Nuclear Factor-E2-related Factor-1 Mediates Ascorbic Acid Induction of Osterix Expression via Interaction with Antioxidant-Responsive Element in Bone Cells*

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We recently found that deletion of the gulonolactone oxidase gene, which is involved in the synthesis of ascorbic acid (AA), was responsible for the fracture phenotype in spontaneous fracture mice. To explore the molecular mechanisms by which AA regulates osteoblast differentiation, we examined the effect of AA on osterix expression via Nrf1 (NF-E2-related factor-1) binding to antioxidant-responsive element (ARE) in bone marrow stromal (BMS) cells. AA treatment caused a 6-fold increase in osterix expression in mutant BMS cells at 24 h, which was unaffected by pretreatment with protein synthesis inhibitor. Sequence analyses of mouse osterix promoter revealed a putative ARE located at −1762 to −1733 upstream of the transcription start site to which Nrf potentially binds. A gel mobility shift assay revealed that nuclear proteins from AA-treated BMS cells bound to radiolabeled ARE much more strongly than nuclear extracts from AA-untreated cells. A chromatin immunoprecipitation assay with Nrf1 antibody confirmed the interaction of Nrf1 with the mouse osterix promoter. A reporter assay demonstrated that the promoter activity of mouse osterix containing an ARE was stimulated 4-fold by a 48-h treatment with AA in spontaneous fracture BMS cells. Treatment of mutant BMS cells with AA resulted in a 3.9-fold increase in the nuclear accumulation of Nrf1. Transfection of mutant BMS cells with Nrf1 small interfering RNA decreased Nrf1 protein by 4.5-fold, blocked AA induction of osterix expression, and impaired BMS cell differentiation. Our data provided the first experimental evidence that AA modulated osterix expression via a novel mechanism involving Nrf1 nuclear translocation and Nrf1 binding to ARE to activate genes critical for cell differentiation.

Osteoporosis, affecting both women and men, is a common disease characterized by a loss of bone mass and strength and a microarchitectural deterioration of bone tissue with a consequent increase in the risk of developing fragility fractures with even minor trauma (1). In order to identify candidate genes that may be important in the development of osteoporotic fractures, we previously studied a mutant mouse that develops spontaneous fracture (sfx)2 at the very early age of 5–7 weeks (2, 3). We and others found that deletion of the gulonolactone oxidase gene, which is involved in the synthesis of ascorbic acid (AA), was responsible for AA deficiency and impairment of differentiated functions of osteoblast, bone fracture, and premature death in sfx mice (2, 4). Treatment of sfx mice with AA in drinking water completely rescued the bone phenotypes in vivo and prevented them from premature death (2). Consistent with our findings, a point mutation from G to A at nucleotide 182 in the L-gulono-γ-lactone oxidase gene in rats, which alters an amino acid residue from cysteine to tyrosine, has also been reported to reduce AA synthesis and impair bone formation (5). In addition, several epidemiological studies have shown that a lower level of blood AA, also known as vitamin C, may substantially increase the risk of hip fracture in smokers and elderly patients, whereas supplementation of antioxidant vitamins greatly reduces the frequency of osteoporotic fractures (6–8). These genetic studies together with clinical observations have reinforced the critical role of antioxidant vitamins in maintaining bone health (2). Although it is well known that AA is required for osteoblast differentiation, the molecular mechanisms by which AA regulates bone formation and mineralization remain to be elucidated.

AA, a key antioxidant vitamin, is a cofactor of prolylhydroxylase shown to be required for hydroxylation and secretion of procollagen to form stable triple-helical collagen both in the growing and in the mature connective tissue (9). Deficiency of AA is responsible for scurvy syndrome, which has characteristics of the defective synthesis of collagen (10). Moreover, it has been shown that AA induces embryonic and mesenchymal stem cells to differentiate into osteoblasts (11, 12). The potential mechanisms by which AA directs the multipotent progenitor cell commitments are believed to be mediated through collagen matrix synthesis, cell-matrix interaction, and activation of integrin signaling (11, 13). The interaction of type I collagen and α2β1 integrin activates mitogen-activated protein kinase, which then phosphorylates and activates the osteoblast-specific transcription factor Cbfa1 to induce cell differentiation (14, 15). However, recent in vitro studies have provided con-

* This work was supported by National Institutes of Health Grant AG19698 and the Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: sfx, spontaneous fracture; AA, ascorbic acid; BMS, bone marrow stromal; siRNA, small interfering RNA; βGP, β-glycerophosphate; AA-2P, ascorbic acid 2-phosphate; RT, reverse transcription; ChIP, chromatin immunoprecipitation; mOsx, mouse Osx; ALP, alkaline phosphatase.
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Vincing evidence that AA also influences differentiation of other cell type lineages, such as neuronal cells, that produce little or no matrix (16). Furthermore, the gulonolactone oxidase knock-out mice have normal levels of proline hydroxylation and collagen production in the tail, mammary gland, and tumors, whereas these mice show widespread abnormalities in multiple organs besides the bone (2, 17). In addition, it has been demonstrated that AA can stimulate the expression of a number of osteogenic marker genes in the presence of collagen synthesis inhibitors and can induce chondrocyte hypertrophy independent of production of a collagen-rich matrix (18). These studies strongly suggest that additional mechanisms besides collagen-mediated signaling may be involved in mediating AA effects in a variety of the target cells.

