Published in final edited form as:

Ann N Y Acad Sci. 2007 November ; 1117: 367–376. doi:10.1196/annals.1402.038.

Osteogenic Differentiation of Human Mesenchymal Bone Marrow Cells in Silk Scaffolds Is Regulated by Nitric Oxide

Petros D. Damoulis^a, Dimitrios E. Drakos^a, Eleni Gagari^b, and David L. Kaplan^c

^aDepartment of Periodontology, School of Dental Medicine, Tufts University, Boston, Massachusetts, USA

^bDepartment of Oral and Maxillofacial Pathology, School of Dental Medicine, Tufts University, Boston, Massachusetts, USA

^cDepartment of Biomedical Engineering, School of Engineering, Tufts University, Medford, Massachusetts, USA

Abstract

Bone marrow-derived mesenchymal stem cells (BMSC) are a powerful tool for tissue engineering and can be used in the regeneration of bone and other tissues. Nitric oxide (NO) produced by the endothelial NO synthase (eNOS) plays an important role in bone development and healing. We hypothesized that NO plays a role in osteogenic differentiation of BMSC cultured in three-dimensional silk scaffolds. eNOS protein was measured by Western Analysis and its activity was assessed by measuring nitrite in culture supernatants. Mineralization was evaluated through calcium deposition and the expression of genes associated with osteogenic differentiation (collagen I, RUNX2, and osteocalcin) was quantified using real-time RT-PCR. eNOS was consistently expressed with minor fluctuations, but NO production significantly increased at later time points (weeks 4 and 5). Addition of a competitive NOS inhibitor (L-NAME) resulted in a modest decrease in calcium deposition, which became statistically significant in week 5. This was preceded by a dramatic decrease in RUNX2 and osteocalcin expression in week 4. These results support our hypothesis and implicate NO as an important player in bone tissue engineering.

Keywords

nitric oxide; mesenchymal stem cells; cell differentiation; osteogenesis; tissue engineering

Introduction

Tissue engineering involves the combination of cells, scaffolds, and mediators for the development of three-dimensional biological structures. These structures can be used as research tools, to explore cellular mechanisms under conditions approximating more closely the *in vivo* environment, as well as therapeutically, to repair defects resulting from trauma and/or disease. Engineering of osseous tissue has been the target of extensive research, using a variety of cells, scaffolds, and mediators. Bone marrow mesenchymal cells (BMSC) are undifferentiated cells with high proliferative activity and with the potential to differentiate into various cell types, including bone and cartilage.^{1,2} Because of these characteristics, BMSC have been extensively used for bone tissue engineering.^{3–5} A variety of different scaffold

Address for correspondence: Petros D. Damoulis, D.D.S., D.M.Sc., D.M.D., Department of Periodontology, School of Dental Medicine, Tufts University, Rm. 639, One Kneeland Street, Boston, MA 02111. Voice: 617-636-3743; fax: 617-636-3401. E-mail: petros.damoulis@tufts.edu.

P. D. Damoulis and D. E. Drakos have contributed equally for the submission of this manuscript.

materials have been used to develop three-dimensional osseous-like structures using BMSC, including collagen, demineralized bone, and silk.^{5,6} Silk appears to be particularly suited for bone tissue engineering due to its biocompatibility, slow degradability, and mechanical properties.^{7,8} Even though significant progress has been made in describing cellular growth and differentiation of BMSC in those scaffolds as well as characterization of the resulting osseous-like structure, the intricate pathways that regulate this process remain largely unexplored. One such pathway may involve nitric oxide (NO), a ubiquitous free radical that plays a significant role in bone development *in vitro* and *in vivo*.^{9,10} NO is produced through the L-arginine/nitric oxide synthase (NOS) pathway from the terminal guanidino nitrogen of L-arginine, yielding L-citrulline as a byproduct.^{11,12} This reaction can be inhibited by substituted arginine analogues, such as N^G-nitro-L-arginine methyl ester (L-NAME).¹³ There are three main isoforms of NOS, two constitutive (type I/neuronal, type III/endothelial) and one inducible (type II/macrophage), the latter primarily expressed in inflammatory responses.^{13,14} Endothelial NOS (eNOS) is the isoform most associated with bone development, since targeted disruption of the eNOS gene results in impaired osteoblast function and osteopenia.¹⁰ Endothelial NOS expression in human BMSC was recently reported,¹⁵ but its role in osteoblastic differentiation of these cells is unknown. The purpose of this study was to investigate eNOS expression and activity in human BMSC during their transition toward a mineralizing phenotype in three-dimensional silk scaffolds, as well as the effect of eNOS inhibition on the differentiation process.

