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Downregulation of Gnas, Got2 and Snord32a following tenofovir exposure of primary osteoclasts

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Abstract

Clinical observations have implicated the antiretroviral drug tenofovir with bone loss during the management of HIV infection. The goal of this study was to investigate the in vitro effects of tenofovir exposure of primary osteoclasts in order to gain insights into the potential mechanisms for the drug-induced bone loss. We hypothesized that tenofovir may alter the expression of key genes involved in osteoclast function. To test this, primary osteoclasts were exposed to physiologically relevant concentrations of the prodrug tenofovir disoproxil fumarate (TDF), then intensive microarray analysis was done to compare tenofovir-treated versus untreated cells. Specific downregulation of Gnas, Got2 and Snord32a were observed in the TDF-treated cells. The functions of these genes help to explain the basis for tenofovir-associated bone loss. Our studies represent the first analysis of the effects of tenofovir on osteoclast gene expression and help to explain the basis of tenofovir-associated bone loss in HIV-infected individuals.

Keywords

HIV; HAART; osteoblast; G-protein; AST; snoRNA

Introduction

Human immunodeficiency virus (HIV) is a major pandemic, with over 30 million infected people worldwide. Highly active antiretroviral therapy (HAART), which combines several antiretroviral drugs, is effective in managing the viral infection but is associated with viral drug resistance and toxicities. Tenofovir and its prodrug, tenofovir disoproxil fumarate (TDF), is a

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nucleotide analog reverse transcriptase inhibitor (NtRTI) that competes with deoxynucleoside triphosphates (dNTPs) during HIV reverse transcription, leading to chain termination due to its lacking a 3'-hydroxyl group on the deoxyribose required to form a new 5'-3' phosphodiester bond that extends the DNA chain. TDF has advantages over the nucleoside analogs used to treat HIV infection. First, the activation of TDF is more rapid compared to that of nucleoside reverse transcriptase inhibitors (NRTIs) since it is phosphorylated, which abbreviates the intracellular activation pathway for a more rapid and complete conversion from the prodrug to the active drug. Second, TDF has minimal cellular and mitochondrial toxicity compared to nucleoside analogs [1;2;3;4]. Nonetheless, TDF has been reported to be associated with loss of bone mineral density (BMD), particularly in young children and adolescents [5;6;7;8].

Bone homeostasis relies on the balance of bone formation and resorption, which are conducted by osteoblasts and osteoclasts, respectively. Osteoblasts regulate osteoclast differentiation by expressing two factors that are necessary and sufficient for osteoclast formation: M-CSF and RANKL. M-CSF is required for survival and proliferation of early osteoclast precursors. Binding of RANKL and the RANK receptor on osteoclasts stimulates expression of genes necessary for osteoclast differentiation, cellular fusion and bone resorption. Osteoblasts also express osteoprotegerin, a soluble decoy receptor for RANKL, which can inhibit the activation of RANK by RANKL. The ratio of RANKL to osteoprotegerin produced by osteoblasts helps to determine osteoclast forming activity within the bone microenvironment and is involved in the close coordination between bone formation and bone resorption under normal physiological conditions.

For children and adolescents, the rate of bone formation exceeds that of bone resorption, allowing for the size and mass of bone to increase over time. Studies in rhesus monkeys have demonstrated the inhibition of cortical bone mineralization and bone toxicity following the administration of TDF [5;6]. Furthermore, clinical studies have found that TDF therapy in HIV-infected children resulted in bone abnormalities such as unfused epiphyses and decreased trabecular bone [8;9]. TDF-induced bone abnormalities such as osteopenia, osteoporosis and spontaneous fractures may lead to the loss of alveolar crestal height and oral bone loss [10; 11]. Little is known about the mechanism(s) of TDF-associated bone loss in HIV-infected individuals.

In this study, we investigated the in vitro effects of tenofovir exposure of primary osteoclasts in order to gain insights into the potential mechanisms for the drug-induced bone loss. We tested the hypothesis that tenofovir may perturb the expression of key genes involved in osteoclast function that would increase bone resorption. To test this, we isolated primary murine osteoclasts, exposed to physiologically relevant concentrations of TDF, and then purified total RNA for intensive microarray analysis. Comparison of TDF-treated versus untreated cells revealed specific downregulation of *Gnas*, *Got2* and *Snord32a* were observed in the TDF-treated cells. The functions of these genes help support a model to explain the basis for tenofovir-associated bone loss. Our studies represent the first analysis of the effects of tenofovir on osteoclast gene expression and help to explain the basis of tenofovir-associated bone loss in HIV-infected individuals.