We previously carried out a microarray analysis and found that expression levels of a number of genes related to energy and drug metabolism were down-regulated in the bones of sfx mice compared with control mice (2). Interestingly, many of these genes contain putative antioxidant-responsive element (ARE) in their proximal promoter regions to which Nrf (nuclear factor-E2-related factor) can potentially bind. In this study, we focused on AA regulation of transcription factors known to be critical for osteoblast differentiation (19, 20). We found that AA induced acute osterix expression during bone marrow stromal (BMS) cell differentiation via stimulating interaction of Nrf1 with ARE in the promoter region. Our studies have provided the first experimental evidence for a novel mechanism by which antioxidant vitamins directly stimulate the expression of ARE-containing genes, leading to osteoblast differentiation and bone formation.

MATERIALS AND METHODS

The sfx mutant mice in a BALB/cBy genetic background were discovered and characterized as described previously (2, 3). The mice were housed at the Jerry L. Pettis Memorial Veterans Affairs Medical Center Animal Research Facility (Loma Linda, CA) under standard approved laboratory conditions with controlled illumination (14 h light, 10 h dark) and temperature (22 °C) and unrestricted food and water. The animals were fed with either a Harlan Tekland S-2335 mouse breeder diet-7004 or with a Harlan Teklad 4% mouse/rat diet 7001 (Harlan, Indianapolis, IN) (2). Both of the diets are free of AA.

BMS cells were isolated from sfx mutant mice compared with control mice (2). The cells were plated at a density of 2 × 10^6 cells/120-mm dish and 3 × 10^5 cells/well in a 6-well plate and grown to 80% confluence (normally 1 week) in AA-free α-minimal essential medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The cells were treated with mineralization media containing β-glycerophosphate (βGP) and ascorbic acid 2-phosphate (AA-2P) (experimental) or βGP without AA-2P (control) for the indicated period of time.

Real time RT-PCR—The total RNA was extracted from BMS cells using Trizol (Invitrogen). An aliquot of RNA (500 ng) was reverse-transcribed into cDNA by oligo(dT)12–18 primer. Real time PCR contained 100 ng of template cDNA, 1× SYBR GREEN master mix (Qiagen), and 100 nm specific forward and reverse primers in a 25-μl volume of reaction. Primers for peptidyl prolyl isomerase A were used to normalize the expression data of test genes. Sequences of the primers were as follows: osteix, forward (5’-tggctgctctcctgtctgta) and reverse (5’-TCACTGAGGAAAGGTGGGT); Nrf1, forward (5’-TACGTGCCTGCATTACACC) and reverse (5’-ACCTTCTGTCTCATCTGCG); alkaline phosphatase (ALP), forward (5’-ATGGATAACGGGCTGCTGCTACA) and reverse (5’-ATGTCGTCCTGAGCGCGTG); peptidyl prolyl isomerase A, forward (5’-CATGGAATATGCCCTGGACCCA) and reverse (5’-TCTGGACGACAAAAACCTGCC).

Transient Transfection—Nucleoporation was carried out as reported previously (21). Our transfection efficiency in BMS cells is more than 80%, as observed using fluorescein-labeled mock siRNA. Briefly, BMS cells isolated from sfx mutant mice were cultured in 120-mm dishes and grown to 80% confluence before they were removed by trypsin digestion for transfection. Undifferentiated BMS cells (1.5 × 10^6) were resuspended in 100 μl of fibroblast nucleofector buffer (Amaxa, Gaithersburg, MD) containing 5 μg of plasmid or 1.25 μg of siRNA. The cells were then transferred into a 2-mm gap width electroporation cuvette and electroporated at 135 V for 15 ms, using a Gene Pulser (Bio-Rad). After electroporation, the cells were plated at high density (150,000 cells/cm^2) in 6-well plates and grown to 80% confluence in AA-free α-minimal essential medium.

Nodule and ALP Activity Assays—The nodule assay was carried out as described previously (2). BMS cells transfected with siRNA were plated at a density of 150,000 cells/cm^2 in 6-well plates and cultured in a mineralization media supplemented with 300 μg/ml AA-2P and 10 mm βGP. The medium was changed every 3 days. After 24 days, the cells were washed with phosphate-buffered saline, fixed in cold 70% ethanol, and stained with 40 mM alizarin red (pH 4.2). The mineralized area was measured with the OsteoMeasure system equipped with a digitizing tablet and a color video camera (Osteometrics Inc., Atlanta, GA). Two parallel plates were used for ALP staining and activity assay, respectively, 7 days after transfection (22, 23).
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Preparation of Cytoplasmic and Nuclear Extracts—Cytoplasmic and nuclear extracts were prepared as described previously (24). Briefly, BMS cells were lysed on ice for 5 min in buffer E (0.3% Nonidet P-40, 10 mM Tris–HCl (pH 8.0), 60 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1× phosphatase inhibitor, 1× protease inhibitor mixture (Sigma), and 1 mM phenylmethylsulfonyl fluoride). Nuclei were pelleted by spinning for 5 min at 3000 rpm at 4 °C, and the supernatant was saved as the cytoplasmic extract. Glycerol was added to cytoplasmic extracts to a final concentration of 20%. The pellet was washed in buffer E lacking Nonidet P-40 and resuspended in buffer C (20 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 20% glycerol, 1× phosphatase inhibitor, 1× protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride). NaCl was added to a final concentration of 0.4 M, and the nuclei were gently shaken for 20 min at 4 °C. Nuclear extract was obtained after a 10-min spin at 12,000 rpm at 4 °C.

