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The effect of gamma keratose on cell viability in vitro after thermal stress and the regulation of cell death pathway-specific gene expression

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ABSTRACT

When skin is thermally burned, transfer of heat energy into the skin results in the destruction of cells. Some of these cells are damaged but may be capable of self-repair and survival, thereby contributing to spontaneous healing of the wound. Keratin protein-based biomaterials have been suggested as potential treatments for burn injury. Isolation of cortical proteins from hair fibers results in an acid soluble fraction of keratin proteins referred to as "gamma" keratose. In the present study, treatment with this fraction dissolved in media was able to maintain cell viability after thermal stress in an in vitro model using primary mouse dermal fibroblasts. PCR array analysis demonstrated that gamma keratose treatment may assist in the survival and salvage of thermally stressed cells by maintaining their viability through regulation of cell death pathway-related genes. Gamma keratose may be a promising biomaterial for burn treatment that aids in spontaneous wound healing from viable tissue surrounding the burn.

1. Introduction

Transfer of the heat into tissue during thermal burning of the skin results in cell destruction and damage depending on several factors, including their distance from the thermal source. Complete tissue necrosis is often observed in the region in direct contact with the source, beginning with the surface of the skin and radiating downward into the tissue and outward. The tissue surrounding this necrotic zone can be termed a "thermally stressed zone," which has received the energy as an initiating factor, but is susceptible to several further insults that result in cell death [1]. Some damaged cells in this peripheral zone may be capable of self-repair and survival, if properly treated, thereby contributing to spontaneous healing of the wound. This phenomenon can be replicated in cell culture by models that utilize mild heating (i.e. 42–48 °C), resulting in a heterogeneous population of cells, some of which die as a direct result of the thermal injury and others that die secondary to the thermal insult due to the inflammatory cytokines released into the media. There are also some cells that survive both insults and are capable of self-repair and therefore, warrant closer examination as they may be able to contribute to spontaneous wound healing. Use of such cell culture models has been shown to induce various cell death pathways [2–8]. Depending on the intensity and duration of the exposure, apoptosis, necrosis or autophagy pathways are triggered. Apoptosis is a genetically programmed cell death mechanism that is associated with the activation of cysteine-dependent aspartate-specific proteases called caspases and is involved in the development of homeostasis [9]. Necrosis is morphologically characterized by cellular swelling, swelling of organelles, plasma membrane rupture and loss of intracellular contents [10]. Until recently, necrosis was considered to be non-programmed and unregulated cell death, but recent evidence indicates that programmed necrosis occurs through the activation of death receptors in a caspase-independent manner termed "necroptosis" [11]. Autophagy is a catabolic process involving degradation of the cell's own components and is usually activated by oxidative, nutritional or toxic stresses [12]. In contrast to other cell death pathways, autophagy reflects a cell survival mechanism by maintaining normal cellular function during nutrient deficient conditions or by removing damaged organelles and aggregated proteins [8,13].

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Numerous studies have been published in which in vitro models have been used to investigate thermally-induced cell death. In one study the log-phase cultures of mouse mastocytoma cells exposed to heat from 42 °C to 44 °C showed an increase in apoptosis; at 45 °C both necrosis and apoptosis were observed, and at 46° and 47 °C only necrosis was observed [2]. In another study, prolonged energy deprivation followed by thermal stress (43 °C for 10 min) of murine P_3 O_1 myeloma and Ehrlich ascites carcinoma cells induced high expression levels of heat shock protein 70 (Hsp70) that protected the cells from necrosis [4]. A third study used osteoblasts exposed to 48 °C for 10 min and showed a 15–20% increase in necrotic cells within minutes after heating. After a 12 h recovery at 37 °C, there was a 10% increase in apoptotic cells compared to control cells maintained at 37 °C, indicating that thermal stress induced necrosis in the early phase and apoptosis in the late phase [5]. These studies have shown that mild heating of cells in culture can induce both necrosis and apoptosis at different stages, thus serving as a basis for further investigation of these pathways at the cell and molecular level.

Our previous work has shown that gamma keratose, an acid soluble fraction of keratin biomaterial, was able to maintain cell viability post thermal stress compared to other keratin and control treatments in an in vitro model using primary mouse fibroblasts [14]. It was evident in these experiments that a large number of cells died from the initial heat treatment, additional cells died within hours after heating, and surviving cells re-established the culture. Thus, we concluded that all three pathways of cell death may be present in this cell culture model, and that keratin treatment may affect one or more of these pathways. In the present study, we hypothesized that treating primary dermal fibroblast cells with gamma keratose after thermal stress wound induce differential expression of genes related to the cell death pathways. A PCR array was used to investigate cell death pathway-related gene expression in thermally stressed cells treated with gamma keratose dissolved in the media compared to cells treated with fibroblast growth media only.

2. Methods

2.1. Fibroblast cell isolation

Primary mouse fibroblasts were isolated from adult CD1 mouse ear pads. The ear pads were incubated in dispase II (Sigma–Aldrich, St. Louis, MO) overnight and then the dermis was peeled off using forceps. The remaining tissue was minced before incubating in 0.35% type I collagenase (Sigma–Aldrich) reconstituted in DMEM high glucose (Invitrogen, Carlsbad, CA) for 30 min at 37 °C on a shaker. The suspension was filtered using a 100 micron meshed filter and the cells were pelleted by centrifugation. The cell pellet was washed once with Hank's balanced salt solution (HBSS; Invitrogen) to remove the residual collagenase. Finally, the fibroblast cells were seeded in a 150 mm tissue culture plastic (TCP; Fisher Scientific, Pittsburgh, PA) dish at 2.5×10^6 cells/mL in fibroblast growth media [DMEM high glucose plus 10% Fetal Bovine Serum (Hyclone® Laboratories, Inc., Logan, UT)] and 1% Antibiotic/Antimycotic solution (Hyclone® Laboratories).

