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The effect of hydrocortisone on angiogenesis

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Abstract. Although there are several known inhibitors and stimulators of angiogenesis, the exact choreography of this multi-step process remains unknown. The interaction and implications of increased hydrocortisone concentration during this process is also unknown. This study aimed to examine the effects of different hydrocortisone concentrations on angiogenesis. The hypothesis of this study was that increased concentrations of hydrocortisone would lead to a greater degree of angiogenesis. A co-culture of human umbilical vein endothelial cells was used to analyze the process of angiogenesis. Three levels of hydrocortisone were applied to the cells to determine the extent of angiogenesis: a mid-range physiologic level, an upper-range physiologic level, and an elevated level. The increased levels of hydrocortisone did not inhibit or stimulate angiogenesis to a greater degree than the mid-range physiologic level.

Introduction

Angiogenesis is the process by which new blood vessels are formed from pre-existing vasculature. The process begins as endothelial cells (ECs) fuse to and invade a permeable basement membrane. The ECs migrate through the membrane into the interstitial space before forming new tubule structures (Jain et al., 1997; Figure 1). Complete comprehension of the precise mechanisms and chemical interactions involved in the process of angiogenesis is limited (Battegay, 1995) due to the multitude of mechanisms involved (Folkman and Shing, 1992) and the vast complexity of the process.

Angiogenesis is commonly involved in routine physiological processes such as the regen-

eration of the uterine lining, embryonic development (Battegay, 1995; Saladin, 2004), and wound healing (Ausprunk and Folkman, 1977; Battegay, 1995). Angiogenesis is also involved in pathological processes such as atherosclerosis, diabetic retinopathy, rheumatoid arthritis, tumor growth (Bussolati et al., 2001), metastasis (Gulec et al., 2001; Auerbach et al., 2003), psoriasis (Wang et al., 2004), polyarthritis, endometriosis, and retrolental fibroplasia of premature babies (Jain et al., 1997).

The calculated density of the microvessels in the microcirculatory system can be an indicator of the extent to which angiogenesis occurs in either physiological or pathological processes. Folkman (2001) defined microvessel density (MVD) as a count of vessels in a specific field of vision being determined by intercapillary distance.

Given the physiologic and pathologic importance of angiogenesis, it is important to develop assays that closely resemble the process *in vivo*. Though *in vivo* assays provide the most accurate representation there is notable expense, time,

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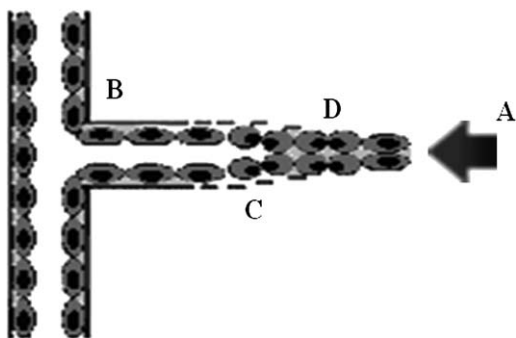


Figure 1. A) angiogenic stimulator acts to weaken the vessel wall which allows the ECs to fuse to and invade the basement membrane, B) ECs differentiate, C) ECs migrate in the direction of the angiogenic stimulus, D) ECs proceed through cellular division to build a new tubule structure (Protein Sciences Corporation, 2004).

and training required. Therefore, in an effort to determine the most accurate *in vitro* assay, Donovan et al. (2001) compared three *in vitro* assays: the Matrigel assay, a growth factor reduced Matrigel assay, and a co-culture assay. The results of this study indicated that the morphological features exhibited in the co-culture assay most closely resemble that of the physiologic environment *in vivo*.

Hydrocortisone is a glucocorticoid released by the zona fasciculata layer of the adrenal gland. The release of hydrocortisone is controlled by the pulsating release of adrenocorticotrophic hormone (ACTH) which exhibits a circadian rhythm (Adams, 1995). Under non-stressful conditions, hydrocortisone is produced at a rate of about 1 mg/kg body mass per day (Adams, 1995).

At any given time the concentration of hydrocortisone can be measured either in serum or in saliva. Most of the hydrocortisone found in serum is bound to proteins, 60% to transcortin and 30% to albumin (Aardal and Holm, 1995). Aardal and Holm (1995) determined that the concentration of hydrocortisone in serum and saliva samples at 8 am ranges from 3.5 to 27.0 nmol/L; they also determined that the hydrocortisone concentration at 10 pm is <6.0 nmol/L.