Western Blot—An aliquot of 30 μg of protein was resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was incubated at 4 °C overnight in a buffer containing 5% dry skim milk, 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), and 0.05% Tween 20. Immunoblotting was performed in the same buffer containing 0.2 μg/ml anti-Nrf1 or anti-osterix at room temperature for 1 h. Nrf1 and osterix proteins were detected using appropriate secondary antibody and a Western blotting detection system. After exposure to chemiluminescence reagent, the membrane was dried and visualized by autoradiography.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides were labeled as described previously (25). Nuclear extracts (4 μg) from BMS cells were incubated in a binding buffer containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 3 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, 0.1 mM ZnCl2, 50 μg/ml poly(dI-dC), and 20 fmol of labeled DNA probe. Excess unlabeled DNA competitors were added into the reaction 5 min before the addition of radiolabeled probe. The reactions were incubated at room temperature for 20 min and analyzed on 5% nondenaturing polyacrylamide gel in 1× TBE buffer (50 mM Tris borate-EDTA, pH 8.0). Gels were dried and visualized by autoradiography.

Chromatin Immunoprecipitation—The chromatin immunoprecipitation (ChIP) assay was performed using commercial ChIP assay kit (Upstate Biotechnology). Briefly, 2 × 106 BMS cells derived from sfx mice were treated with 300 μM/ml AA-2P and 10 mM βGP for 24 h. The cells were then cross-linked in 1% formaldehyde at 37 °C for 10 min and lyzed in SDS lysis buffer supplemented with protease inhibitor mixture (Sigma) for 10 min on ice. The lysate was sonicated and precleared with protein A agarose/salmon sperm DNA prior to immunoprecipitation with 2 μg of anti-Nrf1 or rabbit IgG (Santa Cruz Biotechnology) overnight at 4 °C. Following wash and elution steps, cross-links were reversed at 65 °C for 4 h. The DNA in the immunoprecipitated samples was purified by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. An aliquot of purified DNA was amplified by PCR with specific primers to the mouse osterix gene and analyzed on 4% agarose gel.

Reporter Constructs and Luciferase Assay—An osterix promoter from −2965 to +80 relative to the transcription start site was generated by PCR from C57BL/6j mouse DNA and inserted into the KpnI and XhoI sites of the pGL3-basic vector (Promega, Madison, WI). DNA sequences of primers are listed below: forward, 5′-CATGGAGATCTGCGTCTTC; reverse, 5′-GTCCCAAGGAGTCAGGCAGAT. A synthetic 3× ARE repeat from −1762 to −1733 of the mouse osterix promoter was inserted into pTAL-Luc vector (Clontech, Palo Alto, CA). The luciferase assay was carried out as described previously (25). Briefly, BMS cells were transfected with 5 μg of reporter plasmid. After electroporation, the cell suspension was split into 24-well plates with 0.5 ml each and cultured in a humidified 37 °C incubator with 5% CO2 in a growth medium. After a 24-h incubation, the transfected cells were treated with 10 mM βGP alone or 300 μg/ml AA-2P and 10 mM βGP. The cells were cultured for another 48 h and then lysed in 120 μl of 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM dithiothreitol and 0.5% Nonidet P-40. An aliquot of 100 μl of cell extract was used for luciferase assay, and 5 μl of cell extract was taken for protein measurement. The light units from the luciferase constructs were normalized to protein concentration.

Statistical Analysis—Data are presented as means ± S.D. and analyzed by Student’s t test or analysis of variance as appropriate. One- and two-way analysis of variance tests were performed using STATISTICA software (Statsoft, Tulsa, OK).

RESULTS

Expression of Osterix Is Differential Stage- and AA-dependent—In order to determine if the impairment in BMS cell differentiation observed in our previous studies in sfx mice is attributable to decreased expression of osteoblast-specific transcription factors, we examined the effect of AA-2P on the expression levels of the osterix gene. We cultured the BMS cells from both wild-type and mutant mice in a mineralization medium supplemented with βGP alone or βGP and AA and evaluated the expression levels of osterix by real time RT-PCR at days 0, 6, 12, 18, and 24 (Fig. 1, A and B). We set day 0 as the basal expression level of undifferentiated BMS cells without AA-2P treatment. We found that osterix expression was not changed appreciably during the 24-day culture when BMS cells derived from sfx mice were exposed to a medium containing 10 mM βGP alone (−AA) (Fig. 1A). However, there was a steady increase in the expression of osterix during the BMS cell differentiation when BMS cells were treated with 10 mM βGP and 300 μg/ml AA-2P (+AA). A 12-fold and a 22-fold increase in osterix expression were seen at days 6 and 12, respectively, compared with undifferentiated BMS cells without AA-2P treatment. The maximal induction of 40-fold in osterix mRNA levels was reached at day 18. By day 24, a 27-fold AA stimulation of osterix expression was detected in the mature osteoblast cells. A similar AA-dependent expression of osterix was also seen in BMS cells isolated from wild-type mice with an ~20-fold increase at day 24 (Fig. 1B). The lower induction of osterix expression in wild-type BMS cells compared with sfx BMS cells could be due to higher basal level of expression in wild-type BMS cells in the absence of AA at day
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Expression of osterix is dependent on AA during BMS cell differentiation. BMS cells derived from mutant mice (A) and wild-type mice (B) were cultured until 80% confluence and then switched into a mineralization medium supplemented with 10 nM bGPe alone (−AA) or with 300 μg/ml AA-2P and βGP (+AA) (47). At days 0, 6, 12, 18, and 24, total RNA was extracted for RT-PCR. Day 0 represents cultures prior to the addition of AA-2P and βGP and reflects the basal expression level of undifferentiated BMS cells. The results were expressed as fold changes over the expression level of day 0. The data shown are means ± S.D. from six replicates. A star indicates statistical significance of the expression levels of osterix in the cells treated with AA as compared with the corresponding cells without AA treatment (p < 0.01). C, osterix expression was evaluated by Western blot in sfx BMS cells treated with AA for 72 h with antibody specific to mouse osterix (Abcam Inc., Cambridge, MA). D, quantitative data from three replicates as shown in Fig. 1C.

