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2014

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The Role of Heparin in Vascular Smooth Muscle Cell Phenotype Modulation

Introduction

Atherosclerosis is an extremely common disorder around the world, and has become a leading cause of death and disability (Kelley, 2011). Vascular disease is known to primarily affect older patients. However, a study by Kelley et al. has found that the prevalence of atherosclerotic plaques in young and middle-aged people has risen to 8-14% (Kelley, 2011). Atherosclerosis is also one of the most commonly misunderstood diseases by the common citizen. It is a common misconception that atherosclerosis is characterized simply by plaque formation in the vasculature due to poor diet or genetic disposition. It is actually an inflammatory disease (Rudijanto, 2007).

Lesions on the vascular walls were previously thought to consist mostly of lipids; however, they are now known to contain cells, connective-tissue elements, lipids, and debris (Rudijanto, 2007). Atherosclerosis begins after initial injury to the endothelial lining of the vasculature. Injury can be caused by numerous conditions including diabetes mellitus, hypertension, dyslipidemia, and poor flow due to branching of the vasculature. Injured endothelial cells, along with platelets, release growth factors and cytokines. These molecules can promote vascular smooth muscle cells to change from their native contractile phenotype to a more active one – the synthetic, or proliferative, phenotype. VSMCs originally mature from the synthetic phenotype to contractile, which is the phenotype necessary for VSMCs to perform their normal functions. Hayashi posits that the exact cause of the phenotype switching remains
unknown. However, research conducted has shown that molecules such as unsaturated lysophosphatidic acids can cause the modulation (Hayashi et al., 2001).

Additionally, conditions such as hypoxia, along with endothelial injury, may cause the VSMCs to switch back to the synthetic phenotype. A study by Korshunov found that vascular remodeling in mice may even be caused by genetic determinants (Korshunov et al., 2004). This makes it even more imperative that an effective treatment be developed, as factors beyond behavior of the specimen play a large role in vascular disease. The synthetic VSMC phenotype can migrate and proliferate, which causes the formation of a lesion after mixing with areas of inflammation. Additionally, the synthetic VSMCs can synthesize molecules such as collagen and proteoglycans, which can pose further problems. (Rudijanto, 2007).

Yi et al. and Wang et al. have studied the role of hypoxia and high glucose concentration in phenotype modulation. Through their studies, it has been shown that hypoxia plays a large role in VSMC proliferation through phenotype switching (Wang, 2010). However, avoiding these conditions altogether is not feasible, and reversing the damage caused by said problems is not simple. There are several pathways involved in phenotype modulation including PI3K-Akt, MLCK, and PKC, and targeting any single pathway does not reverse the phenotype modulation (Yi, 2012). Thus, researchers such as Yi et al. have decided to target an upstream regulatory pathway, cGMP-dependent protein kinase (PKG). PKG is involved in numerous areas of the vasculature including muscle relaxation, platelet formation, and cell division (Yi, 2012).

A study by Lincoln et al. further examined the role of PKG in phenotype expression in VSMCs. They examined repetitively passaged VSMCs which initially did not express PKG, and found they these cells existed in the synthetic phenotype. However, after gene transfection with PKG-Itα, the cells exhibited higher levels of contractile phenotype markers including calponin.
and SM-α-actin. The cells also exhibited lower levels of synthetic phenotype markers osteopontin and thrombospondin, along with a more contractile morphology. Lincoln further found that exposure of Nitric Oxide to SMCs reduced phenotype expression; this suggests that NO also plays a role in phenotype modulation and vascular injury (Lincoln et al., 1998).

Research by Gilotti et al. has also found that molecules such as heparin and atrial natriuretic peptide (ANP) induce positive changes in the vasculature (Gilotti, 2013). Often thought of as simply a blood thinner, the studies discussed above demonstrate that heparin has an important relationship with PKG in anti-proliferation in the vasculature. The goal of this research is therefore to investigate the exact relationship between heparin the vasculature, and to see whether its effects are similar to those of PKG. Heparin seems to be an important biomolecule in regards to vasculature therapeutics. However, heparin must be investigated carefully and with prudence, as over administering heparin to a patient would have negative effects, such as reducing blood-clotting effects to extreme levels. This makes further heparin research all the more important. There is a dire need for vascular disease therapeutics, and heparin, or treatments based on heparin’s mode of action, could prove to be effective. Heparin may have effects similar to PKG on VSMCs phenotype markers, and these experiments aim to illustrate those effects.