Given the physiologic impact of hydrocortisone and the complexity of angiogenesis, the objective of this study was to examine the effect of a mid-range physiologic level, an upper-range physiologic level, and an elevated level of hydrocortisone on angiogenesis.

Materials and Methods

Assay

Materials for each trial included a co-culture angiogenesis assay of human umbilical vein endothelial cells (HUVECs) with other human cells in a specialized medium. All of these materials were obtained from TCS CellWorks (Botolph Claydon, UK). Each 24-well plate was divided into four groups: three wells to test each of the controls (one known stimulator and one known inhibitor) and six wells to test each of three different concentrations of hydrocortisone. Vascular endothelial growth factor was used as the positive control substance at a concentration of 10 ng/mL. Suramin was used as the negative control substance at a concentration of 2.856×10^7 ng/mL. The three hydrocortisone concentrations represented a mid-range morning physiologic level (5 ng/mL), an upper-range morning physiologic level (10 ng/mL), and an elevated concentration (15 ng/mL).

Upon arrival of each assay kit, the cells were examined to confirm viability. The cells were then incubated overnight at 5% CO₂ and approximately 37 °C. The following day (Day 1), new medium, with the addition of the appropriate amount of hydrocortisone and control substances, was equilibrated to the conditions of the incubator. The original medium was aspirated from each of the wells and replaced with new medium. Following the addition of new medium, to a total volume of 0.5 mL, the cell plate was returned to the incubator at approximately 37 °C and 5% CO₂. The same procedure for removing and applying medium was followed on Days 4, 7, and 9. On Day 11, the medium was aspirated and the cells were washed with Dulbecco's Phosphate-Buffered Saline before being fixed with ice-cold 70% ethanol. The tubule formations were visualized by staining for platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31. The cells were incubated with Dulbecco's Phosphate-Buffered Saline substituted with 1% BSA (Blocking Buffer) for 30 minutes before mouse anti-human CD31, diluted in the Blocking Buffer, was added to each of the wells and then incubated for one hour at 37 °C. Following this incubation period, the

cells were washed with the Blocking Buffer before a goat anti-mouse IgG alkaline phosphatase conjugate was applied and incubated with the cells for one hour at 37 °C. The staining substrate was prepared by dissolving two 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets in 20 mL of distilled H₂O. The cells were incubated with the staining substance until the tubules were dark purple in color. Then, the staining substance was decanted and the cells were washed. Digital images were obtained and analyzed as described below.

Image analysis

For each well, one randomly selected, standard-sized field of view was photographed using a Scalar USB Microscope M2 set at 50x magnification and the associated software program. The images were saved as JPEG files and then converted to TIFF files. The formation of tubules was analyzed using software available from the National Institutes of Health. NIH Image, the Macintosh version, is available for download from <http://rsb.info.nih.gov/nih-image/download.html>. Scion Image, the PC version, is available for download from <http://www.scioncorp.com/>.

Each image was opened in the software program as a black and white image. The image was then converted from stacks to windows and colored to obtain the best contrast between tubule structures and the background. To analyze the extent to which angiogenesis occurred, the number of bifurcation points shown in each image was counted as well as the number of tubules. The tubules were also traced to measure their length in arbitrary units. The numerical data collected from this procedure were copied to a Microsoft Excel file for further analysis.

Statistics

The data collected through the measuring process was statistically analyzed using SPSS 13.0 for Windows. A one-way ANOVA was performed for each of the three types of measurements: number of bifurcation points, number of tubules, and total length of tubules. A Post Hoc Tukey test was performed if a significant differ-

ence was determined by the one-way ANOVA. The mean difference was significant at the $P = 0.050$ level; the mean difference was marginally significant at the $P = 0.075$ level.

Results

The digital images captured for each well were used to count the number of bifurcation points formed, the total number of tubules, and to measure the length of each tubule structure. The vast contrast in growth between the two trials was very apparent in these images.

Number of bifurcation points

The average number of bifurcation points in Round 1 for the hydrocortisone concentrations of 15 ng/mL, 10 ng/mL, and 5 ng/mL were 29.50 +/- 19.69, 30.50 +/- 18.63, and 51.17 +/- 21.23, respectively. The test group concentrations in Round 2 resulted in an average of 3.33 +/- 1.63 bifurcation points for the 15 ng/mL concentration, 6.50 +/- 2.59 for the 10 ng/mL concentration, and 3.33 +/- 2.16 bifurcation points for the 5 ng/mL concentration.