0 (4.7 ± 0.62-fold; p < 0.01). The stimulation of osterix expression by AA was further confirmed by Western blot with antibody specific to mouse osterix (Fig. 1C). We observed an ~4-fold induction at protein level after a 72-h AA stimulation in the mutant BMS cells (Fig. 1D).

To determine if the effect of AA on osterix mRNA levels is acute, we measured expression levels at 4 and 24 h after treatment of BMS cells derived from sfx mice with the combination of βGP and AA-2P or βGP alone (Fig. 2A). We found that the expression of osterix was increased by 2.2- and 6-fold at 4 and 24 h, respectively, after the AA and βGP treatment compared with βGP alone. In a separate experiment, we compared the effect of 300 μg/ml AA-2P versus AA on osterix expression and found that both AA-2P and AA increased osterix expression similarly at 24 h (6.12 ± 1.0 versus 6.72 ± 0.26; n = 3). To further examine whether the AA induction of osterix expression is direct and occurs in the absence of de novo protein synthesis, we pretreated BMS cells with cycloheximide at a concentration of 1 μM that inhibited >80% protein synthesis and then evaluated the effect of AA on the osterix expression after 24 h of treatment (Fig. 2B). We found that pretreatment of BMS cells with cycloheximide did not affect AA induction in osterix expression. Approximately 6-fold AA induction of osterix expression was observed in the presence of cycloheximide. To determine whether AA increased osterix by stabilization of mRNA, BMS cells were stimulated with βGP and AA-2P for 24 h and then treated with 5 μg/ml actinomycin. Total RNA was harvested at subsequent time intervals of 0, 2, 4, 6, and 8 h. The half-life of the osterix mRNA was then measured by real time RT-PCR. Our experiments found that AA treatment did not significantly affect osterix mRNA stability in BMS cells (Fig. 2C). The calculated half-lives of osterix mRNA were 3.5 and 3.7 h in the AA-untreated BMS cells and AA-treated cells, respectively (p > 0.05).

AA Stimulates Transcription of Osterix via Nrf1 Binding to ARE—To examine if AA, like other antioxidants, regulates the transcription of osterix via a mechanism involving protein/DNA interaction in the regulatory region (26, 27), we carried out a computer-based analyses to search for ARE in the osterix promoter. We found that one putative ARE was present at −1762 to −1733 upstream of the transcription start site of the mouse osterix gene (Fig. 3). Similar putative AREs were also found in the promoters of the rat and human osterix genes as well as the human NADPH quinine oxidoreductase 1, a gene known to be regulated by antioxidants via ARE (26, 28). Like the known ARE, the putative AREs in the promoters of the osterix gene contain two directed or inverted AP-1-like repeats with 2–8 bp spacing, followed by a GC box, to which a number of transcription factors, including Nrf and small Maf, can bind. The core binding site of the putative ARE in mouse osterix promoter is completely matched to the ARE core sequence (29).

To determine which family member(s) of Nrf play an important role in mediating AA induction of osterix expression via ARE, we compared expression levels of Nrf1, Nrf2, and Nrf3 in BMS cells using real time RT-PCR. We found that the expression level of Nrf1 was 50-fold higher than that of Nrf2 and 500-fold higher than that of Nrf3 in BMS cells. The expression levels of other Nrf family members and their binding partners, such as Keap-1 and Maf, were not significantly altered upon AA treatment (data not shown).

