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Article

Varying growth hormone levels in chondrocytes increases proliferation rate and collagen production by a direct pathway

Illiana Bennett, Leya Joykutty
American Heritage School, Plantation, Florida

SUMMARY

The purpose of this project was to test whether growth hormone directly or indirectly affected the rate at which cartilage renewed itself. Growth hormone could exert a direct effect on cartilage or chondrocytes by modifying the expression of different genes, such as c-myc proto-oncogene, which in return increases the proliferation rate of the cells. In contrast, an indirect effect comes from growth hormone stimulating insulin-like growth factor. The results from this research supported the hypothesis that growth hormone not only increases proliferation rate but does so using the direct pathway. Out of the different levels of growth hormone that were tested, it was found that 0.300 µg/ml had the greatest effect since those samples had the highest collagen concentrations and reached confluency in T75 cell culture flasks in the least amount of time. This research can be used in the medical sciences for people who suffer from joint damage and other cartilage-related diseases, since the results demonstrated conditions that lead to increased proliferation of chondrocytes. These combined results could be applied in a clinical setting with the goal of allowing patient cartilage to renew itself at a faster pace, therefore keeping those patients out of pain from these chondrocyte-related diseases.

INTRODUCTION

Chondroblasts are the unmatured version of chondrocytes, or cartilage cells. These cells, chondroblasts, secrete the matrix that makes up cartilage. Eventually, these cells become embedded in this cartilage, mature and stop matrix production. The extracellular matrix that these cells produce is extremely important because it is what allows cartilage to serve its functions within the body, which include providing structural integrity, protecting the ends of bones that meet at joints, and making up body parts like the ear and nose. In order to fulfill these roles, cartilage is dependent on the amount of water that is found in its fibers. The protein aggrecan is only found in cartilage and is responsible for producing the high water content that is located in it; the water levels in cartilage can be up to 75% at any given time. Cartilage holds these aggrecan molecules by using collagen fibers, these fibers have a very high strength and they use this to trap the aggrecan inside the cartilage (1).

Growth hormones are the factors in the human body that stimulate this cartilage growth, mostly via indirect pathways. The growth hormones influence the chondrocytes indirectly through the use of insulin-like growth factor-I (IGF-I). IGF-1 is a protein-peptide hormone whose main purpose is supporting cellular division and growth (2). IGF-I is secreted by the liver and contributes to most of the effects caused by growth hormones. Although this model of indirect stimulation has been the main mechanism proposed to explain how growth hormones influence chondrocytes and the growth of cartilage, there have been a few studies suggesting that growth hormones may also directly stimulate chondrocytes. One way this could occur is through increasing the expression of c-myc proto-oncogene (*MYC*) (3). Proto-oncogenes are genes that encode for many proteins that control aspects of cell growth and proliferation, and include genes such as p53 (4). The increased expression of this gene, and other genes similar to it, would be induced directly by the growth hormone.

Previous research has demonstrated that, under normal conditions, approximately 1 mg of growth hormone (5) is released to all body parts where it is needed. For this project that daily amount of growth hormone was divided among an approximate amount of all the places this growth hormone travels in a day, which came to about 0.250 µL.

The first assay used is a cell counting assay, this type of method is used to quantify the amount of viable cells in a sample. This is important because one has to know the amount of cells expected in the outcome of the experiment to perform the test with accuracy and to receive accurate results. There are two main types of cell counting assays, manual and automated. Automated cell counting uses image cytometry, which is used to analyze individual adherent cells (6). On the other hand manual cell counting is the method used in this experiment and consists of using the human perception of the cell when counting. For this project the manual cell counting method was used due to limitations in budget and accessibility.

The second assay used is a Collagen IgG assay, a form of the Enzyme-linked immunosorbent assay or ELISA. This specific type of ELISA kit tests for collagen type II antibody in plasma and serum. The reason for the use of this assay is due to collagen type II being a gene product of some pathways that the growth hormone should directly affect. This means that where there is an increase in the concentration of Collagen it corresponds to an increase in the expression of those genes.

