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Adipocyte induced arterial calcification is prevented with sodium thiosulfate

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ABSTRACT

Background: Calcification can occur in fat in multiple clinical conditions including in the dermis, breasts and in the abdomen in calciphylaxis. All of these are more common in patients with advanced kidney disease. Clinically, hyperphosphatemia and obesity are risk factors. Thus we tested the hypothesis that adipocytes can calcify in the presence of elevated phosphorus and/or that adipocytes exposed to phosphorus can induce vascular smooth muscle cell (VSMC) calcification.
Methods: 3T3-L1 preadipocytes were induced into mature adipocytes and then treated with media containing high phosphorus. Calcification was assessed biochemically and PCR performed to determine the expression of genes for osteoblast and adipocyte differentiation. Adipocytes were also co-cultured with bovine VSMC to determine paracrine effects, and the efficacy of sodium thiosulfate was determined.
Results: The results demonstrated that high phosphorus induced the calcification of differentiated adipocytes with increased expression of osteopontin, the osteoblast transcription factor Runx2 and decreased expression of adipocyte transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (CEBPα), indicating that high phosphorus led to a phenotypic switch of adipocytes to an osteoblast like phenotype. Sodium thiosulfate, dose dependently decreased adipocyte calcification and inhibited adipocyte induced increase of VSMC calcification. Co-culture studies demonstrated that adipocytes facilitated VSMC calcification partially mediated by changes of secretion of leptin and vascular endothelial growth factor (VEGF) from adipocytes.
Conclusion: High phosphorus induced calcification of mature adipocytes, and adipocytes exposed to elevated phosphorus can induce calcification of VSMC in a paracrine manner. Sodium thiosulfate inhibited this calcification and decreased the secretin of leptin and VEGF from adipocytes. These results suggest that adipocyte exposure to elevated phosphorus may be a pathogenic factor in calcification observed in the skin in calciphylaxis and other diseases.

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1. Introduction

Patients with advanced kidney disease such as those with end stage renal disease requiring dialysis are prone to extraskeletal calcification. A lot of attention has been paid to arterial calcification, but other soft tissues are also calcified including the dermis of the skin, breasts and the abdomen [1]. The latter is a common site for calciphylaxis and is characterized by small arteriolar calcification in the dermis with surrounding necrosis and calcification of tissue. Patients often present with livido reticularis that progress to ulcerative lesions with necrotic centers and violaceous borders [1,2]. The disease has a very high mortality rate. Clinically, case controlled studies have demonstrated major risk factors to be hyperphosphatemia, obesity, white race, and the use of Coumadin [3–5]. Treatment with sodium thiosulfate has become common, although the mechanism by which it appears to halt progression of lesions is unknown [6–8]. Studies evaluating vascular calcification in other tissues have found that calcification is the result of an active cellular process mediated in part by upregulation of the osteoblast transcription factor Runx2 to induce a phenotypic switch of vascular smooth muscle cells (VSMC) to an osteochondrocytic phenotype [9]. Given the fact that osteoblasts, adipocytes, and vascular smooth muscle cells have a common mesenchymal stem cell (MSC) origin, we hypothesized that transformation of adipocytes to mineralizing cells may be a cause of calcium deposition in the subcutaneous fat. Alternatively, such transformed cells may potentiate calcification of adjacent arterioles in calciphylaxis.

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2. Materials and methods

2.1. Cell culture

The mouse 3T3-L1 preadipocytic cell line was a gift from Dr. Robert Considine at Indiana University School of Medicine and bovine vascular smooth muscle cells (BVSMC) were isolated from the descending thoracic aorta by the explant method as previously described [10]. Cells were cultured in growth medium consisting of Dulbecco's modified Eagle medium (DMEM, Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS). To induce the 3T3-L1 pre-adipocytes to differentiated adipocytes, confluent 3T3-L1 cells were switched to DMEM with 10% FBS containing 10 μM dexamethasone, 1 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX) for 7 days [11]. The differentiation of adipocytes was confirmed by the increased expression of adipocyte markers PPARP γ and lipoprotein lipase (LPL) and Oil Red O staining as described below. To induce calcification, 3T3-L1 adipocytes were treated with 5 mmol/l β-glycerophosphate (high phosphorus), 1 U/ml fetal alkaline phosphatase, 10^{-7} mol/l insulin and 50 μg/ml ascorbic acid in the presence of 15% fetal bovine serum [12] for 7 days. In some experiments, adipocytes or BVSMC were treated with various dose of sodium thiosulfate (Sigma, St. Louis, MO).

2.2. Calcium deposition

Cells were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complex one method (Calcium kit; Pointe Scientific) and normalized to protein content as previously described [13].

