INTRODUCTION
An abdominal aortic aneurysm (AAA) is weakening of the abdominal aortic wall, which causes the aorta to expand in diameter. AAA has a high risk of fatality due to the possibility of AAA rupture, which results in the rapid loss of a substantial amount of blood. In fact, 65-85% of all AAA ruptures are fatal, which causes approximately 15,000 deaths in the U.S. each year [1,2]. Often in AAA, an intraluminal thrombus (ILT) forms. The ILT is a large blood clot-like structure that contains blood-borne cells including inflammatory cells and extracellular matrix (ECM) components. The ILT may play a complex role in the matrix remodeling and pathogenesis of AAA. Computer models and previous experiments have demonstrated that the ILT creates a hypoxic environment, or area of low oxygen concentration, at the AAA wall [2].

The adventitia, or outermost layer of the blood vessel, contains fibroblasts, which are cells responsible for ECM homeostasis and repair. In normal conditions, fibroblasts secrete collagen I and collagen III, which are proteins normally found in the ECM. Fibroblasts also express discoidin domain receptor 2 (DDR-2), which is a marker specific to fibroblasts and myofibroblasts. Normally, fibroblasts do not express α-smooth muscle actin (α-SMA), which is expressed in smooth muscle cells [3]. However, in response to vascular injury or hypoxia, fibroblasts can transform to a different phenotype called myofibroblasts, which are responsible for excessive ECM remodeling. During an excessive remodeling process called negative remodeling, myofibroblasts proliferate, begin to express α-SMA, and secrete excessive amounts of collagen. This response may contribute to thickening of the adventitia [3].

Although the transformation from fibroblasts to myofibroblasts has been observed in other injury models, little is known about the response of the adventitial cells to hypoxia in AAA. These cells are of particular interest because they secrete ECM proteins. ECM proteins may be degraded or abnormally formed in AAA, which may contribute to wall weakening and the eventual rupture of the aneurysm [2]. Therefore, studying the response of AAA adventitial cells to hypoxic conditions may give insight on the progression of AAA.

Objective: The objective of this study was to isolate cells from the adventitial layer of AAA tissue, and to characterize these cells by evaluating α-SMA, DDR-2, collagen I, and collagen III expression using immunofluorescence staining under either hypoxic (defined as 1% O2) or normoxic (defined as 21% O2) conditions.

Success Criteria: The adventitial cells will express α-SMA and an increase in collagen I and collagen III expression when exposed to hypoxic conditions. In addition, all cells should express DDR-2, since this is a marker for fibroblasts and myofibroblasts. If three or more of the four samples analyzed show an increase or decrease in a particular protein’s expression when in hypoxic conditions, this can be considered to be representative of a general trend in expression change.

METHODS
AAA tissue samples were collected from four patients undergoing elective AAA repair surgery. The four samples were each given a unique identification tag: AAA 0410, AAA 0424, AAA 0529, and AAA 0710. To isolate the adventitial cells, the adventitial layer was peeled away from the rest of each sample. Next, the adventitial layer was cut into small pieces and digested by the enzymes collagenase and elastase for 30 minutes in order to break down the extracellular matrix and expose the cells. After digestion, the adventitial pieces were placed in media containing fibroblast growth factors and incubated at 37 °C to promote the outgrowth of cells from the tissue. Once cells had grown from the adventitial tissue pieces, the cells were passaged into flasks and grown in confluency, or until the cells were nearly covering the bottom of the flask. After cells had reached confluency, they were plated into 6-well plates with coverslips. Only cells from passages 3 through 5 were used for the experiments, since higher passage numbers can cause damage to the cells.

Next, 6-well plates were exposed to either a hypoxic (1% O2) or a normoxic (21% O2) condition. For the normoxic environment, 6-well plates were simply placed in a 37 °C incubator, which contained air maintained at 21% O2. To create a hypoxic environment, 6-well plates were placed in an airtight container, which was purged with gas containing 1% O2 for 5 minutes. Because of the small size of the chamber, at the end of 5 minutes, it could be assumed that the chamber contained air with only 1% O2. Tubing to the chamber was then clamped, and the chamber was placed in a 37 °C incubator. After 24 hours, the chamber was re-purged with 1% O2 in order to rid the chamber of any oxygen that had been dissolved in the media. The experiment was stopped after a total of 2 days, and the coverslips from both experimental groups were fixed with 4% paraformaldehyde.

