeply grateful to Dr. Shirley Neitch from the Marshall University, Huntington, WV, for the skin biopsies. We are indebted to Dr. Stacey Tobin.

Funding Sources: This research was supported in part by the Intramural Research Program of The Blanchette Rockefeller Neurosciences Institute at West Virginia University, Morgantown, WV (T.K.K.), and by the National Institute of Health (CA031845 and CA031841 to P.A.W.).

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Fig. 1. Chemical structure of bryostatin-1 and its synthetic analogue (picolog)
Bryostatin-1 and the most potent new class of 'B-Ring' analog, picolog.

Fig. 2. Preparation of human skin equivalents (HSEs)

Schematic diagram of human skin equivalents (HSEs) preparation on collagen matrix. HSEs were constructed that contained skin keratinocytes (epidermal equivalent) layered on top of skin fibroblasts on a collagen coated basement matrix (dermal equivalent). Cultured dermal fibroblasts (AG04560/AG09555) were added first on collagen matrix and allowed to settle for one day. Next day culture medium was removed cultured skin keratinocytes were added. The dermal fibroblast layer and epidermal keratinocyte layer was allowed to form HSE in keratinocyte basal medium supplemented with appropriate growth factors (keratinocyte basal medium-gold supplemented with BPE-, 2 ml; hEGF, 0.5 ml; insulin, 0.5 ml; hydrocortisone, 0.5 ml; transferrin, 0.5 ml; epinephrine, 0.25 ml; GA-1000, 0.5 ml) in an incubator at 37°C, 5% CO₂, and 90% humidity.

A

B

Fig. 3. Protection of human skin fibroblasts by bryostatin-1 and its synthetic analog, picolog cultured under long-term serum starvation

Skin fibroblast cell line (AG11358) from healthy subjects were cultured on a regular 6-well tissue culture plate. After 15 days of serum starvation, primary cultures of human fibroblasts started to lost cellular integrity while exposure to sub-nanomolar concentrations (0.3 nM) of bryostatin-1 (Fig 3A) and 10 nM of picolog (Fig. 3B) protected fibroblast cellular integrity while under serum starvation. Untreated human fibroblasts did not survive prolonged serum starvation (35 days), but cells treated with bryostatin-1/picolog were maintained under serum-free conditions. Protection of cultured human skin fibroblasts by bryostatin-1/picolog in long term serum starvation (35 days) cultured was tested by cell viability assays after 1

day serum-fed condition. The difference of cell viability between treatment (bryostatin-1/picolog) and non-treatment group was huge. Similar results were obtained from all four skin fibroblast cell lines. Bar=25µm.

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Fibroblast cultures in serum free state for 15 days

A

Fibroblast cultures in serum free state for 35 days

B

Fibroblast cultures in serum free state for 35 days + 1day in 10% serum

C

Fig. 4. Concentration dependence of protection of human skin fibroblasts by bryostatin-1 and its synthetic analog, picolog cultured under long-term serum starvation

Skin fibroblast cell line (AG11358) from healthy subjects were cultured on a regular 6-well tissue culture plate. (A) At 15 days of serum starvation bryostatin-1 is effective to cell survival from 0.03 nM to 30 nM concentration and picolog is effective to cell survival from 0.1 nM to 100 nM. (B) For long term serum starvation (35 days) both bryostatin-1(30nM) and picolog (100nM) are less effective at higher concentrations. Similar results were obtained from all four skin fibroblast cell lines. (C) Concentration dependence of survival of skin fibroblasts after long time serum deprivation and bryostatin-1/picolog treatment. For long term serum starvation (35 days) plus one day 10% serum supplementation both bryostatin-1(30nM) and picolog (100nM) are less effective at higher concentrations in terms of cell survival. Similar results were obtained from all four skin fibroblast cell lines. Bar=25μm.

Fig. 5. Protection of human skin fibroblasts by picolog (synthetic analog of bryostatins-1) cultured under long-term serum starvation conditions

(A) After serum starvation for 15 days, untreated fibroblasts (AG04560) lost cellular integrity (Picolog-). (B) Treatment with 5 nM picolog kept fibroblast cellular integrity intact after 15 days of serum starvation. (C) Fibroblast cell viability after 15 days in serum-free conditions was higher in the picolog-treated cultures. Data are presented in terms of relative cell viability. Results are mean $\pm$ SEM for 15 wells. A-B: Bar = 10 μm .

