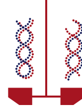
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Hydrocortisone and triiodothyronine regulate hyaluronate synthesis in a tissue-engineered human dermal equivalent through independent pathways

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Hydrocortisone (HC) and triiodothyronine (T3) have both been shown to be capable of independently inhibiting hyaluronate (HA, hyaluronic acid) synthesis in a self-assembled human dermal equivalent (human dermal matrix). We sought to investigate the action of these two hormones in concert on extracellular matrix formation and HA inhibition in the tissue engineered human dermal matrix. To this end, neonatal human dermal fibroblasts were cultured in defined serum-free medium for 21 days in the presence of each hormone alone, or in combination, in varying concentrations. Through a process of self-assembly, a substantial dermal extracellular matrix formed that was characterized. The results of these studies demonstrate that combinations of the hormones T3 and hydrocortisone showed significantly higher levels of hyaluronate inhibition as compared to each hormone alone in the human dermal matrix. In order to gain preliminary insight into the genes regulating HA synthesis in this system, a differential gene array analysis was conducted in which the construct prepared in the presence of 200 μg/mL HC and 0.2 nM T3 was compared to the normal construct (0.4 μg/mL HC and 20 pM T3). Using a GLYCOv4 gene chip containing approximately 1260 human genes, we observed differential expression of 131 genes. These data suggest that when these two hormones are used in concert a different mechanism of inhibition prevails and a combination of degradation and inhibition of HA synthesis may be responsible for HA regulation in the human dermal matrix.

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[**Key words:** Dermal equivalent; Hyaluronic acid; Tissue engineering; Hydrocortisone; Triiodothyronine; Gene array profiling]

Hormones are known to influence the development and function of human skin (1). Both thyroid hormones and various glucocorticoids have known effects on human skin (2,3). Triiodothyronine (T3) is the deiodinated and therefore activated form of thyroid hormone. Myxedema is the skin condition associated with hypothyroidism, resulting from the excessive deposition of hyaluronic acid (HA, hyaluronate) in the dermis (4). Glucocorticoids, widely used as topical agents in the treatment of inflammatory dermatological disorders are known inducers of dermal atrophy, resulting in part from inhibition of hyaluronate synthesis, and a decrease in the expression of collagens type I and type III (5–7). Glucocorticoids and thyroid hormone are also known to coregulate specific metabolic events (8). In specific cases, the addition of both hormones resulted in a synergistic effect, in which the presence of one can facilitate or enhance the action(s) of the other (9).

Previous research in our laboratory, aimed at producing dermal equivalents with varying amounts of hyaluronate, had demonstrated that both T3 and hydrocortisone (HC) at specific concentrations were capable of inhibiting hyaluronate synthesis in a self-assembled human dermal matrix (10,11). Preliminary gene array experiments suggested that T3 and HC may act on different enzymes in the hyaluronate biosynthetic pathway. We therefore posed the following questions: (i) Can higher levels of HA inhibition be induced in the human dermal matrix if combinations of the two hormones are used during maturation in culture? (ii) Are the same enzymes activated and responsible for this inhibitory process as previously observed, or does a different mechanism prevail when combinations of the two hormones are used? We designed experiments to answer these questions. To this end, neonatal dermal fibroblasts were cultured under serum free conditions and in the absence of a three dimensional matrix in the presence of each hormone alone and varying concentrations of combinations of the two (T3 and HC) (12,13). We chose the previously tested T3 concentration of 0.2 nM and two concentrations for HC, 500× of the base value (200 μg/mL) and a new concentration, previously untested, of 350× (140 μg/mL). Combinations of the two hormones in concert were then used at the following concentrations, 0.2 nM T3 + 350× HC and 0.2 nM T3 + 500× HC. We report that combinations of the hormones were able to induce significantly higher levels of HA inhibition in the human dermal matrix as compared to each hormone alone. Differential gene array experiments in which the 500× HC + 0.2 nM T3 construct was compared to the normal (0.4 μg/mL HC + 20 pM T3) construct demonstrated the differential expression of 131 genes; of these, 99 genes were up regulated and 32 genes were down regulated.