From this background research it was concluded that the

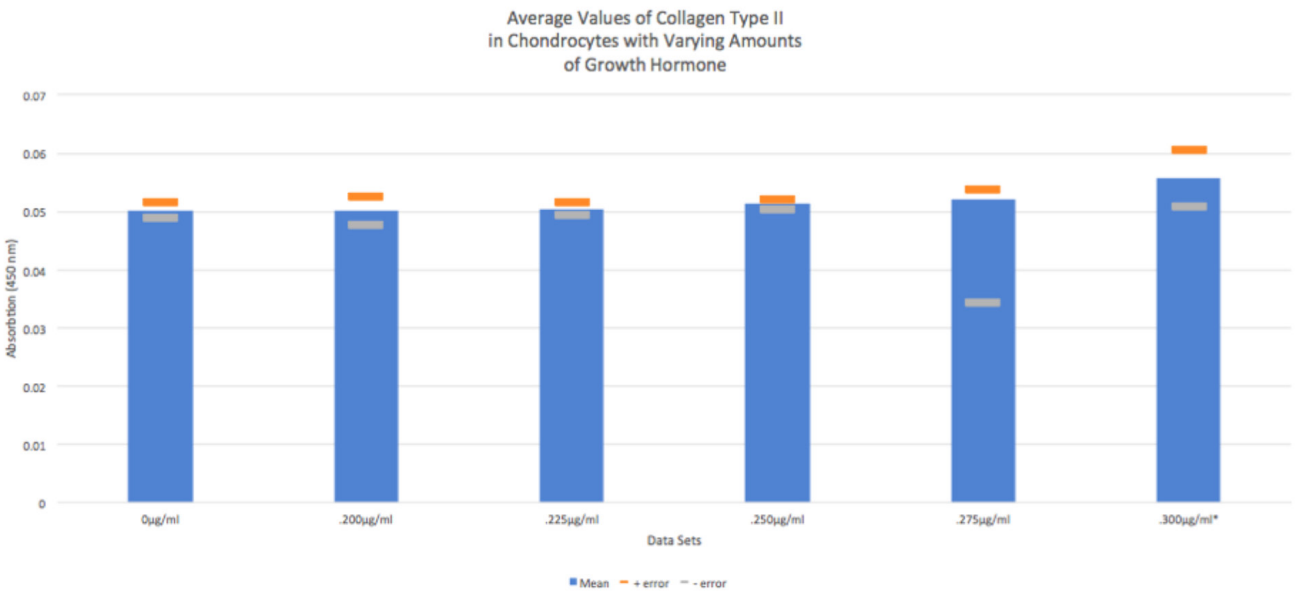

Figure 1. Average values of type II collagen in chondrocytes with varying amounts of growth hormone. Chondrocytes were treated with varying levels (0.200, 0.225, 0.250, 0.275, 0.300, 0 µg/mL) of growth hormone and concentrations of collagen were measured based on absorbance (450 nm). Each bar in the graph represents the average of ten trials. Error bars represent the margin of error for each data set. Experimental groups were compared via a t-test and asterisks (*) represent $p < 0.05$.

cell counting assay was needed to support the hypothesis of the growth hormone increasing the cell proliferation in chondrocytes. It was also concluded that the collagen IgG assay kit was needed to support the hypothesis stating that growth hormone uses the direct pathway of increasing c-myc proto-oncogene expression to increase the cell proliferation. With those assays it was drawn that increasing levels of growth hormone increased chondrocyte proliferation as well. It was also shown that this increased proliferation uses the growth hormone's direct pathways as collagen concentration increases in correspondence to growth hormone level increase.

Therefore, the purpose of this study was to determine whether increased levels of growth hormones could directly increase the rate that cartilage renewed itself through the expression of *MYC*. If this direct effect is indeed occurring, it was expected that when varying concentrations of growth hormone (0.200, 0.225, 0.250, 0.275, 0.300, 0 µg/mL) are added to chondrocytes, the chondrocytes that have a concentration of growth hormone at 0.250 µg/ml added will have the greatest increase of cell proliferation and collagen production. Additionally, it was expected that if the varying levels of growth hormone are added to samples of chondrocytes, then the growth hormone will have a direct effect on the increase of cell proliferation and collagen production in the chondrocytes.

RESULTS

Chondrocytes were treated with different concentrations of growth hormone, after which collagen levels were measured via an ELISA-based Collagen IgG assay. Samples that were treated with increasing concentrations of growth hormone showed an increasing collagen concentration (**Figure 1**). This suggests that growth hormone is able to use a direct pathway to increase collagen production, rather than solely relying on the indirect pathway.

