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ScienceDirect
Antiviral Research 74 (2007) 72–76
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

HIV1 protease inhibitors selectively induce inflammatory chemokine expression in primary human osteoblasts

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Received 20 June 2006; accepted 13 December 2006

Abstract

HIV-infected patients are at increased risk of decreased bone mineral density. Several studies have implicated antiretroviral therapy as a contributor to the decreased bone mineral density seen in treated HIV-1 patients. Whilst the exact molecular mechanisms underlying decreased bone density remain to be elucidated, inflammation has been postulated to be an important pathogenomic mechanism. In this study, we have explored primary human osteoblast gene expression in response to protease inhibitors (PIs), by oligonucleotide microarray analysis. A list of dysregulated genes, correlated with the inflammatory response, increased significantly after NFV and RTV exposure. Analysis of gene and protein expression determined a selectively increase of the pro-inflammatory cytokines monocyte chemoattractant protein (MCP)-1 and interleukin-8 (IL-8) following exposure to a pharmacological concentration of NFV and RTV. These data suggested that generation of local inflammatory cascades may contribute to the development of decreased bone mineral density in highly active antiretroviral therapy (HAART)-treated HIV patients.

Keywords: HAART; Osteoblasts; Inflammation; MCP-1; IL-8; Nelfinavir; Ritonavir

Survival with HIV-1 infection has been prolonged with the institution of highly active antiretroviral therapy (HAART). Combined antiretroviral therapy is associated with a number of metabolic disturbances, including insulin resistance, hyperlipidemia, and significant changes in body composition (Safrin and Grunfeld, 1999). More recently, alterations in bone metabolism (i.e osteopenia) have been recognized to occur in patients receiving HIV-1 protease inhibitors (PIs), key components of conventional combined antiretroviral therapy (Carr et al., 2001; McDermott et al., 2001; Tebas et al., 2000). The proposed pathogenic candidates mediating HAART-induced osteopenia include viral infection (Lawal et al., 2001) and the drug regimen itself. The precise PI osteopenia-inducing mechanisms and the pathway by which it exerts its effect are unknown.

The production of a wide range of cytokines by human osteoblasts *in vitro* has been demonstrated (Walsh et al., 2000). Many of the cytokines produced by osteoblasts may be important local mediators of bone turnover, *e.g.* IL-1, TNF-α and TNF-β, which are potent stimulators of bone resorption *in vivo* and *in vitro* (Walsh et al., 2000). Other cytokines produced by osteoblasts including IL-3, IL-6 and the TGF-β isoforms also affect bone metabolism and turnover.

Monocyte chemoattractant protein-1 (MCP-1), a member of the chemokine family of cytokines, is typically not expressed in normal bone or normal osteoblasts *in vitro*; however, it is upregulated upon stimulation by inflammatory mediators. This expression is temporally and spatially associated with the recruitment of monocytes in osseous inflammation (Graves et al., 1999).

Interleukin-8 (IL-8) is a member of the superfamily of small (8–10 kDa) secreted chemoattractant cytokines that were originally discovered as monocyte-derived factors capable of attracting and activating neutrophils (Baggiolini et al., 1994; Ben-Baruch et al., 1995; Sozzani et al., 1996; Yoshimura et al., 1987). Many cell types are known to synthesize and release IL-8 and other chemokines in response to injury, infection, inflammation, or various pathological conditions, such as rheumatoid arthritis, osteoarthritis, periodontal disease and psoriatic lesions (Zwahlen et al., 1993).

IL-8 and MCP-1 are immediate early stress responsive chemokines important for the activation and chemotaxis of neutrophils and macrophages, respectively. Reduced bone mineral

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doi:10.1016/j.antiviral.2006.12.003

density is a typical hallmark of many inflammatory conditions and is thought to be a consequence of both reduced osteoblast bone formation and increased osteoclast bone resorption. Previous reports showed that IL-8 has suppressing effects on bone remodelling (Rothe et al., 1998) and also increases *in vitro* bone resorption activity by activated monocytes (Liu et al., 2005). Recruitment of circulating monocytes into bone is modulated mainly by monocyte chemoattractants, as MCP-1, and their C–C receptors on monocytes, which interact to initiate and maintain a directed migration of monocytes toward bone tissue (Liu et al., 2005).