Materials and Methods

Primary osteoclast cultures, viability, TRAP staining

Primary osteoclast cultures were prepared from spleen of 5 day old mice as previously described [12;13;14]. Cell viability was determined by measurement of cellular ATP using the CellTiter-Glo Luminescent Cell Viability Assay using the manufacturer's instructions (Promega, Madison, WI). TRAP staining was done using the TRAcP5b kit (Sigma-Aldrich).

Microarray analysis

Primary murine osteoclasts were prepared and treated with 500 nM TDF for 72 h. Total RNA from primary osteoclasts was then extracted using the RNeasy mini plus kit (Qiagen, Valencia, CA). Four independent replicates from TDF-treated primary osteoclasts and untreated cells were analyzed. Two gene chips were used in each replicate experiment. Assistance in RNA quality control, labeling, hybridization, and initial data analysis was provided by Genome Explorations Inc. (Memphis, TN). The integrity of the RNA was examined by capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) with RNA 6000 Neno Lab-in-a-Chip Kit (Agilent Technologies) following manufacturer's instructions. Total RNA was used for cDNA synthesis with the reverse transcription-in vitro transcription (RT-IVT) method [15] using GeneChip WT cDNA Synthesis and Amplification kit (Affymetrix, Santa Clara, CA) according to manufacturer's instructions.

Fragmented and labeled cDNA was hybridized for 17 h at 45°C to GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix). The mouse Gene 1.0 ST array is an expression array featuring whole genome-transcript coverage with 750,000 unique oligonucleotide probes representing a total number of 28,853 mouse genes. There were approximately 27 probes covering the full length of each gene. Background correction, normalization, and signal summarization (per probe set) were calculated accordingly [16]. For each transcript, an independent *t*-test (5% confidence) was applied to assess significance of expression level based on RMA absolute signal log ratios ≥ 1.0 .

Quantitative real-time PCR

Primary osteoclasts were treated with TDF, washed in 1x phosphate-buffered saline (PBS) twice prior to RNA extraction. The RNeasy mini plus kit (Qiagen, Valencia, CA) was used to yield total RNA and to remove traces of genomic DNA contamination from cells based on manufacturer's instructions. Immediately following RNA extraction, 500 ug of total RNA from each sample was used to generate first strand cDNA as templates for quantitative PCR (qPCR) with transcriptor high fidelity cDNA kit (Roche applied science, Indianapolis, IN). SYBR green qPCR reagents from Invitrogen (Carlsbad, CA) were used to detect real-time gene expression. GAPDH and 18S rRNA genes were used as internal controls for normalization. Three independent experiments were performed. Sequences of primer sets used are available upon request.

Results and Discussion

The goal of this study was to determine whether in vitro treatment of primary osteoclasts with TDF, the prodrug of tenofovir (Fig. 1A, B), would alter gene expression as determined by microarray analysis, and provide insights into how drug exposure may influence osteoclast function. We chose primary murine osteoclasts for analysis as they provide a readily tractable model system to assess the mechanism(s) involved in tenofovir-mediated bone abnormalities that can be readily translated into mouse models of HIV infection. Such models would allow for the in vivo analysis of TDF administration on bone morphology in the context of HIV infection.

We first investigated the effect of TDF exposure on cell viability. We sought to determine whether physiologically relevant TDF concentrations corresponding to the dosing of TDF in antiretroviral therapy had any effects on cell viability. A range of TDF concentrations (50 nM to 500 uM) was analyzed for their effects on osteoclast viability. The highest TDF concentrations analyzed, i.e., 50 uM, 250 uM and 500 uM, were found to significantly reduce cell viability (Fig. 1C). The lower TDF concentrations, i.e., 50 nM, 500 nM and 5 uM, had no effect on cell viability. Tenofovir has been shown to cause very little cytotoxicity in human

