Estrogen, obesity, and anticoagulant Protein S contribute to thrombosis in mice

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Introduction

Protein S (PS) is an anticoagulant molecule present in humans and mice. Protein S binds and inhibits coagulation Factor IXa to control thrombin generation[1]. Protein S is imperative for normal hemostasis and the prevention of life-threatening thrombotic events, as demonstrated by numerous studies of patients with PS deficiency[2,3,4].

Pregnant women[5] and women who use estrogen-containing birth control pills [6] have lower levels of PS. Thus, it is postulated that estrogen downregulates PS expression in humans. Because estrogen is synthesized by aromatase, which is highly expressed in adipose tissue[7], we expect that PS will be downregulated in obese mice and in mice treated with exogenous estrogen. Further, we suggest that the downregulation effects of obesity and estrogen on PS abundance will be synergistic. Neither of these relationships have been shown experimentally in a mouse model.

We used a thrombin generation assay (TGA) to measure thrombin production in 24 mice weekly for six consecutive weeks. Half of the mice were obese, and the other half were lean. The obese mice were from a transgenic line, gifted by our collaborator Dr. Laurent Mosnier from Scripps Research Institute. The mice remained obese while being fed a regular diet. Of the 12 obese mice, 6 were treated with a 1.7 mg pellet of estradiol every 21 days, i.e., the obese + estrogen group (OE); the other 6 mice constituted the obese-only (O) group. Similarly, 6 lean mice were treated with estrogen (LE), and the others were the untreated lean control (LC) group. Plasma was collected via venipuncture and centrifugation for each of the 5 weeks of treatment, along with week 0 before estrogen treatment began.

We used SDS PAGE and western blot to compare the level of Protein S in each treatment group to the level in the control group. Week 3 samples were used because there was some premature clotting in many of the weeks 4 and 5 samples.

Finally, we assessed free Protein S in one sample from each category, also from week 3, using capture ELISA.

Figure 1:

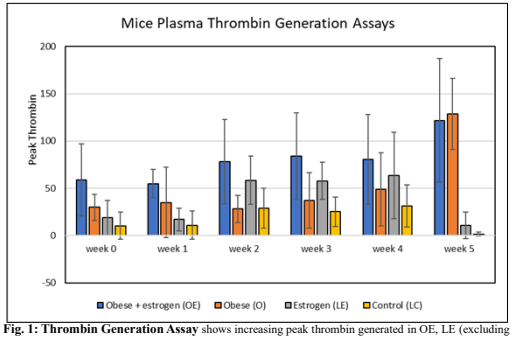

Fig. 1: Thrombin Generation Assay shows increasing peak thrombin generated in OE, LE (excluding week 5), and O as treatment progressed. OE had highest peak thrombin of all groups in every week, except week 5 when O was slightly higher. Peak thrombin in LC remained very low throughout experiment.

Figure 2:

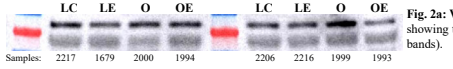

Fig. 2a: Western blot showing total PS (upper bands).

Fig. 2b: Western blot analysis for samples of interest are illustrated in this bar graph. Samples 1993 (OE) and 1679 (LE) had a marked decrease in Protein S compared to control, supporting our hypothesis. Sample 2000 (O) had a level comparable to control, as did 1994 (OE) and 2216 (LE) – although these are not shown in the graph. Since one sample (1999) from the obese treatment group had a high level of Protein S compared to control, we appreciate that many factors are at play in the regulation of coagulation, and we are hopeful that a stronger correlation can be found as we continue to analyze other samples.

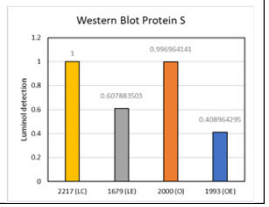

Figure 3:

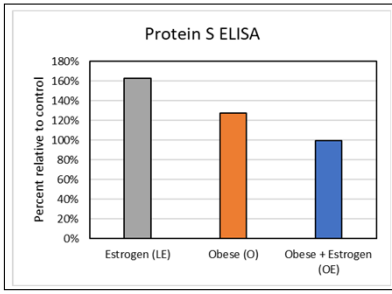

Fig. 3: Here the results of the capture ELISA are demonstrated in a bar graph with amount of Protein S present, measured in percent of control. OE (sample 2743) was lowest, in congruence with our hypothesis, followed by O (sample 1998), then LE (sample 993). However, all of the treatment groups had Protein S levels either similar to or higher than the control (sample 2206). More investigation is needed to confirm the trend seen in this graph.

Results

The results of the TGAs from each category and each week were averaged and compiled into the graph shown in figure 1. Peak thrombin was higher in all treatment groups compared to control, and this difference generally became more pronounced the longer treatment went on. OE had the highest peak thrombin in all weeks, except week 5 when O was slightly higher. This may be due to premature clotting in samples from week 5, which was evident while pipetting. If clots formed in the serum before thrombin was being measured, some of the clotting factors and protein S would not be available for proper measurement. Disregarding week 5, the TGAs show that peak thrombin is abnormally high in obese mice as well as those treated with estrogen, and even higher in the presence of both.

The western blot highlighted lower Protein S in one OE sample (40% of control) and one LE sample (61% of control). The other bands were like the control, except one obese sample which was very high in Protein S (249% of control). Transferrin was used as a housekeeping gene and was accounted for in all calculations.

The ELISA again showed that Protein S was lowest in the OE sample. Here, the O sample was lower than the LE sample. Unfortunately, we found that in this particular set of data, the LC was equal to or lower than all treatment groups. Nonetheless, we have many more samples to measure and are excited to compile a larger pool of data.

Of the 24 mice in the study, 2 died in week 2. Both of them were from the OE group.

Conclusion

In conclusion, this study indicates that PS expression (or activity) may be downregulated by estrogen therapy, and this effect is exacerbated by obesity as seen in thrombin generation assays. Protein S is likely lower in OE mice compared to O and LE, and in some cases, all of these groups are lower than the control. However, we will have to test more samples to draw any final conclusions. This information draws attention to the increased risk of thrombosis in women who use hormonal contraceptives, who are pregnant, or especially women who are obese. The relationships between Protein S, obesity, and estrogen should be further investigated to establish preventative measures for this high-risk group.

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