MONITORING REGULATORY T-CELL ACTIVITY WITH THE FOXP3 GENE

The FoxP3 gene is currently the most reliable marker of suppressor regulatory T-cell activity. FoxP3 is a gene very specific to these cells. Regulatory T-cells are a sub-population of cells that have been shown to be capable of limiting immune responses to self-antigens, playing an essential role in preventing auto-immune diseases. Recent studies have demonstrated that they also serve a role in controlling many other immune responses, including inflammation, infection, allergy, graft vs host disease, organ transplantation, tumor immunity, and immunodeficiency. It is believed that this cell population may exert a ''quality control'' effect in a balanced immune response. In our studies, FoxP3 gene expression was measured with Real-Time PCR to assess regulatory T-cell activity. For any Real-Time PCR work, an endogenous control gene, often called a 'housekeeping' gene, must first be established. The ideal control should maintain a constant level of expression in experiments to give an accurate measure of the changes in the gene of interest. In our studies, ß-Actin proved to be the most stable and abundantly expressed housekeeping gene. Using this technique, regulatory T-cell activity was monitored in patients diagnosed with ENL (erythema nodosom leprosum). It was proposed that the medicine the patients were being treated with, known as thalidomide, was an anti-inflammatory drug therefore aiding the body's own natural defense mechanisms against the bacteria in the infection. It was discovered that the patient's immune system would call more regulatory T-cells in response to the foreign antigens threatening the body's health – proving that this hypothesis was indeed true.

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INTRODUCTION

Measuring gene expression is a fundamental technique in research, especially in the microbiological and immunological fields. To accurately measure a gene of interest, such as FoxP3, a housekeeping gene has to be determined. A housekeeping gene is what a cell uses for a basic or fundamental function, such as a structural gene. In any experimental system, this would require a housekeeping gene that does not fluctuate. This is done through a widely used method known as real-time reverse transcription and polymerase chain reaction (RT-PCR), a very sensitive yet accurate way of monitoring gene expression. This process consists of several steps, including the culturing of cells, RNA extraction, RNA storage, the assessment of RNA concentration, and the reverse transcription of the RNA into cDNA prior to RT-PCR.

This technique is sensitive because it is prone to potential variables during the monitoring of gene expression. To compensate, an endogenous control (the housekeeping gene) is commonly measured alongside the gene of interest, acting as an unchanging standard. The ideal housekeeping gene is both stable and highly expressed independent of the cell's extracellular environment. In this study, the stability of two common housekeeping genes, GAPdH and ß-Actin, was assessed in stimulated Jurkat (regulatory T-cells) cells.

MATERIALS AND METHODS

PBMC Isolation

The starting point of many assays in immunological research is the isolation procedure of peripheral blood mononuclear cells. The seclusion of lymphocytes is used often in clinical investigations to understand their functions, quantify specific cell phenotypes, test any article or drug on the lymphocytes or the drug in vitro, and/or determine the amount of a specific drug within the cells.

Ficoll separation is the method used to isolate lymphocytes from plasma platelets, red blood cells (erythrocytes), and white blood cells (granulocytes) by using the density gradient medium. The separation purifies lymphocytes from human blood by centrifugation. The plasma gained from the process is removed and the remaining components of the blood are then diluted with an equal volume of balanced salt solution (phosphate buffered saline [PBS]). This dilution results in a better lymphocyte yield and reduces the amount of red cell clumps. The diluted blood is then layered on top of the Ficoll medium in a new centrifuge tube without mixing the two solutions. After this is centrifuged, the density gradient medium helps to separate the blood sample that can be visually observed from the top to bottom: plasma/platelets, PBMC's or lymphocytes, the Ficoll medium, and the granulocytes and erythrocytes. Lymphocytes, platelets, and monocytes appear layered above the Ficoll because they are not dense enough to pass

through the medium, just as the red blood cells and white blood cells are post-centrifugation. The layering enables the lymphocytes to be recovered with a high yield in a small volume with little mixing with the Ficoll. The layer of plasma is first removed, frozen, and preserved for other use. The PBMC's are then removed and washed several times with PBS to get rid of any Ficoll contamination and preserved, now ready for clinical investigations.