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MATERIALS AND METHODS

Production of human dermal equivalents Neonatal human dermal fibroblasts (ATCC) (passage 7) were harvested at confluence and seeded at high density (3 million cells/4.52 cm^2) on a porous polycarbonate membrane in a transwell format (Corning, Big Flats, NY, USA) for 21 days in the presence of serum-free medium. Specifically, the formulation contains the following ingredients: a base 3:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) (high glucose formulation, without L-glutamine) and Hams F-12 (Sigma–Aldrich, St. Louis, MO, USA) medium supplemented with 4 mM L-glutamax (Invitrogen, Carlsbad, CA, USA), 5 ng/mL epidermal growth factor (Millipore, Billerica, MA, USA), 0.4 μg/mL hydrocortisone (Sigma–Aldrich), 1 × 10^{-4} M ethanolamine (Sigma–Aldrich), 1 × 10^{-4} M O-phosphorylethanolamine (Sigma–Aldrich), 5 μg/mL insulin (Sigma–Aldrich) 5 μg/mL transferrin (Sigma–Aldrich), 20 pM triiodothyronine, 6.78 ng/mL selenium, 50 μg/mL L-ascorbic acid-2-phosphate (Asc-2-P) (Wako Chemicals, Richmond, VA, USA), 0.2 μg/mL L-proline, and 0.1 μg/mL glycine. The lower chamber was fed 3 mL and the upper chamber 2 mL of medium throughout the 21 day culture period with medium changes every two to three days. Hydrocortisone and T3 in varying concentrations were added to the medium using 95% ethanol as a cosolvent.

Cell count procedure Human dermal matrices were digested with collagenase IV (Sigma–Aldrich) for 1 h at 37°C in collagenase premix (120 mL of PBS, 14 mL of 2.5% trypsin and 1 mL 0.45% glucose) at the end of the 21 day culture period. Serum containing media was added to the digest to stop further enzymatic activity. Cells were diluted 1:1 with trypan blue (Sigma–Aldrich) and counted using a hemocytometer.

Determination of total collagen content Total collagen was quantified using the Hydroxyproline Assay according to previously published procedures (13,19).

Hyaluronic acid inhibition ELISA assay Human Dermal Matrix samples were digested with 0.5 mg/mL Proteinase K (Invitrogen) overnight at 60°C. The digested mixture was boiled for 10 min to inactivate the protease, spun down and the supernatant transferred to a fresh tube. The inhibition ELISA was carried out according to a previously published procedure (13,18).

Glycosaminoglycan disaccharide analysis by FACE Proteinase K digested samples of human dermal matrices were initially digested with 20 U/mL of hyaluronidase SD (Associates of Cape Cod) in 100 mM ammonium acetate, pH 7.00 for 2 h at 37°C followed by chondroitinase ABC (Associates of Cape Cod, East Falmouth, MA, USA) digestion for 3 h. The digests and disaccharide standards (Associates of Cape Cod) were subjected to reductive amination and conjugation with AMAC (2-aminoacridone) (Invitrogen) to produce fluorescent glycosaminoglycan (GAG) disaccharides. The labeled GAGs were subjected to polyacrylamide gel electrophoresis (PAGE) and imaged with a ChemiDoc XRS imager (BioRad, Hercules, CA, USA) to obtain the demonstrated profiles. For quantification, AMAC labeled glucose (Sigma–Aldrich) was used as a standard, Quantity One software (version 4.6.1) (BioRad) was used to determine various GAG disaccharide (ΔDi-HA, ΔDi-4S, ΔDi-6S, ΔDi-4,6S) values.

Histological processing Samples to be analyzed were fixed overnight in 10% phosphate buffered formalin (Thermofisher Scientific, Pittsburgh, PA, USA). The human dermal matrices were stored in vials containing 70% ethanol prior to embedding and processing. Samples were embedded into paraffin blocks, sectioned into 6 μm sections and stained with hematoxylin and eosin (H&E), imaged and photographed at 400× using a Westover Micromaster I microscope equipped with a digital camera.

Transmission electron microscopy Human dermal matrices (4.52 cm^2) were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH = 7.4 (Electron Microscopy Sciences, Hatfield, PA, USA), further processed, and subjected to transmission electron microscopy (TEM) analysis according to a previously published procedure (13).

Measurement of tissue thickness Thickness of Hematoxylin and Eosin stained cross sections of the human dermal matrices were measured by digital image analysis using Micron imaging software (one sample, different field of view). The measurements are made across the sections, perpendicular to the base of the construct. The reported thickness is a mean of 10 measurements ± SEM.

Statistical analyses Statistical analysis of the data was done using a one way ANOVA with equal variances assumed. Multiple comparisons were done by the Bonferroni method. Statistical Package for Social Sciences (SPSS) software was used for conducting the one way ANOVA significance tests. The significance level (α) used was 0.05. Probability values of $p < 0.05$ were considered statistically significant. Experiments were repeated three times, and data are expressed as mean ± standard error (SEM) unless otherwise indicated.

