Kinetics of gene expression during the differentiation of myeloid cells into osteoclasts

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ABSTRACT

Osteoclasts are the cells responsible for the breaking down of bone. Their proper function is integral to bone health. An imbalance will lead to a disorder, causing the bones to become easily breakable and brittle. Their study is therefore very important in biomedical studies, and as with any very specific cell type, can be challenging to acquire in large quantities. Procedures have been designed to derive large amounts of osteoclasts from their progenitor cells, myeloid cells *in vitro,* with relative ease and in a short amount of time. However, the nature of this *in vitro* derivation is not very well studied. Most procedures call for a 48- to 72-hour incubation period to derive these cells, but it is unclear why this amount of time is required. The investigation of whether osteoclasts can be derived in less time will be monitored using the expression levels of RANK, TCIRG1, MMP9, and CSF1R; four genes vital to osteoclast function. Derivation will be achieved in phorbol-12-myristate-13-acetate (TPA) and 1,25-dihydroxyvitamin D_3, or more simply, vitamin D_3. RNA will be extracted from cells after 0, 4, 8, and 24 hours of exposure, and expression levels will be measured. Other time-sensitive mechanisms by which osteoclasts differentiate may be exposed as well.

INTRODUCTION

Osteoclasts are multinucleated cells with a very unique ability to dissolve mineralized cells. Normally, microfractures build up through normal wear and tear of bone cells. In a healthy person, these microfractures on the surface of the bone are dissolved by osteoclasts. New, healthy bone is put in its place by antagonistically-operating cells known as osteoblasts. These two cell types form a very

intricate cycle that must not be disturbed to maintain good bone health. If osteoclasts are functioning at rates that are too high, not enough bone will be able to grow and bones will become weak and porous. This can lead to osteoporosis, a disorder often occurring in post-menopausal women, characterized by weak, easily breakable bones. Osteopetrosis is a much rarer disorder, essentially the opposite of osteoporosis: bones become too thick, generally due to a decrease in osteoclast function. Despite being thicker, the bones are also very brittle, due to the inability to restore bone and remove microfractures that build up normally.

In order to study these disorders, laboratories would require a large amount of osteoclast cells. As there are naturally issues with using primarily derived cells for large scale research on human cells (small number available, high cost, require a human donor), it is much more common in a laboratory environment to derive these cells *in vitro* from their natural progenitors, myeloid cells. Myeloid cells are undifferentiated cells that grow in the bone marrow and can be differentiated, using different signals, into a relatively wide array of cells that serve many important functions. Osteoclasts are one of these products. The specific signals *in vivo* that will cause osteoclast differentiation are NFκB, or RANKL, and CSF1, or colony stimulating factor (Mellis, et al 2011). It has been determined that osteoclasts can be forcibly derived *in vitro* using a combination of TPA and vitamin D_3. According to Bar-Shavit et al., very early developers of a procedure to make osteoclast cells *in vitro*, a 72- hour period is best to allow the growing osteoclasts to fully mature. At this point, approximately 40-50% of the original cells should have been derived (Bar-Shavit, et al., 1983).

Instead of waiting 72 hours, a myeloid cell line will be exposed to TPA and Vitamin D_3 for 24 hours, and various time points will be tested for the expression levels of RANK, TCIRG1, MMP9, and CSF1R. These four genes are very important to osteoclast function and development. RANK is the receptor for RANKL, one of the ligands that signals for osteoclastogenesis. TCIRG1 encodes for the protein subunits of vacuolar-ATP synthetase, an enzyme that acidifies the area of the osteoclast that will

connect to the bone surface. Mutations are associated with the development of recessively-inheritable malignant osteopetrosis (Susani, et al., 2004). MMP9 is one of the many matrix metalloproteinases working in osteoclasts. This group of enzymes has the ability to metabolize components of the bone matrix, playing a major role in the osteoclast's overall function (Bruni-Cardoso, et al. 2010). CSF1R is a tyrosine-kinase receptor for colony stimulating factor 1, which is necessary for osteoclast differentiation (Aoki et al., 1997).

METHODS AND MATERIALS

RNA Isolation: The RNA used in this experiment was donated by Dr. Sylvia Anderson. An HL60 myeloid cell line was exposed to phorbol-12-myristate-13-acetate (TPA) and 1,25-dihydroxyvitamin D_3 for up to 24 hours. At 0, 4, 8 and 24 hours after exposure, cells were removed, and RNA was isolated using a QIAGEN RNeasy© Plus Mini Kit. 10 µg/mL aliquots were prepared for each sample.

Primers: Primers were designed for RANK, TCIRG1, MMP9, and CSF1R, outlined in Figure 1. Each primer extended over multiple introns to ensure that genomic DNA was not being amplified.