HepG2 and skeletal muscle cell lines, with average CC_{50} of 399 and 870 μ M, respectively [1]. It is to be expected that primary culture is more sensitive to drug treatment in comparison to immortalized cell lines. As shown in our cytotoxicity assay, primary osteoclast culture reacts to TDF in a much lower concentration (Fig 1C). Tartrate-resistant acidic phosphatase (TRAP) is a marker commonly used in the identification of primary osteoclasts in culture. Therefore, TRAP staining was next used to further verify osteoclast identity and viability at lower TDF concentrations. Figure 1D shows the results of TRAP staining on osteoclasts treated at TDF concentrations of 500 nM, 1 μ M and 2 μ M. Compared to the no drug control, TDF treatment at the tested concentrations had no effect on osteoclast viability, as well as osteoclast size and numbers (Fig. 1E). Based upon these results, we chose the TDF of 500 nM for analyzing the impact TDF exposure on osteoclast gene expression. This TDF concentration is physiologically relevant based upon the serum concentrations and dosing regimens used in antiretroviral therapy of HIV infected individuals [17;18]. Therefore, the TDF concentration used in microarray analysis of primary osteoclast gene expression is relevant to the concentrations used in anti-HIV therapy.

To investigate the impact of TDF treatment on osteoclast gene expression, primary osteoclasts were treated with 500 nM TDF, and then total RNA was extracted used to generate cDNAs that were then prepared for use in microarray analysis. The microarray analysis was intensive and allowed for the thorough analysis of osteoclast gene expression profiles. As described below, this intensive approach helped to focus the gene expression profile on a relatively limited number of genes. The replicate-to-replicate variability of TDF treatment of primary osteoclasts was likely another variable that led to the resulting focused gene expression profile. The focused gene expression profile could also be a reflection of the limited amount of TDF uptake by osteoclasts in tissue culture. Since TDF is a phosphonate, this could enhance its uptake into cells when associated with bone tissue. Bisphosphonates (ie, diphosphonates) are drugs used clinically to prevent loss of bone density, particularly in diseases such as osteoporosis, bone metastasis, and multiple myeloma. Bisphosphonates target bone and inhibit osteoclast function after their cellular uptake by inducing apoptosis [19;20]. Since TDF is phosphonate, it is possible that their association with bone could enhance their uptake by osteoclasts by a mechanism similar to that of bisphosphonates.

Table 1 lists the resulting gene expression profile identified from intensive microarray analysis of TDF-treated primary osteoclasts. A total of nine genes were identified. Notably, no significantly up-regulated genes were observed from our microarray analysis. Among those candidates, most were non-verified, relatively smaller RNAs (six out of nine). There were only three previously identified genes, Snord32a, Gnas and Got2, two of which encode for proteins. Gnas (guanine nucleotide binding protein, alpha stimulating – also known as GalphaS) is a membrane-bound G-protein receptor that is involved in signaling to MAPK/ERK (Fig. 2A). GalphaS activates the cAMP-dependent pathway by stimulating the production of cAMP from ATP by direct stimulation of the membrane-associated enzyme adenylate cyclase. The cAMP can then act as a second messenger that interacts with and activates protein kinase A, which can phosphorylate a wide array of downstream targets. The MAPK/ERK pathway is important for osteoclast proliferation and for the formation of actin filaments to form the sealing zone on bone tissue prior to bone resorption. Gnas has a highly complex imprinted expression pattern that can result in maternally, paternally, as well as biallelically expressed transcripts derived from four alternative promoters and the 5' exons. There is also an antisense transcript. One of these transcripts and the antisense transcript are both paternally expressed noncoding RNAs, and may regulate imprinting in this region. Due to the complex nature of the gene locus, qPCR analysis of the observed downregulation in the microarray analysis was not done. The downregulation of Gnas gene expression would be expected to result in less MAPK/ERK signaling and ultimately a reduction in osteoclast proliferation and actin filament formation – these reductions would result in less bone resorption. This observation, in the context of TDF-