There was no significant difference among any of the test groups within Round 1 of data collection (ANOVA, $P = 0.137$). However, there was a significant difference among the test groups within Round 2 (ANOVA, $P = 0.034$). This significant difference was found between the 10 ng/mL test concentration group and both the 15 ng/mL and 5 ng/mL concentration groups (15 ng/mL, Tukey, $P = 0.056$; 5 ng/mL, Tukey, $P = 0.056$). When including the known negative control (suramin) and positive control (VEGF) in the comparison analysis, there was a significant difference between the two controls in both rounds of data collection (Round 1, Tukey, $P = 0.001$; Round 2, Tukey, $P = 0.000$). The average number of bifurcation points in the suramin wells in Round 1 of data collection was 1.67 +/- 1.15. The average number of bifurcation points in the VEGF wells in Round 1 of data collection was 99.00 +/- 49.96. Round 2 of data collection resulted in an average of 3.00 +/- 2.00 bifurcation points in the suramin wells and 25.67 +/- 6.66 bifurcation points in the VEGF wells. In

Round 1, there was a marginally significant difference between both of the controls and the 5 ng/mL concentration (suramin, Tukey, $P = 0.060$; VEGF, Tukey, $P = 0.072$). In Round 1 there was also a significant difference between the positive control and the two higher test concentration groups (15 ng/mL, Tukey, $P = 0.005$; 10 ng/mL, Tukey, $P = 0.006$; Figure 2). In Round 2, there was a significant difference between the VEGF and each of the test concentrations (15 ng/mL, Tukey, $P = 0.000$; 10 ng/mL, Tukey, $P = 0.000$; 5 ng/mL, Tukey, $P = 0.000$). The mean number of bifurcation points in the Round 2 suramin wells did not differ significantly from any of the test concentrations (15 ng/mL, Tukey, $P = 1.000$; 10 ng/mL, Tukey, $P = 0.474$; 5 ng/mL, Tukey, $P = 1.000$; Figure 3).

Number of tubules

When considering the mean number of tubules formed, there was no significant or marginally significant difference among the test concentration groups (Round 1, ANOVA, $P = 0.303$; Round 2, ANOVA, $P = 0.782$). The average number of tubules in Round 1 was 147.00 +/- 50.43, 106.83 +/- 40.22, and 125.33 +/- 38.26 for the 15 ng/mL, 10 ng/mL, and 5 ng/mL concentration groups, respectively. The average number of tubules in Round 2 was 83.50 +/- 9.07, 89.83 +/- 18.44, and 85.83 +/- 17.79 for the 15 ng/mL, 10 ng/mL, and 5 ng/mL test groups, respectively.

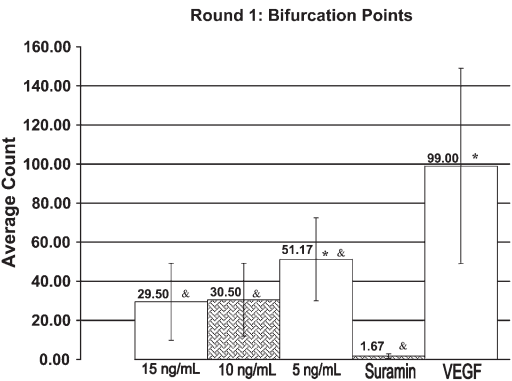


Figure 2. Comparison of the mean (+/- SD) number of bifurcation points found in suramin, VEGF, and each of the test groups in Round 1. (*) indicates a significant or marginally significant difference from suramin; (&) indicates a significant or marginally significant difference from VEGF.

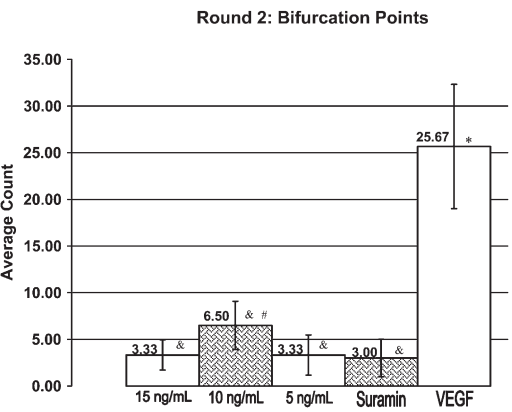


Figure 3. Comparison of the mean (+/- SD) number of bifurcation points found in suramin, VEGF, and each of the test groups in Round 2. (*) indicates a significant or marginally significant difference from suramin. (&) indicates a significant or marginally significant difference from VEGF. (#) indicates a significant or marginally significant difference from other test concentrations.