The aim of this study was to assess phenotypic alterations in primary human osteoblasts exposed to high extracellular concentration of the PIs indinavir (IDV), saquinavir (SQV), ritonavir (RTV) and nelfinavir (NFV). To permit comparison with other published studies (Jain and Lenhard, 2002), we examined gene expression profile in response to concentrations of PIs that are physiologically relevant. Indeed the utilized concentration of 5 μM is the highest concentration reached in blood samples from HIV infected patients treated with HAART (Duval et al., 2004). We then focused on the regulation of individual genes characteristic of the inflammatory response, which is an important pathogenic mechanism contributing to overall alteration in mature human osteoblast biology.

Primary human osteoblasts (Promocell, Germany) were grown in 96-well plates with osteoblast basal media (Promocell, Germany) and exposed to RTV, SQV, NFV or IDV 5, 10, 25 μM for 24 h. To determine the effect of PI on cell activity, medium was aspirated, and 100 μl/well of methylthiazolyldiphenyltetrazolium bromide (MTT, Sigma–Aldrich) solution was added (0.5 mg/ml MTT in serum free DMEM). Cells were incubated at 37 °C, 5% CO_2, for 1 h. Colorimetric responses were determined spectrophotometrically at 450 nm. Furthermore, MTT colorimetric signals were examined 1, 2, 4, 12, 24 and 48 h following addition of 5 μM RTV, SQV, NFV or IDV.

RNA extraction was performed by TRI-reagent/chlorophorm method, whilst cDNA synthesis, *in vitro* transcription and microarray analysis were performed as described previously (Kieran et al., 2003). All the expression analyses were microarrayed in duplicate using Affymetrix Gene Chips U133A_2.0. The image files, obtained through Affymetrix GeneChip Software, were analysed by Robust Multichip Analysis (RMA) (Bolstad et al., 2003). Expression was compared to control and a signal log ratio (SLR) of 0.586 or greater was taken to identify significant differential regulation. Using normalized RMA values, average linkage hierarchical cluster analysis was performed and the cluster dendrograms visualized using TreeView software (Eisen et al., 1998). Gene expression alterations were functionally classified using NIH-DAVID 2.1; http://david.abcc.ncifcrf.gov/ (Douglas et al., 2003).

MCP-1 and IL-8 protein concentration were assessed by colorimetric ELISA assay (Promocell, Germany); mRNA levels assayed by quantitative Real Time PCR and the products were reported as a function of crossing time (Ct), the cycle number at which PCR amplification becomes linear. mRNA expression was normalized to control and GAPDH expression, resulting in mean fold change values or ΔΔCt. The primers used to quantified MCP-1, IL-8 and GAPDH gene expression in Real Time PCR were: MCP-1 Fw 5′-CCC CAG TCA CCT GCT GTT AT; MCP-1 Rv 5′-AGA TCT CCT TGG CCA CAA TG; IL-8 Fw 5′-CTG CGC CAA CAC AGA AAT TA; IL-8 Rv 5′-ATT GCA TCT GGC AAC CCT AC; GAPDH Fw 5′-GAG TCA ACG GAT TTG GTC GT; GAPDH Rv 5′-TTG ATT TTG GAG GGA TCT CG.

The statistical significance of experimental findings was analyzed by linear regression and Pearson statistical analysis to examine microarray data, and two-way ANOVA for colorimetric ELISA assay and quantitative Real Time PCR. A p-value of less than 0.05 for the data has been considered significant. Final data represent the means of four experiments and were obtained as replicate samples from one assay.