mediated bone loss, suggests that the bone formation activity of osteoblasts may also be downregulated so that an overall loss of bone density is observed. Reports in the literature have also linked *Gnas* to bone abnormalities. A recent report described a transgenic mouse model with locally restricted inactivation of *Gnas* to extend studies of the role of *Gnas* function in vivo [21]. In this study, a hRen-Cre transgenic mouse line was crossed with a mouse strain in which exon 1 of the *Gnas* gene was flanked by loxP sites. The most notable phenotype involved marked skeletal malformations of the forelimbs with shortened and fused extremity bones. This phenotype is similar to syndromes in humans associated with *Gnas* loss-of-function, including Albright hereditary osteodystrophy and progressive osseous heteroplasia. Interestingly, loss of *Gnas* has been found in osteoblasts to result in a defect in the formation of the primary spongiosa with reduced immature osteoid, overall length, and trabecular bone volume [22]. The cortical bone was thickened, with narrowing of the bone marrow cavity, and was likely due to decreased cortical bone resorption since osteoclasts were observed to be markedly reduced on the endosteal surface of cortical bone. To date, no specific reports on the effects of reduced or loss of *Gnas* expression on osteoclast function have been reported.

Got2 (glutamate oxaloacetate transaminase 2, also known as aspartate aminotransferase or AST) is a mitochondrial enzyme involved in energy transduction, specifically amino acid metabolism as well as the urea and tricarboxylic acid cycles (Fig. 2B). AST is commonly detected in the blood as a clinical indicator of organ injury and cellular stress – particularly for the liver, with alanine transaminase (ALT). Organ injury typically results in higher serum AST levels, and the AST/ALT ratio is commonly analyzed. Interestingly, a reduction of Got2 expression was found to correlate to a loss in bone mineral density in a knockout mouse model for Canavan's Disease, a neurodegenerative disorder involving a deficiency in aspartocyclase [23]. Although these findings are correlative, this data suggests an association between the general reduction in Got2 expression and loss of bone mineral density. Previous studies of the effects of NRTIs on mitochondrial gene expression in the mouse liver have been studied by microarray analysis and led to the identification of many genes influenced by NRTI exposure [24]. Although Got2 was not one of the genes characterized, liver cells and non-osteoclasts were analyzed and it is formally possible that tissue-specific differences exist. We followed up the microarray data analysis with qPCR analysis. We chose to detect the expression level of several genes (i.e., cathepsin K, NFAT, and TRAP) that are specifically involved in osteoclast differentiation by qPCR, and their gene expression was not significantly effected by TDF, and in agreement with the microarray analysis (Table 2). The general trends from qPCR suggest downregulation of Got2 expression, though our results were not statistically significant (Table 2). This observed discrepancy with the microarray data may be due to the SYBR green method used for qPCR, which is a relatively non-quantitative method for qPCR compared to other methodologies (e.g., TaqMan). Also, more than 1 or 2 housekeeping genes is likely required in order to establish accurate normalization for the verification of the small fold differences observed in the microarray analysis.

Snord32a (small nucleolar RNA, C/D box 32A) is a member of the small nucleolar RNA (snoRNA) group of highly abundant, non-polyadenylated, non-coding transcripts that localize to the nucleus but are thought to have more broad functions (Fig. 2C). Snord32a is located in the second intron of *Rpl13a*, whose gene product is part of the large ribosomal subunit [25]. The snoRNAs are essential for regulating biological functions such as RNA processing and modifications, gene expression, protein trafficking, and genome stability. snoRNAs are highly conserved throughout evolution, and are found in Archaea as well as in eukaryotes [26;27; 28]. The two main classes of snoRNAs include the C/D box snoRNAs, which are associated with methylation, and the H/ACA box snoRNAs, which are associated with pseudouridylation. After transcription, pre-rRNAs undergo processing in order to generate the mature rRNA. A complex pattern of nucleoside modifications occurs prior to cleavage by exo- and endonucleases, which includes methylation and pseudouridylation, that are directed by