The average number of tubules formed in the suramin-treated wells was 52.67 +/- 9.29 for Round 1 and 61.00 +/- 2.65 for Round 2. In the VEGF-treated wells, there was an average of 227.33 +/- 22.94 and 137.00 +/- 14.42 tubules formed for Rounds 1 and 2, respectively. With regards to the mean number of tubules, the difference between the two control groups was significant in both rounds of data collection (Round 1, Tukey, $P = 0.000$; Round 2, Tukey, $P = 0.000$). When comparing each of the controls to the test concentrations in Round 1, there was a significant difference between suramin and the 15 ng/mL concentration (Tukey, $P = 0.022$). There was also a significant difference between VEGF and both of the lower concentration groups (10 ng/mL, Tukey, $P = 0.003$; 5 ng/mL, Tukey, $P = 0.012$). The difference between VEGF and the 15 ng/mL concentration was only marginally significant (Tukey, $P = 0.063$). In Round 2, there was a significant difference between VEGF and each of the test concentration groups (15 ng/mL, Tukey, $P = 0.001$; 10 ng/mL, Tukey, $P = 0.002$; 5 ng/mL, Tukey, $P = 0.001$; Figure 4). The mean number of tubules in the wells treated with suramin did not differ significantly from any of the test concentration wells in Round 2 (15 ng/mL, Tukey, $P = 0.237$; 10 ng/mL, Tukey, $P = 0.080$; 5 ng/mL, Tukey, $P = 0.163$; Figure 5).

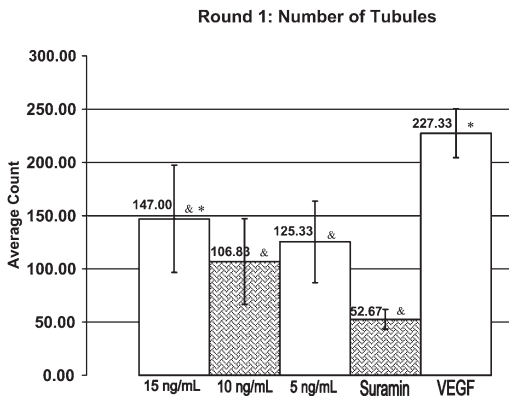


Figure 4. Comparison of the mean (\pm SD) count of tubules in suramin, VEGF, and each of the test groups in Round 1. (*) indicates a significant or marginally significant difference from suramin; (&) indicates a significant or marginally significant difference from VEGF.

Tubule length

Similar to the mean number of tubules, there was no significant difference in average tubule length among the three hydrocortisone concentrations in either round of data collection (Round 1, ANOVA, $P = 0.721$; Round 2, ANOVA, $P = 0.216$). The average length of tubules for each of the three test concentrations in Round 1 was as follows: 57.66 ± 26.13 (15 ng/mL), 46.95 ± 22.42 (10 ng/mL), and 54.62 ± 21.35 (5 ng/mL). In Round 2, the average length of tubules was 19.97 ± 2.34 , 24.15 ± 6.16 , and 25.57 ± 6.81 for the 15 ng/mL, 10 ng/mL, and 5 ng/mL concentrations, respectively.

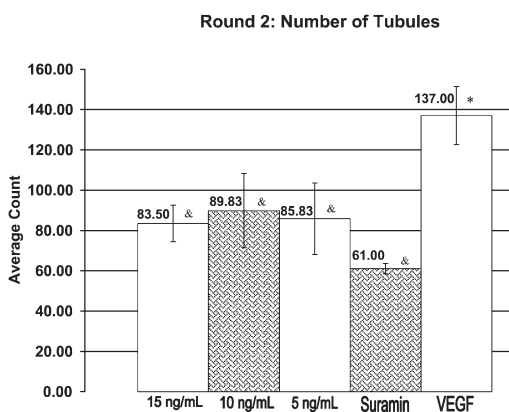


Figure 5. Comparison of the mean (\pm SD) count of tubules in suramin, VEGF, and each of the test groups in Round 2. (*) indicates a significant or marginally significant difference from suramin; (&) indicates a significant or marginally significant difference from VEGF.