To determine the effect of Protease inhibitors on primary human osteoblasts, we examined cell viability at 24 h after exposure to 5, 10 or 25 μM for each PI. Fig. 1 Panel A demonstrates that there are no significative differences in cell cultures exposed 5 and 10 μM PIs, however, both NFV and RTV at 25 μM have a substantial toxic effect on the cells ($p < 0.05$). At 5 μM PI does not significantly alter osteoblasts viability *in vitro* after 24 h exposure; then, to further characterize the response to these drugs we determined the effect of temporal exposure to 5 μM RTV, SQV, NFV or IDV on osteoblast viability. Fig. 1 Panel B shows no significant effect of 5 μM RTV, SQV, NFV or IDV on osteoblast viability.

Human osteoblasts were exposed to NFV, IDV, RTV or SQV 5 μM for 24 h and the osteoblast transcriptome was ana-

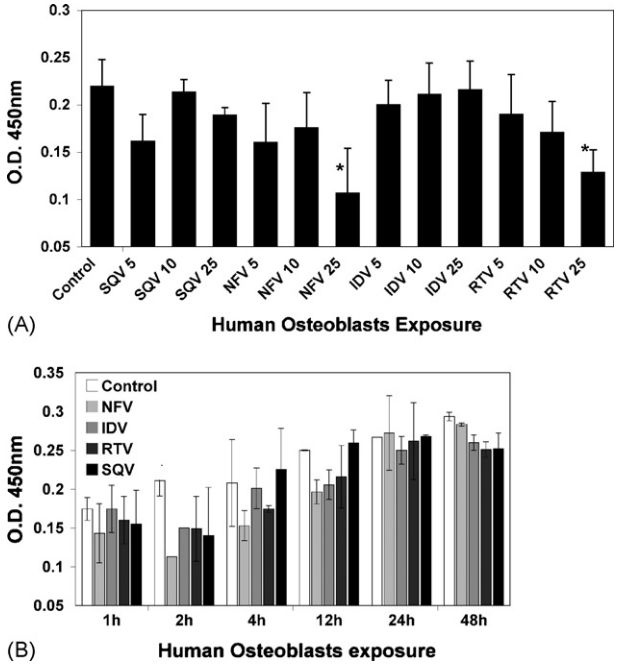

Fig. 1. (Panel A) Cell activity results by MTT assay. Osteoblasts were exposed to 5, 10 or 25 μM PI concentrations for 24 h. Mean values ± S.D. are indicated, resulting from assays carried out in triplicate. For experiments indicated with an asterisk (*), the mean values per each assay and control values were significantly different ($p < 0.05$). (Panel B) Cell activity data resulting from osteoblasts cultures exposed to 5 μM PIs at the indicated time points. Mean values ± S.D. are indicated, resulting from assays carried out in triplicate.

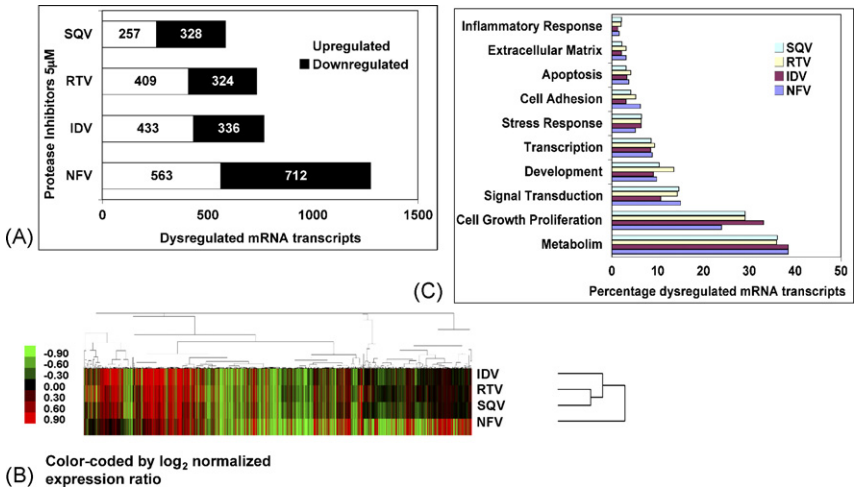

Fig. 2. (Panel A) Genomic expression was assayed by oligonucleotide array analysis after 24 h 5 μM PI exposure. The numbers of the altered genes, for which signal log ratio is 0.6 or greater, is reported on the *x*-axis. Panel B shows unsupervised hierarchical cluster analysis of 1804 osteoblasts genes whose expression is significantly altered. Groups of altered genes are found to cluster together, and in particular the expression profiles after RTV and SQV exposure have a high similarity, as has been reported at the left of the dendogram, despite NFV- and IDV-related expression profile have lower similarity than the other ones. (Panel C) Functional classification of all genes found to be significantly altered in response to NFV, IDV, RTV and SQV exposure.

