The Impact of Dietary Compounds on Osteoclast Differentiation/Function

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Abstract

Many conditions can alter the balance of bone renewal, with osteoporosis being a phenotype resulting from too much break down compared to renewal. Osteoporosis affects a wide variety of people, from transplant patients to postmenopausal women and the elderly. As such, it requires the attention of researchers in order to find safe and effective treatments to prevent and even reverse the bone density loss that results in thousands of deadly fractures each year. Several dietary compounds have been studied in order to replace estrogen, but little has been done to analyze the effects of compounds on actual gene expression in osteoclasts. The purpose of this study was to analyze the effects of fourteen compounds on the expression of RANK, MM9, CSF1R, and CA2, all critical to osteoclast identity and function. Gene expression was looked at using RT-PCR with analysis by agarose gel electrophoresis. Two compounds, digoxin and ground mustard, showed marked decreases compared to differentiated cells with no compounds present. This indicates that these compounds have some ability to interfere with osteoclast differentiation and function. Previous work on digoxin has shown decreased fracture rates, and this study suggests that digoxin's mechanism includes decreasing carbonic anhydrase II expression in osteoclasts. Future work should focus on quantifying and determining the mechanisms of these effects.

Introduction

Osteoporosis represents a major problem for many groups of people, the most well-known being post-menopausal women and the elderly. Although many prescription remedies are currently in use, there is a desperate need for a remedy that can affect the root cause by altering gene expression, without many of the dangerous side effects that can occur with current treatments. In recent years, screening of dietary compounds has resulted in effective treatments for diseases such as Familial Dysautonomia (Anderson & Rubin 2005). Osteoclasts rely on the expression of many genes in order to properly differentiate and function, and genes of interest can be found by looking at the causative mutations for osteopetrotic phenotypes, where bone is not resorbed properly. Among these genes are RANK, MM9, CSF1R, and CA2.

Bone is constantly renewing itself. Osteoclasts break down older bone which has obtained microfractures, then osteoblasts are able to lay down new bone in its place. If the balance of break down and renewal is disrupted, a diseased phenotype develops. In the case of osteoporosis, the ratio favors break down, resulting in brittle and thin bones with increased fracture rates (Kanis et. al 2013). These fractures can be deadly, with over 40,000 deaths due to osteoporosis caused fractures in Europe alone (Kanis et. al 2013).

Osteoporosis is especially prevalent in post-menopausal women due to lowered amounts of estrogen, which plays an important role in maintaining bone mineral density (Riggs et. al 1998)). Because of this role, previous research has focused on using dietary compounds such as genistein to replace estrogen without the harmful effects of estrogen replacement therapy (Lappe 2013). However, this does not approach the problem of osteoporosis from the

level of gene expression in bone-degrading cells. By looking for ways to interfere with osteoclast differentiation and function through changes in gene expression, dietary compounds may be found that can help bring the balance of bone break down and renewal back towards normal.

The four genes analyzed in this study are differentially expressed in precursor cells and differentiated, mature osteoclasts. They also play key roles in osteoclast function. RANK (recedptor activator of nuclear factor kappa-B) and CSF1R (colony stimulating factor-1 receptor) are critical in that they modulates osteoclast differentiation, acting as a receptor to differentiation signals such as RANKL and M-CSF (Nakagawa et. al 1998, Dai et. al 2004). MM9, matrix metallopeptidase 9, plays a role in osteoclast activation as well as cytokine-induced bone matrix breakdown (Kusano et. al 1998). Finally, CA2 (carbonic anhydrase II) plays a key role in bone resorption by modulating proton secretion to acidify bone (Lehenkari et. al 1998).

The purpose of this study was to screen fourteen compounds for their potential effects on the gene expression of osteoclasts derived from HL-60 precursor cells. The compounds were present along with a differentiation agent, so the gene expression levels could be compared to cells treated with only the differentiation agent. Since the agent was present in the same concentration in all samples, any decrease in expression in a compound treated sample could indicate interference with osteoclast differentiation and function. Reverse transcriptase PCR was performed and agarose gel electrophoresis was used to analyze gene expression in all samples, in order to identify such decreases.