Materials and Methods

Silk Scaffolds

Silk fibroin solution was prepared from dried *B. mori* cocoons as previously reported.^{7,16} Scaffolds were prepared by adding granular NaCl to this aqueous silk solution to obtain 5 mm × 5 mm cylinders with a pore size of 500 μm.

Cell Culture

BMSC were isolated from fresh bone marrow aspirate and cultured as previously described,¹⁶ seeded at P2 in the scaffolds at a density of 10⁵ cells/scaffold, and cultured in osteogenic medium over a period of 5 weeks.¹⁷ The NOS inhibitor L-NAME was added at a concentration of 250 μM, starting on day 4. Assays were performed at weekly time points.

Western Analysis

Cells were lysed with a Tris/NaCl/Triton-X/octyl β-D-glucopyranoside buffer with protease inhibitors. Lysates were separated electrophoretically in a 7% Tris-Acetate NuPAGE gel (Invitrogen, Carlsbad, CA, USA) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were labeled with either anti-eNOS polyclonal (EMD Chemicals, San Diego, CA, USA) or anti-β-actin monoclonal (Sigma-Aldrich, St. Louis, MD, USA) primary antibody and a horseradish peroxidase-conjugated secondary antibody (EMD Chemicals) and visualized with enhanced chemiluminescence (Roche Applied Science, Indianapolis, IN, USA). The chemiluminescent signal was captured by a CCD camera and digitized (UVP Upland, CA, USA).

Measurement of Nitrite and Nitrite Ions (NO_x⁻)

Culture supernatants were assessed for the presence of NO_x⁻, which reflect the amount of NO produced by NOS. Nitrate was converted to nitrite and total nitrite was measured fluorometrically after reaction with 2,3-diaminonaphthalene at 355 nm excitation and 405 nm emission.¹⁸

Calcium Quantification

Total calcium was extracted in 0.5% trichloroacetic acid and measured with the QuantiChrom Calcium Assay kit (BioAssay Systems, Hayward, CA, USA). Free calcium forms a stable blue complex with a phenolsulfonephthalein dye, that can be measured spectrophotometrically at 612 nm.⁵

Quantitative Real-Time RT-PCR

RNA was extracted with Trizol (Invitrogen), purified using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA), and quantified spectrophotometrically. Reverse transcription was performed using the High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using reagents from the same manufacturer. cDNA samples were analyzed at least in duplicate for collagen I, RUNX2, osteocalcin, and GAPDH (as the housekeeping gene), using primers, probes, and conditions that have been previously described.^{6,16} Expression levels for each gene of interest were calculated by normalizing the quantified cDNA transcript level (Ct) to the GAPDH transcript level (Ct) ($2^{\Delta\Delta Ct}$ formula).⁶

Statistical Analysis

All experiments were performed in triplicate. Student's *t*-test was used to compare differences between the control and L-NAME groups at each time point.

Results

BMSC Express a Functional eNOS

Western analysis demonstrated that BMSC cultured in three-dimensional silk scaffolds express the endothelial isoform of NOS at all time points examined (week 1 through 5) (Fig. 1A). The amount of eNOS shows some minor fluctuations, but these changes are not dramatic enough to be quantified in the digitized images. In contrast, NOS activity showed clear differences during culture (Fig. 1B). NO_x⁻ levels were relatively low in weeks 1, 2 and 3, with a significant burst (> 10-fold difference) toward the late time points of the culture (weeks 4 and 5). Although there was a concurrent upregulation in cell proliferation between weeks 3 and 4 (≈twofold increase in scaffold DNA content, from 1.0 µg on week 3 to 2.1 µg on week 4), normalized NO_x⁻ values still showed statistically significant differences. This increase in NOS activity followed the initial burst of mineralization, which occurred during the third week (Fig. 2A). Interestingly, NOS activity showed a bimodal pattern, with a transient, small but significant increase on week 2. This pattern of NOS activity potentially indicates different roles for NO at different stages of cell differentiation.