The statistical significance of the data sets can also be seen in this figure. One of the data sets that has the most statistical significance in correspondence to the control of 0 µg/ml of growth hormone is the sample with 0.300 µg/ml of growth hormone. This sample had a *p*-value less than 5% or 0.05 and as seen in the figure it is the only sample that is noticeably different than the control.

Even with 0.300 µg/ml being the only statistically significant treatment group, the hypothesis stating that growth hormone has a direct effect on the proliferation of chondrocytes is still supported since there is an increase in the concentration of type II collagen.

A cell counting assay was also performed to quantify the number of viable cells in each sample and gain insight into the effect of growth hormone on cell proliferation. Similar to the findings in the previous experiment, treatment with higher concentrations of growth hormone led to increased cell proliferation (**Table 1**).

DISCUSSION

The second hypothesis, that growth hormones have a direct effect on collagen production, was supported with the outcome from the Collagen IgG Assay, since as more growth hormone was added to chondrocytes, the concentration of collagen increased. Although only the samples treated with the highest concentration of growth hormone showed

Growth Hormone (µg/ml)	Trial 1	Trial 2	Trial 3
0.000	800,000	812,500	700,000
0.200	850,000	875,000	862,500
0.225	900,000	1,000,000	912,500
0.250	1,025,000	1,080,000	1,000,000
0.275	1,137,500	1,100,000	1,125,000
0.300	1,250,000	1,187,500	1,225,000

Table 1. Number of cells per mL for varying levels of growth hormone across three trials.

collagen levels that were statistically significantly higher than the untreated condition, the positive correlation between growth hormone concentration and collagen amount was consistent across all samples.

However, the first hypothesis was not supported, since 0.250 µg/ml of growth hormone was not the amount that induced gene expression the most (seen through increased collagen levels). It was expected that the results from the collagen IgG assay would produce a bell-shaped curve, with the concentration of collagen increasing until a certain point (hypothesized to be 0.250 µg/ml of growth hormone), and then decreasing until it got to 0.300 µg/ml. This, however, was not the case and the point at which growth hormone has the greatest effect on collagen production and cell proliferation has not been found yet.

The chondrocytes used in this project (murine-derived) took on average four days to reach confluency. However, when growth hormone was added, the average dropped to one day for the cultures that had 0.300 µg/ml of growth hormone added. Growth hormone also had an impact on the proliferation rate with the other samples; for example, the samples treated with 0.250 µg/ml of growth hormone took an average of two days to reach confluency (compared to an average of four days for control samples).

One of the difficulties encountered during this study was finding an affordable source of chondrocytes that would leave a budget for other materials. The cells that were used were sourced from an outside lab. Towards the end of the study, some of the cells were contaminated; this was most likely due to factors in the lab or an error when the protocol was carried out. Since this only happened toward the end of the project and it was the same protocols being repeated since the beginning, it was most likely an environmental factor that caused the contamination.

The research conducted in this study could be applied to the medical side of science for individuals that suffer from joint disorders or malfunction. This is because the results from this research show that growth hormone has a direct effect on the cell proliferation of chondrocytes. This suggests that using growth hormones locally for regenerating cartilage may be more effective than originally assumed, since growth hormone does not necessarily have to go through the secondary messenger of insulin-like growth factor. Our results also showed that growth hormone increased the rate at which chondrocytes reproduced or proliferated, which could be applied to increase the speed at which cartilage renews itself. This increase in efficiency and rate of renewal could guide therapies for these cartilage disorders or malfunctions, since pain occurs at sites where cartilage has worn away, causing bones to grind against each other. Increasing the rate that cartilage renews itself could help produce this cartilage faster and therefore decrease the pain from where the cartilage was lost.

Future studies could test chondrocyte samples with growth hormone concentrations that are higher than 0.300 µg/ml in order to find the point at which the effect that the growth hormone has on chondrocytes plateaus and then eventually starts to decrease. Completing this bell curve and then finding the highest peak of that curve would help to optimize the effect that growth hormone has on chondrocytes. Ultimately, this would show the level at which growth hormone could be added to cartilage and have the greatest effect on renewing or regenerating the cartilage that was lost.

METHODS

The chondrocyte cell line was murine-derived from the European Collection of Authenticated Cell Cultures.