When including the mean tubule length of the suramin-treated wells and the VEGF-treated wells in the Tukey analysis, there was a significant difference, in both rounds of data collection, between these two controls (Round 1, Tukey, $P = 0.001$; Round 2, Tukey, $P = 0.000$). In Round 1, there was also a marginally significant difference between the suramin and the 15 ng/mL concentration (Tukey, $P = 0.067$). The VEGF differed significantly from the 10 ng/mL concentration (Tukey, $P = 0.021$) and marginally from the 5 ng/mL concentration (Tukey, $P = 0.060$; Figure 6). The second round of data collection resulted in a significant difference between VEGF and each of the test concentration groups (Tukey, 15 ng/mL, $P = 0.000$; 10 ng/mL, $P = 0.000$; 5 ng/mL, $P = 0.000$). In Round 2, there was no significant difference between the suramin-treated wells and those treated with the 15 ng/mL concentration (Tukey, $P = 0.653$) or the 10 ng/mL concentration (Tukey, $P = 0.140$). There was, however, a marginally significant difference between the suramin-treated wells and those treated with the 5 ng/mL concentration (Tukey, $P = 0.069$; Figure 7).

Discussion

In an effort to enhance the statistical validity of the experiment, two rounds of data were collected using the same experimental design and

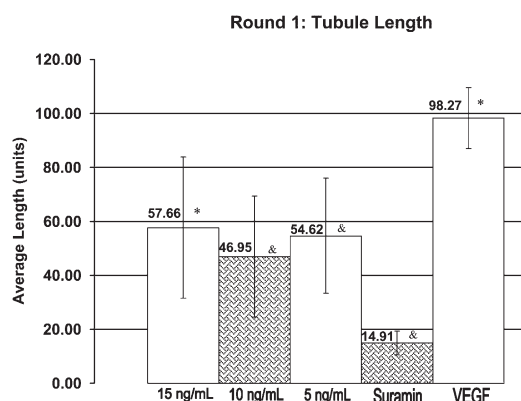


Figure 6. Comparison of the mean (\pm SD) tubule length in the wells treated with suramin, VEGF, and each of the hydrocortisone concentrations in Round 1. (*) indicates a significant or marginally significant difference from suramin; (&) indicates a significant or marginally significant difference from VEGF.

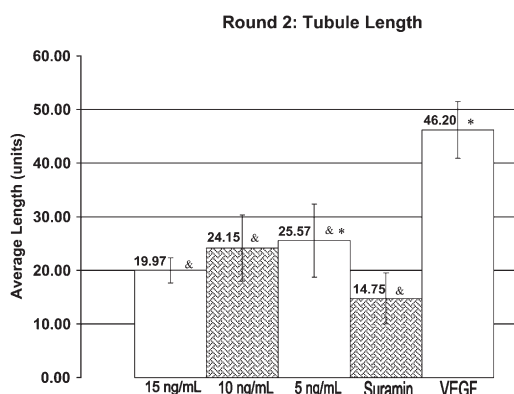


Figure 7. Comparison of the mean (\pm SD) tubule length in the wells treated with suramin, VEGF, and each of the hydrocortisone concentrations in Round 2. (*) indicates a significant or marginally significant difference from suramin; (&) indicates a significant or marginally significant difference from VEGF.

protocol. Given the nature of the analysis, the numbers produced were somewhat subjective. However, the general trends of the results were consistent for each of the measured characteristics, indicating consistency throughout the analysis process. The difference in data between the two rounds can be attributed to the reduced amount of CO₂ in the incubator due to a leak. The researcher analyzed the two rounds independently due to these differing external environmental conditions, which resulted in small sample sizes.

When analyzing the images to determine the number of bifurcation points, the multi-layered composition of the assay influenced the analysis as it was difficult to differentiate overlapping growth in the multiple layers of cells from actual points of bifurcation. Given that there was a significant difference, in both Round 1 and Round 2, between the suramin group and the VEGF group, it can be assumed that the cells were viable and responsive to extreme conditions of inhibition and stimulation with regards to the development of bifurcation points. In both Round 1 and Round 2 the mean number of tubules formed in the wells treated with the different concentrations of hydrocortisone differed with significance or marginal significance from the VEGF group. The total length of tubules formed in both rounds also differed with significance or marginal significance from the VEGF group except for the 15 ng/mL concentration

group in Round 1. These results indicate a need for stronger action of angiogenic stimulators than inhibitors in a system before the system will proceed with the process of angiogenesis. Hence, the test groups yielded results more similar to that of the suramin group with regards to number of tubules formed and tubule length.

Given that there was no difference determined among the test concentrations pertaining to the length or number of tubules formed and only one difference noted with regards to the number of bifurcation points, it appears that increased levels of hydrocortisone do not affect the degree to which angiogenesis occurs. However, since the results for each of the test concentrations are between that of the negative control (suramin) and the positive control (VEGF), it appears that hydrocortisone has a permissive effect on angiogenesis.

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