Methods

Six well plates were prepared with three mL of 1X RPMI Media 1640 with L-Glutamine (Life Technologies, Norwalk, CT, USA). The wells contained 50nM of Phorbol 12-myristate 13-acetate (TPA, TLC Grade ≥99%) and one compound as listed in Table 1 (Sigma, St. Louis, MO, USA). The plates were UV sterilized, and HL-60 cells were added. The cells were incubated at 37 degrees Celsius. After forty eight hours, the cells were observed for phenotypic changes. The RNA of each culture was extracted using the Qiagen RNeasy Plus Mini Kit in the Qiacube automated system according to manufacturer's protocols (Qiagen, Hilden, Germany). RNA concentration was analyzed by UV spectrophotometry for concentration and 260/280 ratio, and 5 ng/uL stocks were made.

Sample	Compound w/ 50nM TPA
1	Untreated, RPMI Media alone (no TPA)
2	EGCG 25ug/uL (3uL)
3	Digoxin 50ng/uL (15uL)
4	Suma Root 1:300 dilution (10uL)*
5	Bupleurum 1:300 dilution (10uL)*
6	Fennel 480ug/mL (30uL) #
7	Echinacea w/ Goldenseal Root 400ug/mL (30uL)*
8	Acai 400ug/mL (24uL) #
9	Ginger Root 100ug/mL (12uL) #
10	Ground Mustard 100ug/mL (30uL) ^
11	Turmeric 100ug/mL (15uL) ^
12	Ambrotose 100ug/mL (30uL) ~
13	1alpha-250dihydroxyvitamin D3, 10nM (6uL, 60uM stock) >
14	TPA Alone, 50nM

Table 1-Compound Screening of HL-60 Cells. Each well contained RPMI media, the listed compound, and 50nM TPA, with the exception of the untreated control which had RPMI media alone.

*=(Nature's Answer, Hauppauge, NY) ^=(McCormick's, Sparks, MD)

~=(Mannatech Inc., Coppell, TX) #=(Vitamin World, Holbrook, NY)

>=(Sigma, St. Louis, MO)

RT-PCR was performed using the One Step RT-PCR Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol, with primer concentration of 0.5uM, 2uL of template RNA, and a total reaction volume of 20uL. Five reactions were performed for each sample, one each for RANK, MM9, CA2, CSF1R, and GAPDH. The primers used are listed in Figure 1, along with the length of the intron spanned to clarify that the product obtained was not DNA contamination. All primers used contained SP6 or T7 (one on forward, one on reverse) on the end for ease of sequencing. The RT-PCR reaction followed a program of 50°C for 15minutes, 95°C for 15 minutes, followed by forty cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for thirty seconds, with a final extension time of two minutes at 72°C.