Cell Culture Medium

In a Biological Safety Cabinet (BSC) 40 mL of DMEM was added to a 50 mL sterile test tube. 0.4 mL of Penicillin Streptomycin, 0.8 mL of L. Glutamine and 4 mL of heat inactivated Fetal Bovine serum was then added to the test tube. For the cells treated with growth hormone, growth hormone (ThermoFisher Scientific, 200 µg/ml) was added to the medium for a final concentration of 0.200 µg/ml, 0.225 µg/ml, 0.250 µg/ml, 0.275 µg/ml, or 0.300 µg/ml.

Cell Counting Assay

Sterile cell culture technique was maintained and cells were grown at 37°C. Cells were checked under a microscope for any signs of bacterial or fungal contamination. Existing media was removed and flasks were washed with 2-5 mL of phosphate buffer saline (PBS). 5 mL of culture medium was added to the cell culture flask and the cells were detached using a sterile cell scraper. Cells were centrifuged for 5 minutes at 1200 RPM and then the supernatant was removed. Cell pellets were then resuspended with 5 mL of culture medium, and 0.5 mL was taken and transferred into a sterile Eppendorf tube. Cells were diluted in Trypan Blue in a 1:5 dilution. 100 µL of the Trypan Blue/cell suspension solution was taken and carefully pipette into the counting well of a hemocytometer and then the cells were viewed under a 10X microscope. The cell concentration was found by taking the average number of viable cells in the four sets of 4x4 grids and multiplying that by 10000 to get the number of cells per

milliliter. This was then multiplied by 5 to correct from the 1:5 dilution of the Trypan Blue. This assay was replicated three times for each of the data sets in the experiment.

Collagen IgG Assay Kit: ELISA Buffer Preparation

10 mL of ELISA Buffer Concentrate (10X) was diluted with 90 mL of UltraPure water.

Wash Buffer Preparation

5 mL of Wash Buffer Concentrate (400X) was diluted to a total volume of 2 L with UltraPure water. 1 mL of Polysorbate 20 was then added to the solution.

Mouse Anti-type II Collagen Polyclonal IgG Standard Preparation

The contents of the Mouse Anti-type II Collagen Polyclonal IgG standard was reconstituted with 1 mL of 1X ELISA buffer. The solution was mixed gently and labeled Solution #1. Seven clean test tubes were obtained and labeled #2 through #8. 500 µL of 1X ELISA Buffer was aliquoted into tubes 2-8. 500 µL from Solution #1 was transferred into tube 2 and was mixed gently. Next, 500 µL from tube #2 was transferred into tube #3 and mixed gently. This process was repeated for tubes #4 through #7, and tube #8 had no antibody added to it.

Anti-Mouse IgG/HRP Conjugate Preparation

A working solution was created by the addition of 0.6 mL of Anti-Mouse IgG/HRP Conjugate to an 11.4 mL Assay Buffer (12 mL total).

Plate Preparation and Reading

100 µL of the standards was added to the appropriate well on the Bovine Type II Collagen Precoated ELISA Strip Plate. The plate was then covered with a 96-well cover sheet and incubated for two hours at room temperature on an orbital shaker. The wells were then emptied and rinsed completely four times with the wash buffer made previously (for complete wash, the wells were filled completely and emptied; after the last rinse, the plate was gently tapped on an absorbent paper to collect residual wash buffer). Next, 100 µL of the diluted Anti-Mouse IgG/HRP Conjugate was added to each well on the plate and the plate was covered and incubated for one hour at room temperature on an orbital shaker. The wells were then emptied and rinsed four times with wash buffer. 100 µL of TMB substrate solution was added to each well of the plate and the plate was covered with a 96-well cover sheet and incubated for 15 minutes at room temperature in the dark. After this time the development of the plate was read at 650 nm and 100 µL of stop solution was added to the plate when Solution #1 had a value of 0.5 through 0.6. The underside of the plate was wiped with a clean tissue to remove fingerprints, dirt, etc. and the plate was read at a wavelength of 450 nm. This assay was replicated with ten trials with each of the six data sets in the experiment.

Statistical Analysis

First, the means of each of the data sets were obtained (0 µL, 2 µL, etc.). With that information, the standard deviation of the different data sets was found. A t-test was then performed to assess the statistical significance of the data sets with the *p*-value found. Finally, the margin of error was calculated for each of the data sets. All statistical analysis was conducted using Excel functions.

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