2.3. Histological analysis

Adipocytes were characterized by Oil Red O (ORO) which stains the lipid droplets in the cytoplasm [11]. Briefly, cells were rinsed in PBS and then fixed in 10% (v/v) neutral buffered formalin. Fixed cells were stained with a working solution of ORO for 30 min at room temperature and counterstained with Harris's hematoxylin for 30 s for nuclear staining. Calcification was determined by Von Kossa staining. Briefly, cells were incubated with 5% silver nitrate solution under ultraviolet light for 60 min. Unreacted silver was removed with 5% sodium thiosulfate for 5 min and counterstained with nuclear fast red for 5 min. Images were collected using a Nikon-inverted microscope with a Nikon D100 digital camera.

2.4. Real time (quantitative) RT-PCR analysis

Total RNA was isolated from 3T3-L1 adipocytes using miRNeasy Mini kit (Qiagen, Valencia, CA). The gene expression in cells was determined by real time PCR using 1 μg of total RNA in TaqMan Reverse Transcription reagent (Applied Biosystems, Foster City, CA). Target-specific PCR primers for Runx2, osteopontin, PPARγ, C/EBPα and LPL were obtained from Applied Biosystems. Real-time PCR amplification was performed using TaqMan Gene Expression Assays (TaqMan MGP probes, FAM dye-labeled) using Applied Biosystems ViiA7 Real-Time PCR system (Applied Biosystems). The cycle number at which the amplification plot crosses the threshold was calculated (C_T), and the $\Delta\Delta C_T$ method was used to analyze the relative changes in gene expression using β-actin as a housekeeping gene [13].

2.5. Co-culture of adipocytes and vascular smooth muscle cells

To determine the paracrine effects of adipocytes and VSMC on each other, co-culture experiments were performed. 3T3-L1 preadipocytes (0.5×10^4/cm^2) were first seeded on the 24-well culture plates and induced to differentiate with adipogenic media for 7 days as detailed above. BVSMC (6×10^4/cm^2) were seeded in BD Falcon cell culture inserts (0.4 μm, BD Biosciences) with growth media. After reaching confluence, inserts with BVSMC were moved into the wells containing 3T3-L1 adipocytes, with common media but no cell–cell contact. Co-cultures were then incubated with high phosphorus media (containing 5 mmol/l β-glycerophosphate) as detailed above and calcification determined after 3 days. BVSMC in the culture insert alone and adipocyte in culture wells alone were used as controls. In some experiments, co-cultures were incubated in the presence or absence of sodium thiosulfate. To determine the mechanisms of paracrine effects, conditioned media was collected from 3T3-L1 adipocytes treated with control or calcification media and leptin, adiponectin and vascular endothelial growth factor-A (VEGF-A) concentration measured using ELISA kits (R & D systems).

2.6. Statistical analysis

Statistical analysis was conducted by ANOVA. The results are expressed as means ± SD, with $p < 0.05$ considered significant (StatView, SAS Institute, Cary, NC).

3. Results

3.1. High phosphorus induced calcification, increased the expression of osteoblastic genes and decreased the expression of adipogenic genes in 3T3-L1 adipocytes

To determine if high phosphorus induces adipocyte calcification, 3T3-L1 preadipocytes were differentiated and then treated with control or calcification media (high phosphorus) for 1, 3 and 7 days. As shown in Fig. 1, at 7 days the adipocytes in cells treated with control or high phosphorus retained oil red staining (Fig. 1A, top). However, the adipocytes treated with high phosphorus media had increased calcification by histological (Fig. 1A, bottom black area) and biochemical analysis (Fig. 1B). By real time PCR, high phosphorus increased gene expression of the osteoblastic transcription factor Runx2 in adipocytes at day 1 (Fig. 2A) and osteopontin at day 1, 3 and 7 (Fig. 2B). In contrast, in adipocytes treated with high phosphorus, the gene expression of the adipogenic transcription factors PPARγ was decreased at day 3 and 7 (Fig. 2C) and C/EBPα was decreased at day 7 (Fig. 2D). These results suggest that adipocytes exposed to high phosphorus can upregulate osteoblastic transcription factors and downregulate adipogenic transcription factors and facilitate calcification.

3.2. Adipocytes enhance calcification of VSMC in a paracrine manner

To evaluate the potential of adipocytes to affect calcification in BVSMC, 3T3-L1 adipocytes and BVSMC co-cultures were incubated with high phosphorus media and calcification determined after 3 days. The results demonstrated that calcification of BVSMC was significantly enhanced when co-cultured with adipocytes compared to BVSMC alone (Fig. 3A). In contrast, there was no difference in calcification of adipocytes in high phosphorus media incubated alone or co-cultured with BVSMC (Fig. 3B). These co-culture data indicate that adipocytes have the potential to enhance the calcification of VSMC but the reverse was not true.

To determine the mechanism of this paracrine effect, conditioned media were collected and analyzed for factors known to be secreted by adipocytes and in turn known to increase calcification in VSMC. High phosphorus modestly increased VEGF-A secretion from adipocytes compared to control media (5.3 ± 0.2

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