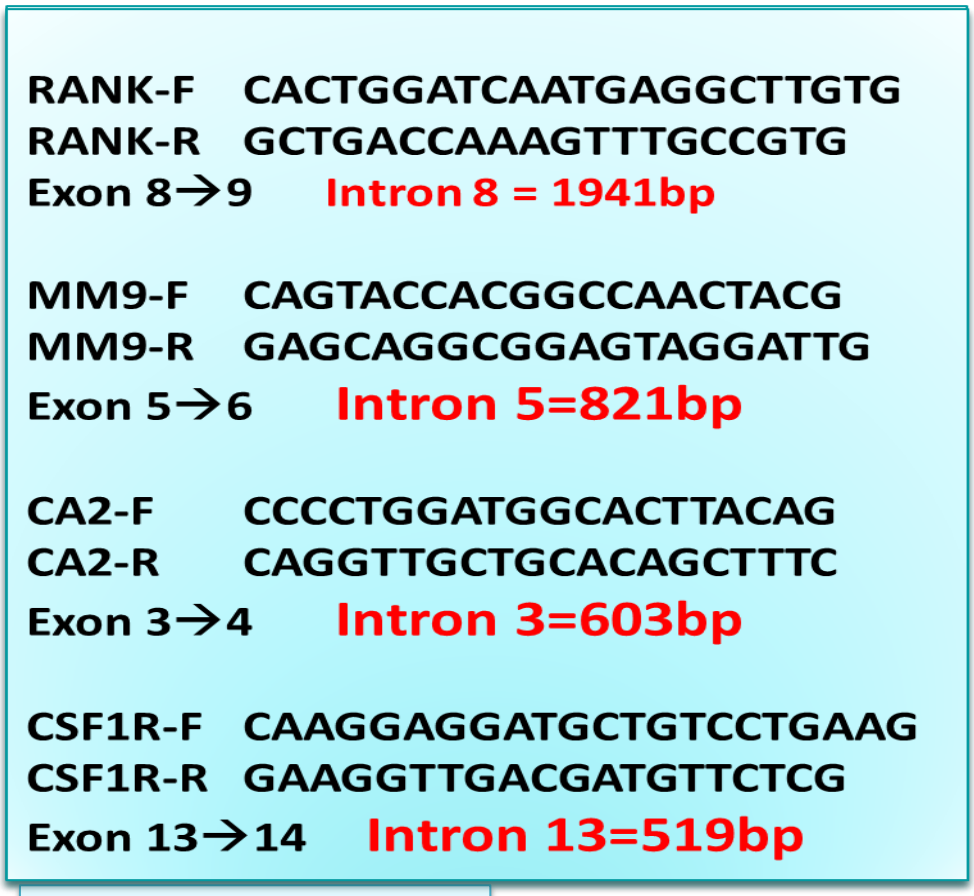

Figure 1-Primers used for RT-PCR reactions, including the lengths of the introns spanned.

Five microliters of loading dye was mixed with each 20uL RT-PCR product. Five microliters of this mixture was analyzed on a Promega Analytical Grade LE 200-well 2% Agarose gel (Promega, Madison, WI, USA). A 100bp Invitrogen TrackIt Ladder was used (Life Technologies, Norwalk, CT, USA). The gel was run at 140V, then visualized using a Carestream Image Station 4000MM, with Carestream MISE Software (Carestream Health Inc., Rochester, NY, USA). Gel analysis was repeated three times, figures presented are representative. Gel images were analyzed for quantitative differences, emphasizing differences between samples with compounds and TPA versus the sample with TPA alone.

RT-PCR products for one sample of each gene product were purified using the QIAquick PCR purification kit according to manufacturer's instructions with a modification using distilled water to elute (Qiagen, Hilden, Germany). All centrifugation steps were carried out for the longest time recommended, and the final elution was performed in two steps instead of one. The purified products were then analyzes using UV spectrophotometry for concentration and 260/280 ratio, and sent out for sequencing using T7 and SP6 primers by Genewiz (Genewiz Inc., South Plainfield, NJ, USA). All sequencing results were used to verify that primers had amplified the desired gene product using the Basic Local Alignment Search Tool (BLAST, NCBI).

Results

RT-PCR products were analyzed using gel electrophoresis with a 2% agarose gel, with a representative image of the results presented in Figure 1. GAPDH controls indicated that equal amounts of RNA were loaded into each reaction. RANK gene expression did not show any notable differences between any compound with TPA and the sample with TPA alone. MM9 expression was again very similar across all samples, though ground mustard does appear to have lower expression than the TPA only sample. In the results for the expression of CSF1R, again, ground mustard appears to have a lower expression than the TPA alone sample. However, in the results for CA2 expression, ground mustard is joined by digoxin, both showing a marked decrease in expression from the TPA alone sample. Sequencing results are shown in Table 2. All gene products were positively identified using BLAST as the desired gene, with a match accuracy to the human transcript of 98% or more (BLAST, NCBI).