Total RNA extraction Total RNA was extracted from Human Dermal Matrix using the Qiagen RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA, USA). Dermal matrices were placed in a 15 mL centrifuge tube with 3 mL RLT buffer (provided in the kit) and 10 μL β-mercaptoethanol (Sigma–Aldrich) and homogenized using a TissueRuptor homogenizer (Qiagen) for 1 min. The homogenized tissue was treated with Proteinase K for 15 min at 55°C and subjected to centrifugation at 3500 ×g for 5 min at 20–25°C. The supernatant was transferred to a new 15 mL centrifuge tube and 0.5 volumes of ethanol (Sigma–Aldrich) was added to the lysate. The sample was then transferred to an RNeasy Midi spin column placed in a 15 mL collection tube, 3 mL at a time, and each time centrifuged for 5 min at 3500 ×g. The flow through was discarded. The column membrane was washed with RW1 wash buffer and centrifuged. DNAse digestion was carried out by addition of 160 μL of DNase I incubation mix (20 μL DNase I stock solution in 140 μL RDD buffer) to the spin column membrane for 18 min at room temperature. The column was washed with RW1 buffer and centrifuged for 5 min at 3500 ×g. Two other washes were also carried out with RPE buffer. The RNA was eluted by adding 150 μL RNAse free water twice directly to the spin column membrane and centrifuged each time for 3 min at 3500 ×g. The RNA samples were monitored with the 2100 Agilent Bioanalyzer, consistently demonstrating high quality RNA as evidenced by the 28S/18S ratio of approximately 2.

Gene array analysis The GlycoV4 oligonucleotide array is a custom Affymetrix GeneChip (Affymetrix, Santa Clara, CA, USA) designed for the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/consortium.shtml). A complete description and annotation for the GlycoV4 array is available at the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/consortium/resources.shtml).

The GlycoV4 focused array includes probes for ~1260 human probe-ids and ~1200 mouse probe-ids related to glyco-genes. This array does not contain mismatched probes.

Data normalization was performed using RMA Express 1.0 with quantile normalization, median polish and background adjustment (14,15).

The Limma package in the R software was used to find transcripts with differential expression (16,17). The fold changes and standard errors were estimated by fitting a linear model for each gene and empirical Bayes smoothing was applied to the standard errors. Results are presented between two or more experimental

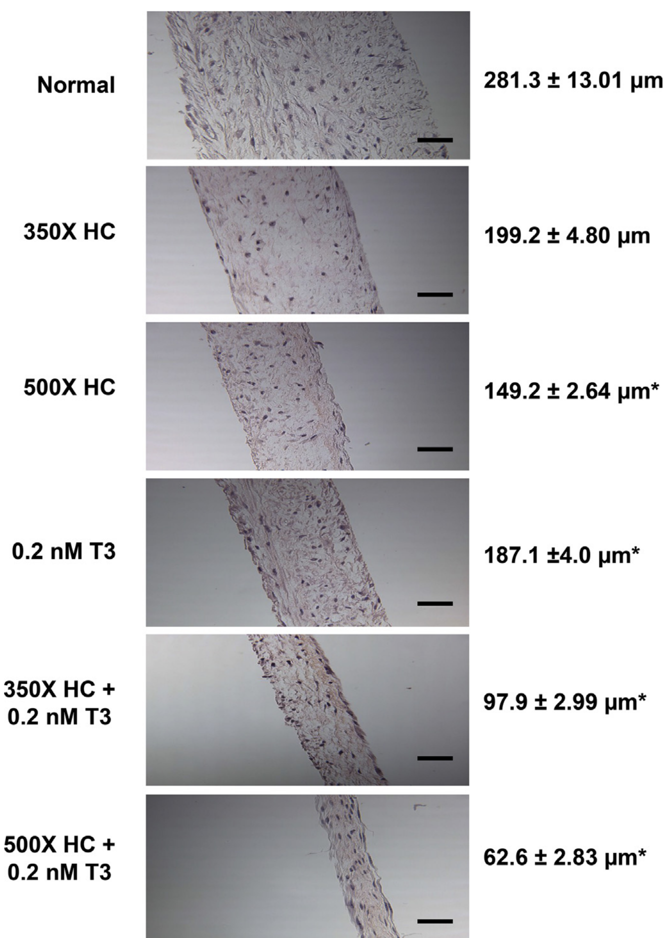

FIG. 1. Hematoxylin and Eosin (H&E) stained sections of human dermal matrices grown in the presence of varying concentrations of hydrocortisone, T3 and combinations indicated. The normal construct is grown in the presence of 0.4 μg/mL HC and 20 pM T3, other concentrations are stated as a multiple of this base value for HC and at 10× for T3, which corresponds to 0.2 nM (shown on the left of the sections). The thickness of the constructs is shown on the right of the H&E sections. * p-value $<$ 0.05 for comparison with normal construct thickness using one way ANOVA at $\alpha = 0.05$; bars 50 μm.

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