In previous studies, it has been shown that antioxidants stimulate nuclear trafficking of Nrf2 to modulate antioxidant gene expression in liver cells (26, 27). We therefore tested if AA treatment also leads to Nrf1 nucleotranslocation to bind to the putative ARE in osterix gene and enhance the transcription in BMS cells. We found that AA treatment increased accumulation of Nrf1 in the nuclei of BMS cells, as measured by Western immunoblotting (Fig. 4A). There was an approximately 3- and 3.9-fold elevation of Nrf1 in the nuclear extracts from the BMS cells treated with AA for 4 and 24 h, respectively, as compared
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FIGURE 2. AA stimulates the expression of the osterix gene in BMS cells. A, real time RT-PCR data. BMS cells derived from mutant mice were cultured until 80% confluence and then treated with 10 mM βGP alone (−AA) or 300 μg/ml AA-2P and βGP (+AA) for 4 and 24 h. Total RNA was extracted and reverse-transcribed for real time PCR. The results were expressed as -fold changes over the expression level of corresponding controls without AA treatment. The data are means ± S.D. from four replicates. A star indicates statistical significance of expression as compared with expression level of corresponding controls (p < 0.01). B, AA induction of osterix expression is independent of new protein synthesis. BMS cells derived from mutant mice were treated with 1 μM cycloheximide for 1 h prior to the addition of 10 mM βGP alone (−AA) or 10 mM βGP and 300 μg/ml AA-2P (+AA). Total RNA was prepared and reverse-transcribed for real time PCR. The results were expressed as -fold changes over the expression level of controls without AA and cycloheximide treatments. The data are means ± S.D. from six replicates. A star indicates statistical significance of expression levels of the cells treated with AA as compared with the expression level of corresponding controls (p < 0.01). C, osterix mRNA stability is not changed by AA. BMS cells from mutant mice were treated with 10 mM βGP alone (−AA) or 10 mM βGP and 300 μg/ml AA-2P (+AA) for 24 h prior to the addition of actinomycin (5 μg/ml). Cells were harvested for RNA extraction and real time RT-PCR at the time points indicated. Data are expressed as mean ± S.D. from three replicates.

We next determined if the effects of AA on osterix expression are mediated via Nrf1 binding to ARE in the mouse osterix promoter. We performed an electrophoretic mobility shift assay. Our electrophoretic mobility shift assay showed that nuclear extract from AA-treated BMS cells from sfx mice bound to radiolabeled ARE derived from mouse osterix promoter and formed a protein-DNA complex much stronger than the nuclear extract from untreated control stromal cells (Fig. 5A). The binding was sequence-specific, since the retarded band was competed away by a 200-fold molar excess of unlabeled mOsx ARE and hNQO1 ARE but not unlabeled Sp1 oligonucleotides. Nrf1 interaction with mouse osterix promoter was further confirmed by a ChIP assay with antibody specific to mouse Nrf1. The binding of Nrf1 to the osterix promoter was also increased by AA treatment as evidenced by the amplified PCR product using genomic DNA immunoprecipitated by anti-Nrf1 (Fig. 5B). In contrast, there was no change for the glyceraldehyde-3-phosphate dehydrogenase gene precipitated by antibody against RNA polymerase II. In subsequent experiments, we amplified a portion of the mouse osterix promoter bearing a putative ARE and cloned into the promoterless pGL3-basic vector. We also inserted a 3× putative ARE from the mouse osterix gene in front of the thymidine kinase minimal promoter of the pTAL-luc reporter. Promoter activities were analyzed in transiently transfected BMS cells derived from sfx mice. We found that AA treatment stimulated the luciferase expression by 4-fold in the BMS cells transfected with pGL3-

with the nuclear Nrf1 from untreated cells (Fig. 4B). In contrast, the cytoplasmic Nrf1 was not affected by AA treatment. The increased Nrf1 in the nuclear extract was not due to increased Nrf1 expression by ascorbate treatment during in vitro differentiation of BMS cells as measured by real time RT-PCR (data not shown). It should be pointed out that full-length Nrf1 (741 amino acids) is primarily a membrane-bound protein localized in the endoplasmic reticulum, which is insoluble under experimental conditions used in this study and therefore could not be detected in our Western blots (30). Therefore, direct comparison of relative amounts of Nrf1 in the cytoplasm versus the nucleus could not be made.

FIGURE 3. ARE in the promoter of the osterix gene is conserved among the species of mice, rats, and humans. The putative AREs consisting of two directed or inverted AP-1-like repeats with 2–8 bp spacing followed by a GC box in the 5′-untranslated region of mouse, rat, and human osterix genes are aligned with a known ARE from the human NADPH quinine oxidoreductase 1 gene (hNQO1). An ARE core sequence is also given at the bottom.

FIGURE 4. AA stimulates nuclear translocation of Nrf1 in BMS cells. BMS cells derived from mutant mice were cultured until 80% confluence and then switched into a medium supplemented with 10 mM βGP prior to experiments. The cells were then treated with 300 μg/ml AA-2P for 0, 4, and 24 h prior to harvesting. The cells were lysed for preparation of cytoplasmic and nuclear extracts. An aliquot of 30 μg of protein was loaded for Western blotting. The intensity of Western blot signal was normalized to the corresponding signal of β-actin and expressed as -fold change over the intensity of controls without AA treatment (0 h). A, representative data of Western blot, B, quantitative data from three independent experiments. A star indicates statistical significance of the expression levels of Nrf1 in the cells treated with AA as compared with the cells without AA treatment (p < 0.01).
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Osterix containing nucleotides from −2965 to +80 relative to the transcription start site as compared with the control cells without AA treatment (Fig. 6A). The activity of the heterogeneous promoter containing 3× ARE was increased 9-fold in the cells treated with AA in comparison with the untreated controls (Fig. 6B). However, the same concentration of AA failed to stimulate the luciferase activity in the cells transfected with pGL3-basic or pTAL-luc plasmids, as expected.