Figure 2-Agarose Gel Electrophoresis Results, red boxes indicate notable differences between samples with TPA versus TPA alone.
U=untreated, T=TPA Alone
Key to the right describes which compound the cells were treated with (Plus 50NM TPA)

1-EGCG
2-Digoxin
3-Suma Root
4-Bupleurum
5-Fennel
6-Echinaceaw/ Goldenseal
7-Acai
8-Ginger Root
9-Ground Mustard
10-Turmeric
11-Ambrotose
12-Vitamin D3

Transcript Name	E Value	Max Ident	Accession
Homo sapiens TNFRSF11A, NFKB activator, transcript variant 1, mRNA (RANK)	3.00E-23	100%	NM 003839.3
Homo sapiens TNFRSF11A, NFKB activator, transcript variant 1, mRNA (RANK)	3.00E-22	100%	NM 003839.3
Homo sapiens TNFRSF11A, NFKB activator, transcript variant 2, mRNA (RANK	2.00E-14	100%	NM 001270949.1
Homo sapiens matrix metallopeptidase 9, mRNA (MM9)	7.00E-52	99%	NM 004994.2
Homo sapiens matrix metallopeptidase 9, mRNA (MM9)	9.00E-46	98%	NM 004994.2
Homo sapiens colony stimulating factor 1 receptor (CSF1R), mRNA	1.00E-45	98%	NM 005211.3
Homo sapiens colony stimulating factor 1 receptor (CSF1R), mRNA	4.00E-46	100%	NM 005211.3
Homo sapiens carbonic anhydrase II (CA2), mRNA	2.00E-60	99%	NM 000067.2
Homo sapiens carbonic anhydrase II (CA2), mRNA	4.00E-62	99%	NM 000067.2

Table 2-Sequencing results showing positive gene identity for RT-PCR Products

Discussion

In this study, both ground mustard and digoxin showed an effect on gene expression in osteoclasts derived from HL-60 precursor cells compared to expression in cells treated with TPA alone. TPA acts as a differentiation agent on the HL-60 cells, causing them to take on an osteoclast identity and function. Ground mustard and TPA treated cells showed less expression of MM9, CSF1R, and CA2 compared to the TPA alone cells. Digoxin and TPA treated cells, on the other hand, showed less expression of CA2 only. These gene expression changes could be indicative of a disruption in osteoclast differentiation and function.

Ground mustard contains many compounds with potential effects in humans, including phytoalexins, steryl esters, and flavonoids such as apigenin and chalcone (Cui et. al 1993). Mustard has been used as a cancer treatment, but the compounds used are removed during the production of ground mustard, and thus are not the likely causative agents of the effects seen in this study (Tseng et. al 2002). Although many of the other compounds in mustard have various anti-inflammatory, antimicrobial, and antioxidant effects, flavonoids have been shown to alter gene expression in cells (Gerritsen et. al 1995). Apigenin specifically has been shown to have a dose-dependent effect on the expression of various cell adhesion proteins such as VCAM-1 and E-Selectin (Gerritsen et. al 1995). This suggests a possible mode of action for the effects observed in this study, though the overall mechanism likely involves multiple compounds present in ground mustard.

Both digoxin and mustard contain glycosides, which are sugars bound to small non-carbohydrate molecules. While digoxin is currently widely used for cardiac problems, mustard

has only been studied in a limited fashion, with a sole focus on antioxidant activity (Singh et. al 1997). Cardiac glycosides work by inhibiting the activity of proton pumps, which are also critical to osteoclast resorption of bone (Blume et. al 2006). However, neither has been extensively studied with regards to their effect on bone. A 2007 study by Rejnmark et. al did show decreased fracture rates in patients over sixty five being treated for cardiac arrhythmias (Rejnmark et. al 2007). This study suggests that the mechanism for digoxin's reduction of fractures is the inhibition of osteoclast activity by causing decreased expression of carbonic anhydrase II. CA2 acts in bone resorption by facilitating proton secretion, which plays a role in dissolving bone (Lehenkari et. al 1998).

Following the results obtained in this study, future research should include the application of real time RT-PCR techniques to verify the gene expression differences observed in the gel electrophoresis analysis. Given the previous work involving cardiac glycosides and their mechanisms, primers should be developed to analyze proton-pump genes within osteoclasts, such as the TCIRG1 proton pump subunit. Dilution studies should also follow, to determine relative toxicity levels *in vitro*. Working within the levels that do not exhibit excessive toxicity prevents effects from being seen that have no chance of working *in vivo*. Further work to determine the mechanisms that each of these compounds use should also be performed. Since, out of the twelve compounds screened in this study, two showed promise, further compounds should also be screened.

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