Gene	Direction	Exon	Code (5' -> 3')	Length
RANK	F	5	GCAGCTCAACAAGGACACAGT	589 bp
	R	9	GCTGTCTTCCTCTATCTCGGTCT	
TCIRG1	F	14	CGTCATCCTGGGCGTCG	582 bp
	R	18	GCGGTGTTGGAGACGCA	
MMP9	F	5	CTTCCCCTTCATCTTCGAGG	579 bp
	R	8	CGGTACATAGGGTACATGAGC	
CSF1R	F	2	CCCTGCTGTTGTTGGTCTGT	539 bp
	R	6	GAAGCGCATGGTGTCCTCC	

Fig 1. Primers used for RT-PCR. These were designed specifically for this experiment.

Reverse Transcriptase PCR: RT-PCR was used to measure the expression levels of each gene. The QIAGEN® One Step RT-PCR Kit was used for this procedure. Each primer pair was used for five reactions, labeled 0, 4, 8, 24, and a control. Deionized water was used for the control in place of RNA. Additionally, GAPDH was used as a positive control with each RNA sample, as it would be expressed in all myeloid or osteoclast transcripts. 20 µL of solution were made, using 4µL 5x QIAGEN® OneStep RT-PCR buffer, 0.8

µL dNTP mix, 0.8 µL each of the forward and reverse primer (to a 0.4µM concentration), 0.8 µL of QIAGEN® OneStep RT-PCR Enzyme Mix, and 10 ng of RNA (1 µL). The remaining 11.8 µL were filled with RNase-free water. The annealing temperature was set at 57°C, the melting temperature at 94°C, and the reaction lasted for 50 cycles.

Gel Electrophoresis: In order to visualize gene expression, a 1% agarose gel was created. This was done using 200 mL of 1x TBE buffer, 2g of agarose, and 16.5 µL of ethidium bromide, an intercalator. 5 sample was run alongside a 100bp ladder. The results were visualized using a UV camera.

PCR Purification/Spectrophotometry: 24 hour samples were purified using a QIAGEN® QIAquick PCR Purification Kit, and the manufacturer's protocol was followed, except that dH_2O was used to elute instead of Buffer EB. A 1:10 dilution was made from each purified PCR product. 100 µL of each dilution was analyzed in the spectrophotometer.

Sequencing: Using the spectrophotometer results, 10ng of product were placed in a 96-well plate and sent out for sequencing. Results were confirmed using a BLAST search.

RESULTS

PCR amplification of each sample showed an increasing level of expression after some amount of treatment. Figure 2 shows the results of the initial gel electrophoresis.

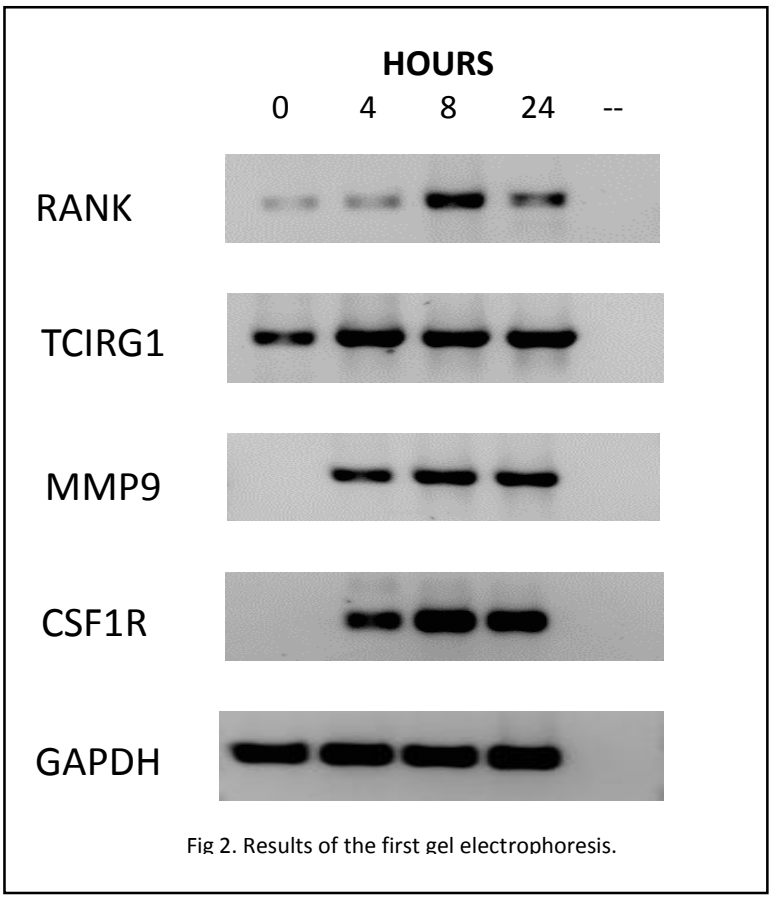

Fig 2. Results of the first gel electrophoresis.

While RANK and TCIRG1 clearly showed low levels of expression even without treatment, MMP9 and CSF1R showed little or no expression without it. GAPDH showed constant expression levels. This experiment showed little, if any, increase in expression between the 4 and 24 hour time periods.