**Knockdown of Nrf1 Expression Impairs AA-induced Osterix Expression and BMS Cell Differentiation**—To establish the cause and effect relationship between AA-stimulated nuclear translocation and target gene expression, we evaluated the effect of AA on osterix expression after suppression of Nrf1 expression by siRNA. We confirmed that treatment with Nrf1 siRNA specifically knocked down expression of osterix by 70% in BMS cells, as measured by real time RT-PCR (Fig. 7A). In contrast, expression of Nrf2 was not altered (data not shown). Consistent with the mRNA data, Nrf1 protein levels in total cell extract were decreased 4.5-fold (75–80%) in BMS cells derived from sfx mice, as measured by Western immunoblotting (Fig. 7B). As expected, AA stimulation of osterix expression of more than 6-fold was seen in the BMS cells treated with nonspecific mock siRNA. However, the AA induction of osterix expression was completely blocked near the basal level when the BMS cells were transfected with siRNA specific to Nrf1 (Fig. 8).

To test if reduced expression of Nrf1 and osterix impairs BMS cell differentiation, we measured the expression of ALP in response to AA treatment in the cells transfected with Nrf1 siRNA, osterix siRNA, and mock siRNA. As expected, transfection of BMS cells with siRNA against osterix attenuated the target gene expression by 90%, as measured by immunoblotting (Fig. 9, A and B). The reduced expression of osterix significantly decreased ALP expression by 50% 3 days after transfection as compared with the mock siRNA controls, as evaluated by real time RT-PCR (Fig. 9D). Consistent with the change of ALP mRNA, the ALP activity was greatly reduced in BMS cells transfected with osterix siRNA in comparison with the mock siRNA control (Fig. 9C). We also found that AA treatment significantly increased ALP mRNA (3.8 ± 0.5-fold over untreated control; p < 0.01) in the mock siRNA-transfected cells.
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FIGURE 7. Nrf1 siRNA knocks down Nrf1 expression in BMS cells. Undifferentiated BMS cells were transfected with mock or Nrf1 siRNA. The cells were lysed 48 h after transfection for RNA extraction or Western blot. A, relative expression level of Nrf1 detected by RT-PCR. The data are means ± S.D. from three replicates. A star indicates statistical significance of expression levels in the cells transfected with Nrf1 siRNA as compared with in the cells transfected with mock siRNA (p < 0.01). B, expression level of Nrf1 by Western blotting. The intensity of signals was normalized to the corresponding signal of β-actin and expressed as an average of -fold change over the intensity of controls from two representative replicates.

FIGURE 8. Nrf1 siRNA blocks AA induction in osterix expression in BMS cells. Undifferentiated BMS cells were transfected with mock or Nrf1 siRNA. The transfected cells were then treated with 10 mM βGP alone (−AA) or 10 mM βGP and 300 µg/ml AA-2P (+AA) for 24 h followed by RNA extraction for RT-PCR. Values are means ± S.D. from three replicates. A star indicates statistical significance of osterix expression in the cells treated with AA compared with the corresponding controls without AA treatment (p < 0.01).

BMS cells derived from sfx mice is due to reduced Nrf1 nuclear localization caused by AA deficiency, we transfected BMS cells with Nrf1 siRNA and induced these cells to differentiate after electroporation. We found that suppression of Nrf1 expression by siRNA specific to Nrf1 significantly reduced nodule formation from the BMS cells of sfx mice (Fig. 10C). The mineralized area in the Nrf1 siRNA-treated cell was significantly diminished as compared with the cells treated with mock siRNA (7.22 ± 0.95% versus 11.35 ± 0.57%; p < 0.01) (Fig. 10D).

DISCUSSION

To identify candidate genes that may be important in the development of osteoporotic fractures, we previously studied a mutant mouse that develops spontaneous fractures at the very early age of 5–7 weeks and found that deletion of the gulonolactone oxidase gene is responsible for the spontaneous bone fracture, reduced body size, thymus, and spleen weights as well as anemia phenotype (2). Similarly, epidemiological studies have provided evidence for the increased risk of bone fractures in patients with vitamin C deficiency as well as in smokers with insufficient intake of other antioxidants, such as vitamin E and selenium (6, 8). The severity of antioxidant depletion and its relevance to the pathogenesis of multiple cell types and the ability of AA to induce differentiation of multipotent progenitor cells to osteoblasts, chondrocytes, cardiac myocytes, and dopaminergic neurons suggested that AA must be acting via other novel mechanisms to regulate cell differentiation at early stages and subsequent bone formation besides its well studied action to promote collagen synthesis/maturation and matrix protein interaction (14, 15, 31). In this study, we provide convincing evidence that AA treatment increased translocation of Nrf1 from the cytoplasm to the nucleus, promoted interaction of Nrf1 with ARE in the promoter of osterix gene, and enhanced promoter activity. We also demonstrate that the effect of AA on the transcription was acute and independent on de novo protein synthesis and mRNA stability. Our data provide the first experimental evidence for the involvement of a novel molecular pathway involving nuclear translocation of Nrf1 and interaction of Nrf1 with DNA for transcriptional regulation of genes critical for early stages of cell differentiation by AA and perhaps other antioxidants as well.