The final step was to perform immunofluorescence staining in order to fluorescently mark and image proteins of interest in the adventitial cells. First, primary antibodies for α-SMA, DDR-2, collagen I and collagen III were added to separate coverslips to bind to those respective proteins. Next, fluorescent secondary antibodies were added to the coverslips to bind to the primary markers in order to image the presence of each protein. Each time immunofluorescence staining was performed, a coverslip was also used as a primary delete, where only secondary antibodies were added to the coverslip. Primary deletes were used to gate the images for non-specific fluorescence, so that only true expression of each marker could be observed. DAPI was also used as a counterstain to mark all nuclei, and thus all cells, on the coverslips. Pictures of the coverslips were obtained, and the pictures were manually analyzed in order to characterize the cells by protein expression.
RESULTS

All adventitial cells showed expression of α-SMA, DDR-2, collagen I and collagen III, regardless of hypoxic or normoxic conditions. In response to hypoxic conditions, three samples (AAA 0410, AAA 0529, and AAA 0710) showed an increase in α-SMA expression. Expression of α-SMA remained constant in response to hypoxia in the AAA 0424 sample. In response to hypoxic conditions, three samples (AAA 0424, AAA 0529, and AAA 0710) showed an increase in both collagen I and collagen III expression. The AAA 0410 sample was unique because it showed a decrease in collagen I expression, and a constant expression of collagen III when exposed to hypoxic conditions. All samples showed a constant expression of DDR-2 in both normoxic and hypoxic conditions.

Table 1: Immunofluorescence staining results for adventitial cells exposed to hypoxic conditions

<table>
<thead>
<tr>
<th></th>
<th>AAA 0410</th>
<th>AAA 0424</th>
<th>AAA 0529</th>
<th>AAA 0710</th>
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<tbody>
<tr>
<td>α-SMA</td>
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<td>DDR-2</td>
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<td>Collagen I</td>
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<td>Collagen III</td>
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Table 2: Legend for Table 1

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<th>Constant Expression</th>
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<tr>
<td>Gained Expression in Hypoxia</td>
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<td>Lost Expression in Hypoxia</td>
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DISCUSSION

The presence of α-SMA and DDR-2 expression indicates that the adventitial cells in all samples may have been transformed to myofibroblasts, even before exposure to hypoxia. Transformation to a myofibroblast phenotype may occur within AAA as a wound-healing response to conditions within the aneurysm; however, the adventitial cells may have transformed to myofibroblasts as a result of stresses from cell culture. Because three of the four samples tested showed an increase in α-SMA, collagen I, and collagen III expression in response to hypoxia, these changes in expression can be considered indicative of a general trend. Increased expression of α-SMA, collagen I, and collagen III may indicate that the myofibroblasts are becoming more activated in response to hypoxic conditions, which may contribute to excessive ECM remodeling and adventitial thickening in AAA. The AAA 0410 sample was unique because collagen I expression decreased and collagen III expression remained the same in response to hypoxia, in contrast to the other three samples. This may have been due to human errors in the immunofluorescence staining process – for example inadequate mixing of the primary or secondary antibody solution. Alternatively, decreased collagen I and collagen III expression may also indicate that this sample was in a different phase of AAA progression, since deformed or degraded collagen and other ECM proteins may contribute to AAA rupture. The AAA 0424 sample was also unique because it maintained a similar level of expression of α-SMA in both hypoxic and normoxic conditions. Since all changes in expression were evaluated by eye, it is possible that the increase in α-SMA expression was not significant enough to be visible, but would be present if the results were quantified.

Limitations of this study include the absence of a control group. Our lab was unable to acquire tissue samples from non-diseased aortas for comparison within the time frame of this study. In addition, it is possible that the 2-D behavior of the adventitial cells in culture differed from their behavior in the patient. Because of these two factors, it is uncertain whether the adventitial cells had been transformed to myofibroblasts as a result of AAA progression, or from stresses in cell culture. Another limitation of the study is the relatively low sample number (n=4), which may not accurately represent the population of AAA patients.

Future work may include isolating adventitial cells from autopsy tissue from non-diseased aortas, and performing the same experiments on these cells. Quantification of the results from this study may also be performed by using image analysis techniques, or through PCR, which will quantify the amount of RNA for each protein of interest in the cells.

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REFERENCES