RNA Extraction

The isolation of RNA is typically done using RNA extraction kits with prepared buffers, materials and protocols. In this instance, the cells that the RNA is extracted from are peripheral blood mononuclear cells, harvested using PBMC isolation. RNA can be extracted from a minimum of 1×10^6 to a maximum of 1×10^7 pelleted cells per tube.

Just before the RNA extraction, ß-mecaptoethanol must be added to buffer RLT in order to make it a lysis buffer. After it is added, the buffer RLT is stable for about one month. After the PBMC's are obtained and thawed, they are disrupted by the addition of BLT. The lysate is then pipetted into a QIAshredder spin column placed in a 2 mL collection tube (included in the RNA extraction kit) and homogenized in a microcentrifuge. The buffer RLT that was added helps the sample bind to the silica membrane that lines the column and when homogenized, the membrane shears any DNA that was released during lysing. After centrifuging, 70% ethanol is added to the homogenized lysate to precipitate the sample, removing unwanted debris. This mixture is then added to a new mini column in a 2 mL collection tube and centrifuged, discarding the flow afterwards. Buffer RW1, used to wash away contaminants, is pipetted in the column and centrifuged, the flow-through discarded once again. A solution of DNase and buffer RDD is pipetted directly onto the silica-gel membrane in the column and place on the bench top at room temperature for 15 minutes. The DNase enzyme and buffer RDD eliminate any extra DNA material left behind in the gel membrane. Afterwards, it is washed again with buffer RW1 and centrifuged. The column is transferred into a new 2 mL collection tube and buffer RPE is pipetted into the column and centrifuged twice. To elute the solution, the column was transferred into a new 1.5 mL collection tube, RNAase-free water pipetted onto the silica gel membrane, and centrifuged. The eluted solution left in the collection after centrifugation is the cleaned mRNA sample.

Real-Time PCR

The mechanism used to run this real-time PCR protocol is an ABIPrism 7700, which includes the preparation process of the RNA known as reverse transcription. Since the method of PCR cannot be run on mRNA that was extracted from the Jurkat cells, reverse transcription is first run on the template in order to translate the mRNA back into their original DNA sequence. This is because when a gene is activated, it copies itself (using its mRNA) exponentially to make proteins. By measuring this amount of mRNA, gene activity can also be monitored. The RNA template is first thawed out on ice. The primer solutions (forward and reverse primers specific for the gene), 10× buffer RT, dNTP mix, and RNase-free water are thawed out at room temperature. All the solutions are immediately stored on ice after vortexing and centrifuging briefly to collect residual liquid from the sides of the tubes.

After reverse transcription is performed, the newly replicated cDNA is added to a MasterMix of buffers, manganese acetate, RNase-free water, dNTPs (one for each nucleotide), Taq polymerase, and forward and reverse primers made specifically for the gene sequence being researched. The cDNA is heated to denature and separate the strands at 50°C for 2 minutes. When this happens, the forward and reverse primers run along their associated 5′ and 3′ strands. Taq polymerase then attaches itself to the strands, searching and copying specific gene sequences (30 minutes at 60°C). The process is repeated through 30–40 cycles, the gene sequence replicating exponentially each time (5 minutes at 95°C).

RESULTS

In the standard curves produced with our data, the slope of the linear regression equation is carefully examined. The slope of the housekeeping gene that is closest to the numerical value to the FoxP3 slope would represent the best-controlled housekeeping gene. The R value also tells us how accurate the equation line is compared to the original data points plotted. The closer the R value is to 1.0, the more accurate the equation to back up the results of the data given.

The slope of the FoxP3 data is −1.6679 while the slopes of housekeeping genes are −1.6708 (ß-Actin) and −1.7161 (GAPdH). Since ß-Actin's slope is closer to FoxP3s, it is determined to be the better housekeeping gene marker for FoxP3.

Using this information, we were able to test ENL patients treated with thalidomide for 21 days, taking skin biopsies and testing for the presence of ß-Actin. It was discovered that the levels of ß-Actin increased during the treatment period meaning that regulatory T-cells were called to the infection site with the aid of the thalidomide, meaning that it is indeed an anti-inflammatory drug.

DISCUSSION AND IMPLICATIONS

With the knowledge that ß-Actin is the marker for the FoxP3 gene, we can further monitor and understand more about regulatory T-cells and the human immune system. Not only that, but this information can also be used to study the immune systems of people who are diagnosed with viruses/illnesses/diseases such as HIV or tuberculosis.