2.2. Gamma keratose extraction

The starting keratin biomaterial was extracted from Chinese human hair using the oxidative protocol specified by de Guzman et al. [15]. The extracted keratin biomaterial was termed crude keratose. The gamma keratose fraction was further separated from crude keratose by isoelectric precipitation. Briefly, concentrated hydrochloric acid was added dropwise to the crude keratose solution with stirring until pH 4.2 is reached. At this pH, the alpha keratose precipitates leaving the acid-soluble gamma fraction in the supernatant. The insoluble alpha keratose was separated by fixed angle centrifugation at 1500 rpm for 15 min at 4 °C. After neutralizing the gamma-containing supernatant to pH 8.4, it was dialyzed against endotoxin free water using a 5 kDa nominal low molecular weight cutoff (NLMWCO) spiral wound cartridge (Millipore, Billerica, MA) for 5 volume washes. Finally, the solution was concentrated to minimal volume, pH adjusted to 7.4, lyophilized, and sterilized by gamma irradiation at 1 MRad prior to use.

2.3. In vitro thermal stress model

For this experiment, primary mouse fibroblasts between passages 3–6 were seeded at approximately 8×10^5 cells/mL in 100 mm TCP dishes. The cells were allowed to grow in fibroblast growth media until they were 95% confluent. Media changes were performed every 3 days. For a thermal stress experimental run, a media change was performed a day prior to heating the cells. On the day of the experiment, cultures were moved from a 37 °C, 5% CO_2 incubator to a 44 °C, 5% CO_2 incubator and heated for 150 min. After the heat treatment, culture dishes were returned to the 37 °C, 5% CO_2 incubator to allow re-attachment of cells and the media temperature to equilibrate. Six hours post heat treatment, cultures were randomized and treatment was applied in fresh media. Treatments included 0.1 mg/mL gamma keratose solubilized in fibroblast growth media and fibroblast growth media alone (control containing 10% FBS). The former will be termed gamma keratose treatment and the latter growth media treatment in the rest of this paper.

2.4. Trypan blue assay

Cell counting with a hemocytometer was performed by first staining the cells using 0.4% trypan blue reagent (Invitrogen, Gibco, Grand Island, NY), which stains cells with a disrupted plasma membrane. Cells were visualized under a microscope and counted at the 6hr, 12hr, 18hr and 24 h post heat treatment time periods. Out of two separate in vitro experiments performed, three cultures showing the highest percent cell viability after gamma keratose treatment and three cultures showing the lowest percent cell viability with growth media treatment were selected for further analysis using PCR arrays. Cell viability data from these samples was presented as mean ± SEM (standard error of the mean; Fig. 1).

2.5. RNA extraction & quantification

RNA extraction was performed using the 5 Prime Perfect Pure RNA cultured cell kit (5 Prime Inc., Gaithersburg, MD 20878). Briefly, after trypsinization and cell counting, the cell pellet obtained after centrifugation was lysed with a solution provided in the kit. The suspension was loaded onto a column with a filter and centrifuged, followed by two washes with wash solution and RNA elution from the column filter using an elution buffer. Extracted RNA was quantified using a Thermoscientific Nanodrop 2000 spectrophotometer (Fisher Scientific). Out of the two separate thermal stress experiments that were performed, RNA was extracted from three individual (10 cm) tissue culture plates for each treatment.

2.6. PCR arrays

cDNA synthesis was performed with the RT^2 First Strand Kit (SABiosciences, Valencia, CA) using the Veriti® 96 well thermocycler (Applied Biosystems, Foster City, CA) following the protocol specified by the manufacturer. After the cDNA synthesis was performed, the RT^2 Profiler ™ PCR array mouse cell death pathway finder (SABiosciences) was used to determine the expression of genes that are

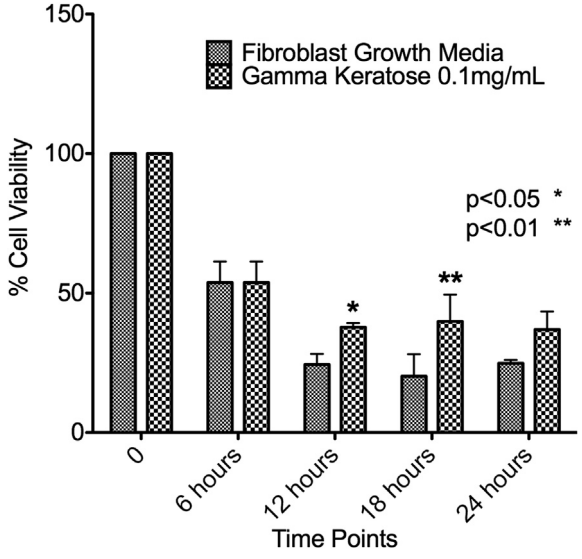

Fig. 1. In vitro Heat shock Model. 0 h and 6 h represent immediately pre- and post-heat shock, respectively; the two columns of data represent cells assigned to control and treatment groups, respectively, and were cultured in growth media until treatment began immediately after the 6 h equilibration period. Gamma keratose treated cells were able to maintain cell viability at 12 h, 18 h post-thermal stress compared to the fibroblast growth media treated cells with statistical significance. $P < 0.01^{**}$, $P < 0.05^{*}$.