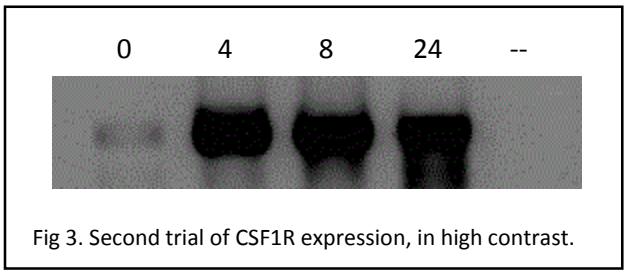

Fig 3. Second trial of CSF1R expression, in high contrast.

Additional investigations into the CSF1R gene, which, along with RANK, plays a major role in the initial differentiation of osteoclasts, showed a miniscule amount of expression at the 0 hour, but clearly existent, as shown in Figure 3.

An interesting result occurred with the expression of RANK. Low expression levels were detected at the 0 and 4 hour time points, but a clear peak happens at the 8 hour mark, followed by a decline in expression. In order to test the validity of this result, the RT-PCR reaction was repeated, and RANK was run on another 1% agarose gel. Figure 4 shows the comparison between the first and second tests. While RANK expression seems to be higher in the first strands, there is a clear increase at the 8 hour point, followed by a noticeable dip at the 24 hour point. For the remaining genes, expression looks as if it will plateau somewhere between the 8 and 24 hour point.

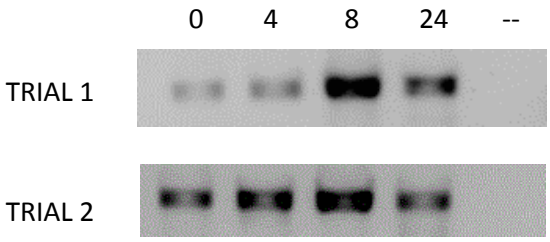

Fig 4. Comparison of RANK expression between two trials.

A BLAST search confirmed the presence of each gene, as shown by Figure 5.

GENE	LENGTH	COVERAGE	MATCH	ACCESSION
Homo sapiens tumor necrosis factor receptor superfamily, member 11a, NFKB activator (TNFRSF11A), transcript variant 1, mRNA (RANK)	1024	57%	99%	NM_003839.3
Homo sapiens T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3 (TCIRG1), transcript variant 2, mRNA	898	48%	100%	NM_006053.3
Homo sapiens matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase), mRNA (MMP9)	1020	58%	100%	BC006093.1
Homo sapiens colony stimulating factor 1 receptor (macrophage) (CSF1R), transcript variant 1, mRNA	396	27%	99%	NM_005211.3

Fig 5. Sequencing results following a BLAST search

DISCUSSION

It would seem that all expression has reached a plateau somewhere between the 8^{th} and 24^{th} hour. Unless the expression of another gene is playing a major role in the differentiation of osteoclasts that requires more time, it would seem that following 24 hours of exposure to TPA and Vitamin D_3, a

myeloid cell has sufficient time to become a fully formed osteoclast. This can be investigated further by examining the living cells at each time point.

Knowing the amount of time required to derive osteoclasts from myeloid cells in a laboratory environment will save a good deal of time when experimenting with osteoclasts. If, for example, it takes 24 hours instead of 48 hours for osteoclastogenesis to complete, the experiment can continue an entire day earlier.

The increases in the expression of all four genes supports the claim that the combination of TPA and Vitamin D_3 will cause the differentiation of an osteoclast. According to these results, MMP9 seems to be the last gene to be expressed. Seeing as this is the enzyme that allows the cell to dissolve the bone matrix, this makes sense that it would be the final step of osteoclast formation, and that it would not be expressed in undifferentiated myeloid cells. TCIRG1 is expressed at higher rates in osteoclasts, as a higher acid content is required in the organelles of an osteoclast in order for the cell to be able to dissolve the bone matrix.

CSF1R, the receptor to the colony stimulating factor, is required to differentiate the cell into the osteoclast. The fact that it is not expressed at high levels at the 0 hour could be evidence for the role that either the combination of TPA and Vitamin D_3 or the signaling of the RANK pathway has on the differentiation of the osteoclast. Further investigation of this mechanism could be completed by knocking out the RANK gene and exploring the effects on differentiation.

RANK certainly requires further investigation. If there truly is a peak in expression at around the 8^{th} hour of exposure to TPA and Vitamin D_3, this has important implications for the differentiation of osteoclasts. It is a possibility that after the peak recedes, the cell may be a fully functioning osteoclast. Additionally, the evidence supports the hypothesis that RANK's primary job is to cause differentiation, followed by a steep decline in RANKL signaling. Kido et al. pointed out that RANK works through a positive feedback mechanism using its own (RANKL) signaling pathway (Shinsuke, et al.) As mentioned,

RANKL signaling may also play a role in increasing the expression of the CSF1R gene, although there is no evidence beyond the chronological order in which they are expressed. Further investigation could be able to expose the exact mechanism through which this differentiation process is working, and how these genes are interacting.

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