The AP-1-like repeats with 2–8 bp spacing followed by a GC box in the mouse osterix promoter are located at −1762 to −1733 upstream of the transcription start site. In fact, a similar sequence containing putative ARE is also found in the comparable regions of the rat and human osterix promoters. The presence of conserved putative ARE in three mammalian species suggests that transcription factors binding to this regulatory element may play a critical role in osterix gene regulation. There are six members of the basic leucine zipper (bZip) family of transcription factors, including p45 NF-E2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2, that can recognize AP-1-like repeat sequences. Among them, p45 NF-E2 and Bach2 are specifically expressed at low levels in hematopoietic progenitor cells and differentiated cells of the erythroid, megakaryocyte, and mast cell lineages, whereas Nrf1, Nrf2, and Nrf3 are ubiquitously expressed (32–34). In our experiments, we have demonstrated that Nrf1 was predominantly expressed in the cells of osteoblas-
Ascorbic Acid Stimulates Osterix Expression via Nrf1

Suppression of osterix expression impairs osteoblast differentiation. BMS cells derived from mutant mice were cultured until 80% confluence. The undifferentiated BMS cells were then transfected with mock or osterix siRNA and plated at high density (150,000 cells/cm²). The cells were immediately cultured in mineralization medium containing 10 μM βGP and 300 μg/ml AA-2P after transfection. A, Western blot analyses of transfected BMS cells 2 days after transfection. B, quantitative data of Western blot. The data shown are means ± S.D. from three replicates. A star indicates statistical significance of osterix siRNA-treated samples versus mock siRNA controls (p < 0.01). C, representative ALP staining in BMS cells transfected with mock or osterix siRNA 3 days after transfection. D, ALP mRNA expression analyzed by real time RT-PCR 3 days after transfection. Values are means ± S.D. from three replicates. A star indicates statistical significance of mineralized areas from the mock siRNA-treated cells compared with the osterix-treated cells (p < 0.01).

FIGURE 9. Suppression of osterix expression impairs osteoblast differentiation. BMS cells derived from mutant mice were cultured until 80% confluence. The undifferentiated BMS cells were then transfected with mock or Nrf1 siRNA and plated at high density (150,000 cells/cm²). The cells were immediately cultured in mineralization medium containing 10 μM βGP and 300 μg/ml AA-2P after transfection. A, Western blot analyses of transfected BMS cells 2 days after transfection. B, quantitative data of Western blot. The data shown are means ± S.D. from three replicates. A star indicates statistical significance of osterix siRNA-treated samples versus mock siRNA controls (p < 0.01). C, representative ALP staining in BMS cells transfected with mock or osterix siRNA 3 days after transfection. D, ALP mRNA expression analyzed by real time RT-PCR 3 days after transfection. Values are means ± S.D. from three replicates. A star indicates statistical significance of mineralized areas from the mock siRNA-treated cells compared with the osterix-treated cells (p < 0.01).

FIGURE 10. Suppression of Nrf1 expression impairs osteoblast differentiation and mineralized nodules formation from BMS cells. BMS cells derived from mutant mice were cultured until 80% confluence. The undifferentiated BMS cells were then transfected with mock or Nrf1 siRNA and plated at high density (150,000 cells/cm²). The cells were immediately cultured in mineralization medium containing 10 μM βGP and 300 μg/ml AA-2P after transfection. A, representative ALP staining in BMS cells transfected with mock or Nrf1 siRNA 7 days after transfection. B, quantitative data of ALP activities in the BMS cells transfected with mock or Nrf1 siRNA. The data shown are means ± S.D. from three replicates. A star indicates statistical significance of Nrf1 siRNA-treated samples versus mock siRNA controls (p < 0.01). C, representative mineralized nodules with Alizarin red staining from the BMS cells transfected with mock or Nrf1 siRNA 24 days after transfection. D, ALP mRNA expression analyzed by real time RT-PCR 3 days after transfection. Values are means ± S.D. from three replicates. A star indicates statistical significance of mineralized areas from the mock siRNA-treated cells compared with the Nrf1-treated cells (p < 0.01).

Knockdown of Nrf1 expression by siRNA led to a decrease in mineralized nodule formation after 24 days in culture. In this regard, we plated mock- or Nrf1 siRNA-treated cells at high density and immediately treated the cultures with mineralization media to induce differentiation. We felt that the confluent nature of cultures along with the addition of mineralization media would allow cells to directly enter differentiation without the need to undergo proliferation, thereby ensuring that the siRNA would stay in the transfected cells for a few days. Accordingly, we detected green cells after 3 days in >80% of transfected BMS cells using fluorescein-labeled mock siRNA under our culture conditions. Knockdown of Nrf1 expression by siRNA decreased ALP mRNA after 3 days, ALP protein after 7 days, and nodule formation after 24 days. In terms of how the transient

tic lineage and BMS cells, whereas Nrf2 and Nrf3 were expressed at much lower levels. Blockage of 80% Nrf1 expression by RNA interference diminished Nrf1 competition of ARE sites with other repressors (26), therefore abolishing AA induction of osterix expression and significantly impairing BMS cell transfection of BMS cells with Nrf1 siRNA produced long term effects on mineralization, it is possible that inhibition of Nrf1 expression and subsequent osterix expression led to a delay in the differentiation and mineralization process in siRNA-treated cultures.
Ascorbic Acid Stimulates Osterix Expression via Nrf1

In our studies, we used 300 μg/ml AA-2P to stimulate differentiation of sfx mutant BMS cells rather than the 50–100 μg/ml AA that is normally used in the mineralization medium for a number of reasons. 1) AA is readily oxidized and metabolized, whereas AA-2P has been shown to be stable. AA-2P needs to be converted into AA by phosphatases in the extracellular matrix before it can be transported into cells for subsequent action. The intracellular level of AA in AA-2P-treated cells rises slowly, reaching a plateau at 24 h (41). 2) The molecular weight of AA-2P is greater than that of AA (290 versus 179). Thus, the 300-μg/ml AA-2P dose used in this study (~1 mM) is comparable with the dose of AA (50–100 μg/ml) traditionally used in the mineralization media. 3) In our dose-response experiments with AA-2P (0.1–1 mM), we found that, although 100 μg/ml produced effects comparable with 300 μg/ml in some experiments, 300 μg/ml AA-2P produced consistent data. We did not observe any toxic effects of AA-2P in our experiments, since the cells were healthy.

