Determining an Endogenous Control within Jurkat Cells for Evaluation of IMiD-1 Analogue

Thalidomide is a controversial anti-inflammatory medication known to cause severe malformations to the unborn children of pregnant women. It was introduced in the 1950s as a sedative and treatment for morning sickness and was banned until 1965, when it was discovered that patients with ENL (erythema nodosum leprosum), a painful skin condition associated with leprosy, lessened the severity of their condition by taking thalidomide and aiding their body's own natural defense mechanisms against the bacteria in the infection. The thalidomide-derived drug analogue under investigation in this study will be identified as immunomodulatory drug-1 (IMiD-1).

In this research, we examined the exact mechanism of IMiD-1, its effect on Jurkat cells, and these cells' gene expression of interleukin-2 (IL-2). We measured IL-2 expression with real-time reverse transcription polymerase chain reaction (RT-PCR) to assess the effect of the drug analogue IMiD-1 on the immune system's Jurkat cells. For any RT-PCR trial to work, an endogenous control gene, most often a housekeeping gene, must first be established. The ideal housekeeping gene is both stable and highly expressed independent of the cell's extra-cellular environment. The two candidates as endogenous controls in our experiment were GapdH and β-Actin. Ultimately, GapdH proved to be the most constant and abundantly expressed housekeeping gene. When GapdH was used in our studies, we found that IL-2 gene expression was increased by almost 200% when compared to other known IL-2 immunomodulatory stimulants such as PHA and PMA environments, indicating that IMiD-1 may be a promising IL-2 stimulant in the Jurkat cells of our immune system and a potential alternative to thalidomide.

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Background

In the microbiological and immunological fields, measuring gene expression is a fundamental technique in research. To accurately measure a gene of interest, such as IL-2 expression in Jurkat cells stimulated with IMiD-1, an endogenous control must first be established. The widely used method of real-time reverse transcription polymerase chain reaction (RT-PCR), a very sensitive yet accurate way of monitoring gene expression, is also used to evaluate potential housekeeping genes which may serve as controls. This entire process consists of several procedures including: cell culturing, RNA extraction, RNA storage, the assessment of RNA concentration, and the reverse transcription of the RNA into cDNA prior to RT-PCR.

Housekeeping genes are required by cells in order to maintain basic cellular functions. When RNA levels are being measured within cells through the use of RT-PCR, housekeeping genes provide a standard or a constant for comparison against the gene of interest. GapdH and B-actin are two common housekeeping genes which are potential candidates for the endogenous control in our experiment. Both of these genes code for scaffold proteins that allow cells to maintain their shape and also play a role in intracellular transport.

The reason for establishing a control is to ensure that one has accurately added exactly the same levels of template for all samples to each reaction in RT-PCR. A problem could have arisen pertaining to contamination, or the samples used may not have contained the exact same volume throughout. Due to these numerous potential variables in the experiment, GapdH and B-actin genes are essential standards to which we will compare our results. It has been shown, however, that not all housekeeping genes are ideally suited for all experimental conditions. Some genes have been shown to be more stable in certain tissues than in others when the cells are experimentally manipulated. Therefore, it is imperative that before embarking on an investigation of gene expression, a stable housekeeping gene is established.

Methods

Jurkat cells were cultured in RPMI with 10% fetal calf serum (FCS) media at 5% CO2, 37°C until sufficient numbers were attained. Cells counts were performed using a hemocytometer with trypan blue dye. Cells were stimulated with either 5 ug/mL phytohaemagglutinin (PHA) or 1 ng/mL phorbol 12-myriastate 13-acetate (PMA) and 1 μM ionomycin at $1e^6$ cells/mL and cultured in slant tubes for 0, ½, 1, 2, 4, 6 hours and overnight. Un-stimulated cells were also cultured as controls. All sample tubes were then placed in a 37°C incubator until the directed time frame was fulfilled. Cells were pelleted, supernatants removed, and stored at −80°C. RNA isolation was performed using QIAGEN RNeasy Mini Kit according to manufacturer's protocol with an on-column DNase digestion.[1,2] RNA concentration and purity was assessed