lyzed. Distinct patterns of gene expression were observed, with significant altered expression following 24 h PI exposure. Of the 22,233 gene sequences represented on the Affymetrix HG-U133A_2 oligonucleotide microarray, 5.73% (1275 genes), 3.46% (769 genes), 3.29% (733 genes) and 2.63% (585 genes) were significantly altered following NFV, IDV, RTV or SQV exposure, respectively (Fig. 2 Panel A). Fig. 2 Panel B shows the result of unsupervised hierarchical cluster analysis of osteoblasts genes whose expression is significantly altered in response to PIs. These data demonstrate co-regulation of gene response to PI exposure.

All significantly altered genes were used as input in functional characterization of the PI associated transcriptome. A classification by functional analysis was curated by DAVID 2.1 database (Fig. 2 Panel C). This analysis identified inflammatory genes as being co-ordinately dysregulated in response to PIs. The 2, 5, 0 and 10 inflammation associated genes were found to be dysregulated after SQV, RTV, IDV or NFV exposure, respectively (Table 1). This selective alteration of inflammation-associated genes was of note, given the role of inflammation in altering osteoblast function that has previously been described.

Having delineated the aberrant transcriptomic response to PI exposure, the dysregulated inflammatory chemokines MCP-1 and IL-8 expression was identified as being significantly upregulated in human osteoblasts in response to NFV and RTV. MCP-1 protein secretion was significantly enhanced in NFV- and RTV-treated cells (46 pg/ml, $p < 0.05$; 51.4 pg/ml, $p < 0.01$; Fig. 3 Panel A). Increased MCP-1 protein level was correlated with enhanced mRNA expression in the NFV-exposed cells ($\Delta\Delta$Ct 2.58, $p < 0.01$; Fig. 3 Panel B), demonstrating that

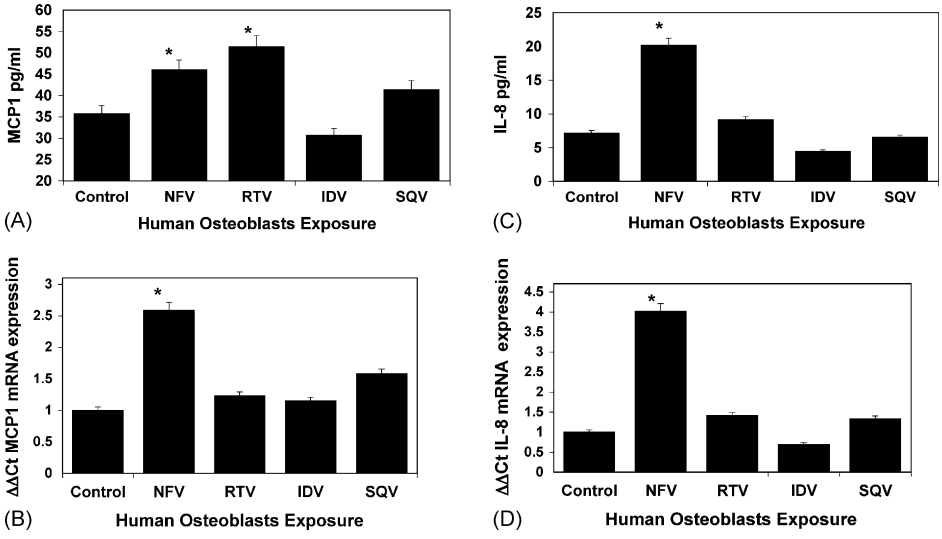

Fig. 3. (Panels A and B) MCP-1 protein and gene expression after 24 h 5 μM PI exposure. (Panel C and D) IL-8 protein and gene expression after PIs 5 μM 24 h exposure. Mean values ± S.D. are indicated, resulting from assays carried out in triplicate. Experiments indicated with an asterisk (*), mean values per each assay and control values were significantly different ($p < 0.05$).

