Summer Research Proposal 2011

Circannual Variation in Gene Expression of Thrombotic and Thrombolytic Plasma Proteins

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Background:

Circannual variation of thrombotic (clot forming) and thrombolytic (clot degrading) factors rests on the back burner of modern biochemical research. Scientific interpretation of the clotting cascade is rather static since natural selection tends to act very selectively in favor of the currently understood mechanism. The proteins and fibrins that construct a clot must be present in a delicate balance. The slightest deviation from that balance implies much larger implications. While the processes resulting in successful hemostasis may not change on a molecular level in the lifetime of an organism, there is lingering ambiguity about consequences from disrupting the harmony resting between clotting factors.

Several sub-microscopic structures hold critically indispensable responsibilities in the intrinsic and extrinsic pathways that function together in sight of platelet aggregation. Contrarily, thrombolytic factors must be also present in order to prevent widespread, and essentially reckless coagulation of the blood. It is hypothesized that seasonal variation of thrombotic and thrombolytic structures is observed, primarily in torpor-engaging (hibernating) species and as vestigial traits in non-torpor engaging species. Endogenous thrombin, tissue plasminogen activator (TPA), TPA inhibitor (TPAI), fibrinogen, and heparin cofactor-II (HC-II) are purported to vary and to produce quantifiable variance in hemostasis over the seasons.

Further, the proposed hypothesis under study is formulated assuming gene expression is in concordance with fluctuating plasma concentrations of coagulation factors, suggesting that mRNA transcripts for each factor will rise and fall in concordance with the change in protein presence in blood. These transcripts are the means by which genetic analysis of the coagulation system may be carried out. No current research addresses such hypotheses. While coagulation factor gene expression rests on the forefront of clinical research, a 2009 study of forty human

participants reports substantial variation in fibrinogen and endogenous thrombin plasma concentrations; observing troughs during winter and peaks during summer (*1*). The results are biologically sensible. Decreased blood activity during winter facilitates decreased thrombotic factor gene expression to prevent thrombosis in sedentary subjects (*3*). It should be noted that only one of six quantitative hemostatic marker measurements in this experiment was repeated, despite subjects' diversity in age, gender, smoking habits, and physical activity.

Contributing to growing dissonance of conclusions, conflicting research has been just as successfully published. Studies on patients with deficiencies of clot suppressing protein C and S indicated onsets of deep-vein thrombosis (DVT) increased during winter. Conclusions were attributed to increased blood viscosity, decreases in antithrombin III, and colder temperatures (*Clinical and Applied Thrombosis/Hemostasis*). DVT diagnosis on these parameters would be legitimate, but only is disregard of previously observed observed decreases in thrombotic fibrinogen from *Biological Variation on Inflammatory Markers*, expressing necessity for more research.

More problematically, the 1997 Rotterdam study, conducted at Netherlands' Erasmus University Medical School elucidates wintertime increases in Fibrinogen. In contrast to the study on DVT, decreased plasma fibrinogen concentration was proven to be independent of decreased temperatures of winter times. Experimental value of these conclusions is sufficient because data collected at Rotterdam covered a subject population of 7,983 (all over fifty-five years old). Of this group, 2,325 were selected as an analytical standard for comparison against test groups of varied ages. Therefore, the ambiguity between experimental conclusions regarding plasma clotting factor concentration requires a deeper analysis at the genetic level.

By analyzing mRNA transcripts of endogenous thrombin, TPA, TPAI, fibrinogen, and HC-II, the precise relationship between each factor may be diligently studied and interpreted down to the genetic level to assign some answers to the numerous discrepancies. Since the proposed research is characteristically pioneering, coagulation factor mRNA transcripts will be analyzed from *Homo sapiens*, *Ratus norvegicus* (rat), *Mus musculus* (mouse), and *Gallus gallus* (chicken) to provide a sound set of observations between vertebrate species. Simultaneously, this lays a foundation for more extensive future experimentation regarding circannual variation of coagulation mechanism parameters.

Purpose:

The purpose of this experiment is to determine the extent to which variation in gene expression of endogenous thrombin, TPA, TPAI, fibrinogen, and HC-II correlates to their observed variation of plasma protein concentrations. My data will be complemented by the work performed in Dr. Field's lab on human plasma proteins. While I am hypothesizing that the respective increases and decreases will parallel one another at both the protein and transcript level, data that is contradictory to my hypothesis will lead into investigation of circanually activated compensatory mechanisms, epigenetic factors, transcript stability, and post-translational degradation. Regardless of the data agreement or disagreement with my hypothesis, medical implications of conclusions reside in improved time selection for procedures. Specifically, giving a benefit to patients with history of DVT, hyper-coagulation, hemophilia, or numerous other clotting/bleeding disorders.