after extraction through use of a Becton Dickinson DU 530 Spectrophotometer. One microgram (μg) of each sample was reverse transcribed with QIAGEN Omniscript Reverse Transcription Kit using random hexamers according to manufacturer's protocol but at half the suggested volume.[3,4] Real-time polymerase chain reaction was run on an ABI 7700 with the following reaction conditions: 20 mL reaction volume, Taqman DNA polymerase enzyme (Applied Biosystems), 4.0 m MgCl2.

The following primer and probe sets were used:

B-actin

200 mM forward primer: 5′-CCTGGCACCCAGCACAAT-3′

200 mM reverse primer: 5′-GCCGATCCACACGGAGTACT-3

100 mM probe: 5′-VIC-ATCAAGATCATTGCTCCTCCTGAGCGC-3′

GapdH

200 mM forward primer: 5′-CCACATCGCTCAGACACCAT3′

200 mM reverse primer: 5′-GGCAACAATATCCACTTTACCAGAGT-3′

SYBR green dye used at 10× concentration

IL-2

200 mM forward primer: 5′-CTCACCAGGATGCTCACATTTA-3′

200 mM reverse primer: 5′-TCCAGAGGTTTGAGTTCTTCTTCT-3′

100 mM probe: 5′-VIC-TGCCCAAGAAGGCCACAGAACTG-3′

Results from the RT-PCR experiment were recorded in the form of standard curves graphed logarithmically. The fluorescence (delta Rn) of the RNA samples was graphed in relation to the cycle threshold value. The results show that GapdH is the ideal endogenous control for evaluation of Jurkat cells stimulated with IMiD-1 as GapdH levels were highly expressed and remained constant whether in a PHA, PMA, or unstimulated environment. B-actin levels trended upwards in the PHA and PMA environments.

RESULTS

By analyzing the RT-PCR data, we found that GapdH is the ideal endogenous standard for evaluating the effect of IMiD-1 on Jurkat T-cells. After an incubation time window of six hours prior to RT-PCR analysis, the cycle threshold was reached for GapdH at approximately 14.5 hours and for B-actin at approximately 21.5 hours. This point in time indicates when the RT-PCR machine recognized significant gene expression from the RNA within the machine's wells. The fact that all four extracellular environments (two unstimulated, PHA and PMA stimulated) for GapdH did not alter the amount of GapdH expressed within the cell qualify the housekeeping gene as the ideal endogenous control for the future procedures of this experiment. Regarding B-actin, it was seen that the two stimuli (PHA and PMA) caused the cell to yield a slightly higher amount of the housekeeping gene after six hours of incubation while the two unstimulated controls of the B-actin trial showed lower quantities of the housekeeping gene. Using this information, we were able to evaluate IL-2 expression levels within Jurkat T-cells that were stimulated with the thalidomide-derived IMiD-1 analogue. It was discovered that IMiD-1 resulted in almost 200% greater IL-2 gene expression in Jurkat cells stimulated with the analogue when compared to other known IL-2 stimulated environments such as PHA and PMA.

FUTURE IMPLICATIONS

With the knowledge gained that GapdH is the ideal housekeeping gene for evaluating IL-2 expression in Jurkat cells stimulated with IMiD-1, we can further monitor and understand more about the effect of this thalidomide-derived drug on the body's immune system. In addition, this information regarding GapdH as an ideal endogenous control can also be used in future studies specifically concerning the expression of IL-2 by Jurkat cells. At this stage in the experimental process, further studies will most likely be conducted to determine whether IMiD-1 really is as potent as thalidomide without its teratogenic effects. Additionally, studies are being conducted as to whether the longer periods of heightened levels of IL-2 due to IMiD-1 stimulation is a result of IMiD-1 activating gene transcription (resulting in more mRNA for a longer period of time) or simply keeping the mRNA within the cell from degrading.

REFERENCES

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