Methods and Materials

To examine the effect of heparin on phenotype modulation, Western Blots were performed to compare protein levels between treatment groups. Western blots were ran according to standard protocol. A7r5 cloned rat smooth muscle cells, along with primary bovine VSMCs, were treated in the experiments. DMEM or MEM and 2.5% trypsin/EDTA was used for cell culture stages. The phenotype markers that were examined are calponin and SM-α-actin, and polyclonal rabbit and goat antibodies were used for the blots.

Various treatment groups were used to illustrate the effects of heparin on the VSMCs. Control group VSMCs were incubated under standard conditions for 24, 48, or 72 hours. The
first experiment also had a set of cells incubated with heparin (to saturation) for 24, 48, or 72 hours. These cells were harvested and run through a western blot, stained for the specific proteins, and developed. An experiment was also performed to illustrate the effects of adding more heparin after 24 and 48 hours – this was done to show whether the heparin is absorbed or degraded; adding more heparin would theoretically upregulate the phenotype markers even more after the specified timespan. Finally, these experiments were also run with primary cells, as they may respond differently to the treatments than cloned cells do.

**Results**

Multiple sets of gels were completed to attempt to show maximal effects of heparin.

Figure One, A7r5, SM-α-actin, *From left to right: 24 Hours, 24 Hours w/ Heparin, 48 Hours, 48 Hours w/ Heparin, 72 Hours, 72 Hours w/ Heparin*
Figure Two, A7r5, Calponin, *From left to right*: 24 Hours, 24 Hours w/ Heparin, 48 Hours, 48 Hours w/ Heparin, 72 Hours, 72 Hours w/ Heparin

The following two gels involved adding more heparin two the plates of cells after 24 and 48 hours. The experimental group involving this change is marked with “C” below (i.e. 48C and 72C). Groups where heparin was added only at time of cell splitting are marked with “H.”
The final gel run was stained for SM-α-actin and loaded with both primary (bovine VSMCs) and cloned (A7r5) cells.
Figure five, SM-α-actin, *From left to right, A7r5, A7r5 w/ Heparin, A7r5, A7r5 w/ Heparin (second set) Bovine VSMCs, Bovine VSMCs with Heparin

Discussion

The first set of gels (Figures 1 & 2) seem to show upregulation of the phenotype markers at 24 hours. However, it is difficult to conclude definitively whether the darker protein regions on the gel are due to actual upregulation of the protein or due to loading differences in amount of cells between the lanes. ImageJ was used to attempt to discern whether the difference in band intensity was due to loading discrepancies, and the data showed that the difference between the lanes was not significant (i.e. that there were not major differences in loading amounts between the lanes). The results were inconclusive after further repeat experiments. There are several explanations behind this data: the heparin may have been degraded or absorbed by the cells after a specific time period, the heparin may not have an effect as initially hypothesized, or the specific cells used (A7r5) were not responding as hypothesized. To rule out the first explanation, the second set of gels was run. A group of cells were continually treated with heparin after 24 and 48 hours to illustrate whether additional heparin would have any effect on the cells. Figures 3 & 4 do not show any significant upregulation of the phenotype markers in the lanes treated with additional heparin. At this point, the hypothesis was that A7r5s, due to their highly synthetic nature, would not respond to heparin as primary cells would. Therefore, the third set of gels (Figure 5) were run with primary cells. However, the primary groups do not show significant
differences in protein levels. The initial hypothesis may have been incorrect. Heparin may not induce powerful enough changes in cellular mechanisms to upregulate these phenotype markers and induce a switch back to the contractile phenotype. Further directions involve using heparin for a longer time period, trying different cell types, or examining different phenotype markers.

References


Rudijanto, A. The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. Department of Internal Medicine, Faculty of Medicine Brawijaya University (2007). 86-93.