Table 1
Inflammation response correlated genes assessed by functional and comparative analysis of four dysregulated gene groups after PIs exposure

Gene symbol	Gene title	SQV SLRs	Gene symbol	Gene title	RTV SLRs	Gene symbol	Gene title	NFV SLRs
CXCL-12	Chemokine (C–X–C motif) ligand 12	−0.648	MCP-*1*	**Chemokine C–C motif ligand 2**	0.680	*IL-8*	**Interleukin-8**	1.561
LY96	Lymphocyte antigen 96	−0.658	*PTGS2*	Prostaglandin-endoperoxide synthase 2	0.630	*MCP-1*	**Chemokine C–C motif ligand 2**	1.462
			NR3C1	Nuclear receptor subfamily 3, group C, member 1	0.606	*CXCL2*	Chemokine (C–X–C motif) ligand 2	1.246
			AOX1	Aldehyde oxidase 1	−0.592	*PTGS2*	Prostaglandin-endoperoxide synthase 2	1.169
			CXCL-12	Chemokine (C–X–C motif) ligand 12	−0.731	*NR3C1*	Nuclear receptor subfamily 3, group C, member 1	0.752
						IL-1B	Interleukin-1, beta	0.625
						PROCR	Protein C receptor, endothelial (EPCR)	−0.617
						AOX1	Aldehyde oxidase 1	−0.789
						NFATC4	Nuclear factor of activated T-cells, calcineurin-dependent 4	−0.828
						CXCL-12	Chemokine (C–X–C motif) ligand 12	−1.214

MCP-1 (CCL2) and IL-8 transcripts are highlighted as bold text in RTV and NFV groups.

NFV-associated proinflammatory chemokine induction is correlated with *de novo* gene expression. Upregulated MCP-1 protein level in RTV exposed osteoblasts supernatant is due to post-translational effects, despite normal gene expression at the same time point. Furthermore, IL-8 protein level was enhanced in response to NFV exposure (20.2 pg/ml, $p < 0.01$; Fig. 3 Panel C), a concurrent effect with significant enhanced IL-8 gene expression ($\Delta\Delta$Ct 4.0, $p < 0.01$; Fig. 3 Panel D).

In recent years, osteopenia and osteoporosis have become increasingly frequent among HIV-1 patients. Their pathogenesis is not clear, and the reduced bone mineral density may be either a direct consequence of the viral infection *per se* or an effect of antiretroviral therapy. The etiology of osteoporosis is multifactorial, but increasingly researchers have focused on the potential role of local regulatory factors including cytokines and growth factors. In literature, data obtained by gene-chip microarray analysis have been used to determine transcriptomic alterations in primary human osteoblasts after PI exposure (Malizia et al., 2007).

Using gene chip microarray analysis we identified three groups of dysregulated genes that are potentially associated with NFV-, RTV- and SQV-associated inflammatory responses resulting from intrinsic transcriptomic alterations.

Further, we have demonstrated that osteoblasts, key cellular players in active bone formation, activate inflammatory response to PI exposure and release the pro-inflammatory cytokines MCP-1 and IL-8 in response to NFV and RTV exposure. The enhanced protein expression was due to *de novo* gene expression induction as demonstrated by quantitative Real Time PCR and oligonucleotide array analysis. These data demonstrate that *in vitro* exposure to NFV can activate transcription of the inflammatory responsive chemokine genes IL-8 and MCP-1 in primary human osteoblast cells, suggesting that induction of local inflammatory responses may contribute to the development of decreased bone mineral density in HAART-treated HIV-1 patients.

This work is performed in part with the support of the Health Research Board of Ireland, the Mater College and the Irish Governments Programme for Research in Third Level Institutions. PIs were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

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