Objectives:

The primary objective of this experiment is to quantify and analyze gene expression through mRNA analyses of endogenous thrombin, TPA, TPAI, fibrinogen, and HC-II in

hepatocytes of humans, rats, mice, and chickens. Sources of mRNA will be hepatocytes of the listed organisms, except for humans. . Pure hepatocyte cell lines for humans are available through ATCC and increase reproducibility of data due to minimal impurity of the source. Hepatocytes are a major contributor in coagulation factor production. Two types of polymerase chain reaction (PCR) will be employed to derive quantifiable material from the cell lines and tissue samples. Although I do not need IRB approval to work with cell lines, I will obtain IRB approval of human specimen study prior to beginning any human tissue experimentation.

Techniques:

The primary outline of the proposed research follows techniques of mRNA extraction and purification, reverse transcriptase PCR (RT-PCR), and quantitative PCR (qPCR). The starting materials for experimentation are hepatocyte cells and tissues of species *Homo sapiens*, *Ratus norvegicus*, *Mus musculus*, and *Gallus gallus*. Samples of tissue will be acquired chronologically, being that the basis of study is to determine gene expression between winter and summer. Hepatocytes are identified as expression sites for endogenous thrombin, fibrinogen, TPA, TPA-I and HC-II, making them ideal vehicles for the proposed research. The genes for these proteins, once expressed, are responsible for concentrations of cytoplasmic mRNA transcript in proportion to protein production.

Synthesizing these transcript levels from the various loci we will be testing will provide accurate data for circanually fluctuating gene expression analysis. I will perform extraction of the transcripts using a purchased RNeasy kit from Qiagen Technologies. In the kit processes, cells are lysed and their contents homogenized. Ideal binding conditions for mRNA are established by addition of ethanol and a chaotropic salt. The lysate solution is passed through a silica membrane for selective binding of mRNA transcripts to rid the solution of contaminants. Pure concentrated

RNA is eluted in water. Due to the ubiquity of mRNA harming enzymes, I will need to exercise special precautions to safe guard the integrity of the mRNA (purified or not). The next step towards gene analysis is RT-PCR. Beginning from the mRNA strand, a reverse transcriptase enzyme builds cDNA, complementary to the transcripts found in the cell. The oligo-dT primers in the kit selectively bind to the poly-adenosine tail added to the mRNA transcript as the first step of eukaryotic RNA processing.

Everything that would be completed up to this point is a general purification and stabilization process. The inherent specificity in the measuring of particular mRNA transcripts is elucidated by qPCR. In qPCR pair of primers is used to initiate PCR in the locus of interest in combination with a fluorescent probe. The probe is a short sequence of synthetic DNA with a fluorophore at one end and a quencher at the other. An intact probe will not fluoresce because of the proximity of the fluorophore and the quenching complex. In encountering Taq-polymerase, the probe is dismantled, releasing a fluorescent molecule freed from the quencher. A laser driven fluorescent detection fiber optic array is used to detect the change in fluorescence over time, resulting in a plot that can be analyzed for levels of gene expression. In normalizing our analysis, we will analyze non-fluctuating genes (housekeeping genes) in order to normalize our data. By comparing and contrasting my data with the data derived from Dr. Field's lab on fluctuation of the level of clotting factors in blood, a clearer picture of the genetic control of these factors should emerge.

Works Cited

1) Rudez, G. Et. Al – *Biological Variation in Inflammatory and Hemostatic Markers*, 2009 Journal of Thrombosis and Haemostatis, 7:1247-1255

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3) Van Den Burg, P.J. Et. Al – *Effect of Endurance Training and Seasonal Fluctuation on Coagulation and Fibrinolysis in Young Sedentary Men*, 1997 The American Physiology Society, 0161-7567/97

4) Van der Bom, JG. Et. Al – *Seasonal Variation in Fibrinogen in the Rotterdam Study*, Department of Epidemiology & Biostatistics, Erasmus University Medical School, 1997 Journal of Thrombosis and Hemostasis, 78(3):1059-62