We utilized undifferentiated BMS cells as an in vitro model system to study transcriptional regulation of AA on its target gene for a number of reasons. First, these cells do not bear the gulonolactone oxidase gene and therefore cannot produce AA endogenously. The sfx mutant BMS cells are proliferating and maintain fairly low levels of basal osterix expression in the absence of exogenous AA compared with the BMS cells derived from wild-type mice. Second, the endogenous promoter of osterix in the primary BMS cells is active. The sfx BMS cells express higher levels of osterix mRNA in response to AA treatment than the cells isolated from wild-type mice. Third, unlike the immortalized preosteoblast cell lines, the BMS cells retain the potential to differentiate progressively and form nodules in vitro. Upon treatment with βGP and AA, these undifferentiated BMS cells start to produce extracellular matrix and undergo mineralization similar to the progressive development of the rat osteoblast phenotype (31, 42). In our experiments, we observed the AA induction of osterix expression as early as 4 h after treatment and independent of new protein synthesis and mRNA stability. We do observe that osterix expression continues to increase with time during in vitro differentiation of bone marrow stromal cells with nearly a 40-fold increase seen in mutant cells at day 18. Although our data provide a convincing demonstration that the effects of ascorbate on osterix expression at the early stage of osteoblast differentiation is due to the direct effect of ascorbate on osterix gene transcription via a pathway involving increased binding of Nrf1 to ARE in the osterix promoter region, it is very likely that collagen-mediated integrin/mitogen-activated protein kinase signaling may play a significant role in regulating osterix expression at late stages of osteoblast differentiation (14, 15, 31).

Further time course experiments with inhibition of collagen-mediated signaling pathway are needed to identify the relative contribution of direct versus collagen-mediated indirect effect of AA on osterix expression and osteoblast differentiation.

The effect of ascorbate on osterix promoter activity of the reporter construct was smaller compared with the much larger effect of ascorbate on osterix mRNA levels in the time course experiments. One potential explanation for the small effect in the promoter assay is that our assay utilized transient transfection of a reporter construct that contained only a 2.1-kb length of mouse osterix promoter containing a single ARE in a nonnucleosomal context. However, endogenous osterix regulatory regions may contain additional AREs and other regulatory elements that have not been fully evaluated, and the AA stimulation of osterix expression in the time course experiments was based on the nucleosomal template in which the DNA is wrapped around histones. In addition, it is likely that the expression of other stage-specific transcription factors, such as Runx2, also contribute to full activation of the osterix gene during osteoblast differentiation (43–45). Some of these genes expressed in differentiated osteoblasts may also be regulated via ARE-dependent mechanisms, since putative AREs are present in the proximal promoter regions of Runx2. Therefore, AA appears to be one of the key regulators in osterix gene expression. Recently, there is a report that members of the Nrf family can interact with small Maf proteins to remodel chromatin structure at the β-globin locus control region, suggesting that interaction of Nrf1 with DNA may also remodel nuclear architecture and therefore facilitate other nuclear factors binding to their cis-elements of the osterix gene (43). However, it is also possible that some of the later increase in osterix expression is mediated via other alternative mechanisms, such as matrix interaction, post-transcriptional processing, and protein degradation pathways. Our future studies will sort out the additional pathways and/or mechanisms that contribute to the nearly 40-fold increase in osterix expression seen at day 18 in sfx bone cells.

We focused in this study on the transcriptional regulation of AA upon osterix expression from a number of candidate transcription factors that may be regulated by AA based on the findings that AA-induced osterix expression, and disruption of osterix has been shown to cause maturation arrest of osteoblasts (19). However, other transcription factors, such as Runx2 and Dlx3, may also be stimulated by AA and may contribute to osteoblast commitment. Therefore, it remains to be determined whether other factors are important mediators of the effect of AA on osteoblast differentiation and bone formation.

Based on our data and the data from other published ARE-related studies, we predict that, in the absence of antioxidant, Nrf1 is sequestered in the cytoplasm (26). Upon antioxidant stimulation, however, Nrf1 is released from the cytoplasm and trafficked into the nucleus. The abundance of activated, and possibly phosphorylated, Nrf1 then interacts with small Maf or other transcription factors, binding to ARE as a heterodimer to activate the target genes (28, 43, 46). We will test this model in bone-specific Nrf1 conditional knock-out mice. Our further studies will also focus on identification and characterization of other AA target genes responsible for osteoblast cell differentiation and bone formation.

Acknowledgments—We thank Sean Belcher for editorial assistance. All work was performed at facilities provided by the Jerry L. Pettis Memorial Veterans Affairs Medical Center.

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