Fig. 6. Activation of Erk by bryostatin-1 protect dermal fibroblasts

Inhibition of Erk reverses the protective effect of bryostatin-1 on human skin fibroblasts (AG04560) cultured under long-term serum starvation conditions. (A) After 5 days of culture under serum-deprived conditions, untreated skin fibroblasts (Bry- Inhi-) showed poor cellular integrity. Fibroblasts treated with bryostatin-1 (Bry+ Inhi-) showed greater survival and fewer dead cells after 5 days of serum-free culture compared to untreated cells. Treatment of cells with the Erk inhibitor PD98059 (Bry- Inhi+) decreased cellular integrity under serum deprived conditions, similar to untreated cells. Blocking Erk activation decreased cellular integrity even in the presence of bryostatin-1 (Bry+ Inhi+). Images are representative of experiments conducted in triplicate. Immunoblot analysis of activated Erk in response to 0.3 nM bryostatin-1 in serum-deprived cultured human skin fibroblasts. (B) Bryostatin-1 induced prolonged activation of Erk measured by immunoblot analysis. Cultured dermal fibroblasts were serum starved for 24 hours and then treated with 0.3 nM bryostatin-1 (Bry) for 0, 1, 2, 4, 8, and 24 hours in serum-free conditions. Representative immunoblot showing activated p-Erk1 and p-Erk2, unphosphorylated Erk1/2, and β-Actin as a loading control. (C) Activated Erk1/2 (p-Erk1/2) was quantified by normalizing against total Erk1/2 (p-Erk/Erk). Results are mean±SEM from three independent measurements starting from cell culturing. (D) Representative immunoblot of Erk activation in fibroblasts treated with bryostatin-1 (Bry) in the presence or absence of the Erk inhibitor PD98059 (Inh; 30 µM). PD98059 blocked bryostatin-1 induced Erk activation in cultured human fibroblasts. Results are mean±SEM for three different experiments; Bar=10µm.

Fig. 7. Prolong bryostatin-1 treatment protects dermal fibroblasts by reducing apoptosis
 Skin fibroblasts from AG09555 cell line were cultured and specific treatments were conducted. (A, B) Prolong bryostatin-1 treatment reduces caspase-8 expression levels. Caspase-8 (57–60 KDa) and pro-caspase (41–43 KDa) bands were found in Western blot analysis. After 3 days of bryostatin-1 treatment reduces the expression levels of caspase-8 was significantly low (5 measurements in cells isolated from three individuals). Prolong treatment showed similar results. (C) Quantification of caspase-8 bands with different days of bryostatin-1 treatment. The expression levels of caspase-8 were significantly lowered by bryostatin-1 (0.3nM) treatment (3, 7, 15 days) compare to no treatment (0 day). This study was repeated with culture skin fibroblasts from AG04560 cell line and found similar results. Results are mean±SEM for three different experiments.

Fig. 8. Bryostatin-1 promotes cell survival within human skin equivalents (HSEs)

Human skin equivalents (HSEs) were prepared according to Figure 2. (A) HSEs treated with a single dose of bryostatin-1 (0.3 nM) (Bry+) showed less cell death than untreated HSEs in culture (Bry-). (B) HSEs treated with multiple doses of 0.3 nM bryostatin-1 (Bry+) showed greater cell survival than HSEs treated with a single 0.3 nM dose or untreated HSEs (Bry-). Images are representative of experiments conducted in triplicate with fibroblasts from AG04560. This study was repeated with culture skin fibroblasts from AG09555 cell line and found similar results. A: Bar=10µm, B: Bar=40µm.

Table 1

In-depth description of skin biopsy samples

Stains of dermal fibroblasts (n=4)	
Cell ID	Race, physical condition, age, sex and time of biopsy taken
0078F45 *	Caucasian, healthy 45 yo, F. Skin biopsy was taken ante-mortem.
AG09555 **	Black, healthy 53 yo, F. Skin biopsy was taken ante-mortem.
AG11358 **	Caucasian, healthy 73 yo, M. Skin biopsy was taken ante-mortem.
AG04560 **	Caucasian, healthy 59 yo, M. Skin biopsy was taken ante-mortem.

* Fresh dermal fibroblasts were isolated from a skin biopsy

** Banked skin fibroblasts obtained from the Coriell Institute for Medical Research (Camden, NJ)