snoRNAs. The C/D box snoRNAs specifically guide site-specific 2'-O-methylation of RNAs. 2'-O-methylation of rRNA is required for ribosome function, likely by affecting proper rRNA folding, maturation and stability. It is thought that rRNA modifications act to optimize rRNA structure for the production of accurate and efficient ribosomes. Overall, snoRNA binds to target RNA via base pairing and acts as a scaffold for its protein partners to be recruited and therefore triggers the enzymatic reactions. The qPCR analysis suggested downregulation of gene expression, but the results were not statistically significant (Table 2). The lack of microarray analysis verification of Snord32a is likely due to the limitations in the qPCR analysis (as described above). The reduction in Snord32a expression could result in a decrease of fully functional ribosomes, which would lead to a reduction in gene expression. Three previously uncharacterized snoRNAs were identified in our analysis that had significantly altered expression from the TDF-treated primary osteoclasts as well as two small nuclear RNAs (snRNAs) and one unknown transcript (Table 1). Two of these snoRNAs had higher levels of gene expression, while there was a reduction in expression for the other four snoRNA genes. Many studies have revealed some snRNAs do not possess sequence motifs, nor are complementary to known target RNAs [29]. It is likely that there are many putative targets of snoRNAs that are yet to be identified. The function of snoRNA (and many downstream reactions affected by them) is therefore more complex than anticipated. It is plausible that these other snoRNAs may play some roles in altering gene expression due to TDF treatment.

In summary, we report that TDF-exposure of primary osteoclasts results in a striking alteration of a distinct gene expression profile. Our findings suggest that the clinical observations of TDF-mediated bone density loss observed in children and adolescents could be due, in part, to changes in osteoclast gene expression that result in osteoclast dysfunction. Future studies should focus on extending our observations to in vivo analysis of gene expression alterations due to TDF treatment. In addition, it would be of interest to analyze the specific alteration of gene expression for the genes identified in this study in osteoclasts in vivo to assess their impact on bone formation and resorption. Finally, extending these studies to osteoblasts would provide greater insight into the effects of TDF exposure on bone mineral density.

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Figure 1.

Tenofovir structure and primary osteoclast cell viability following drug exposure. The structure of tenofovir (A) and tenofovir disoproxil fumarate (TDF, the prodrug of tenofovir) (B) is shown. C. Viability of primary osteoclasts following exposure to TDF. A TDF dilution series was added to primary osteoclast cultures for 72 hr, refreshing every 24h. Cell viability was determined by ATP detection. Tartrate-resistant acid phosphatase (TRAP) staining of untreated (D) and TDF-treated (E) primary osteoclasts and analysis (F) is shown.

Figure 2.

Target genes, Gnas, Got2 and Snord32a, following tenofovir exposure of primary osteoclasts.

A. Gnas or stimulatory G-protein alpha subunit ($G\alpha S$) is a key part of the classic signal transduction pathway linking receptor-ligand interactions with the activation of adenylate cyclase and a variety of cellular responses (including the MEK-Erk signaling pathway). B. Got2 (glutamate oxaloacetate transaminase 2) is a mitochondrial enzyme involved in energy transduction, specifically amino acid metabolism as well as the urea and tricarboxylic acid cycles. C. Snord32a (small nucleolar RNA, C/D box 32A) is a member of the small nucleolar RNA (snoRNA) group of highly abundant, non-polyadenylated, non-coding transcripts. The C/D box snoRNAs are associated with methylation, and specifically guide site-specific 2'-O-methylation of RNAs, which impact gene expression.

Table 1

Intensive microarray analysis identifies a distinct gene expression profile from TDF-treated primary osteoclasts.

Gene Accession	Gene Symbol	mRNA Accession	T test p-value	log2 fold change
---	---	ENSMUST00000083033	0.033268	0.667533
---	---	GENSCAN00000035529	0.010961	0.984285
NR_000002	Snord32a	NR_000002	0.01074	-0.673546
---	---	ENSMUST00000082834	0.006467	-0.770904
---	---	ENSMUST00000083804	0.003076	-0.765541
NM_010309	Gnas	NM_010309	0.02253	-0.617939
---	---	ENSMUST00000083338	0.028608	-0.643234
---	---	ENSMUST00000083981	0.00119	-0.955095
NM_010325	Got2	NM_010325	0.005533	-0.633033

Table 2

Real-time PCR analysis of selected genes from TDF-treated primary osteoclasts.

Replicate	Relative Gene Expression ^a				
	Cathepsin K	NFAT	TRAP	Got2	Snord32a
1	1.188	1.077	1.210	0.672	0.399
2	1.212	1.115	1.341	0.983	1.231
3	1.033	1.537	0.767	1.017	1.159
Avg.	1.144	1.240	1.106	0.890	0.930
SD	0.097	0.255	0.301	0.190	0.460

^aGene expression in TDF-treated primary osteoclasts compared to untreated osteoclasts. Gene expression was normalized to GAPDH and 18S rRNA.