NOS Inhibition Influences Mineralization and Expression of Genes Associated with Osteogenic Differentiation

BMSC growth pattern was similar to what has been reported in the literature, with an initial period of quiescence, followed by a period of rapid growth.¹⁷ The respective average DNA values for weeks 1 through 5 were 0.9/1.0/1.0/2.1/2.0 µg/scaffold. This pattern probably represents an initial adaptation period of BMSC in the three-dimensional structure. Addition of a selective NOS inhibitor (L-NAME) had no effect on DNA content or alkaline phosphatase (ALP) activity (data not shown). There was, however, a mild but consistent decrease in calcium content, which became significant at the last time point (week 5) (Fig. 2A). Scaffold calcium content increased significantly between weeks 2 and 3 and again between weeks 4 and 5. This could potentially be explained by the presence of BMSC subpopulations when the scaffolds are seeded, which get activated at different times in culture. This hypothesis is corroborated by the significant drop in collagen I (and to a lesser degree, RUNX2) expression seen on week

2 (Fig. 2B, C). BMSC in silk scaffolds strongly expressed collagen I and showed a significant upregulation of RUNX2 and osteocalcin expression on week 4 (Fig. 2B–D). NOS inhibition resulted in a strikingly similar pattern for all three genes studied. Gene expression was upregulated on week 2 compared to control, whereas on week 4 there was a highly significant downregulation. This downregulation of collagen I, RUNX2, and osteocalcin expression preceded a statistically significant decrease in scaffold calcium content. These findings further support a potential bimodal role for NO in BMSC differentiation and/or differential regulation of BMSC phenotype in separate cell subpopulations.

Discussion

In this study, the expression and activity of eNOS in longitudinal 3D cultures of human BMSC is reported for the first time. We decided to use BMSC as our model, since they have been well characterized, can be induced to differentiate along the osteogenic pathway, and are widely used for tissue engineering applications.^{5,17} The growth and differentiation patterns of these cells in the silk scaffolds were very similar to those previously reported. NOS expression and activity have been reported in mouse¹⁹ and human¹⁵ BMSC, but its association with cell differentiation has not been explored. Our findings indicate that although BMSC express eNOS at relatively constant levels, the enzyme's activity shows dramatic changes, with a significant upregulation seen at later stages of culture, when cells establish a more differentiated phenotype (Fig. 1). These findings also underscore the importance of measuring enzymatic activity when assessing the functional role of eNOS, since it is well known that cellular localization of the protein and presence of activating factors can have a profound effect on its NO output.²⁰ The apparent bimodal pattern of upregulation in eNOS activity (Fig. 1B) is intriguing and could indicate distinct functional roles for NO during BMSC differentiation. Alternatively, it could represent eNOS activation in different cell subsets present in a heterogeneous culture.

Exploring the potential NO-mediated effects presents another challenge. NO can have multiple cellular targets and its effects depend upon the target cells, chemical interactions within the cytoplasm, the amount of NO produced, and the site of its production.²¹ Nitric oxide donors are often employed, which usually decompose in solution releasing relatively high levels of NO over a short period of time. Since we wanted to study the effects of endogenous NO over a prolonged period, we used L-NAME, a soluble arginine analogue, which is a competitive inhibitor of eNOS with an IC₅₀ of 500 nM.²² The concentration used (250 μ M) is well within the range reported in the literature.¹⁹ Even though the inhibitor was constantly present in the culture medium, we did not observe any difference in cell growth (data not shown). This is in agreement with the findings of Pan *et al.*,¹⁹ who did not observe any change in unstimulated mouse BMSC proliferation after the addition of 3 mM L-NAME. However, genistein-induced NO production and cell proliferation decreased after L-NAME addition.¹⁹ Furthermore, osteoblasts from eNOS knockout mice demonstrate a significant reduction in proliferative activity compared to their wild-type controls.²³ It is therefore plausible that low-level eNOS activity, even in the presence of a competitive inhibitor, is adequate for normal cell growth. The effects of NO on osteogenic phenotypic markers are equivocal in the literature, primarily because of inconsistencies in research design. Earlier studies exploring the effects of NO donors or iNOS-mediated NO production reported that NO could promote²⁴ or inhibit²⁵ osteoblastic differentiation. When the issue of NO bioavailability was addressed, an important role for NO emerged as an inducer of osteoblastic differentiation, especially in mesenchymal progenitor cells.^{9,19} This role was further supported from examination of animals lacking eNOS, which demonstrated markedly reduced bone volume and bone formation, due in part to impaired osteoblastic function.¹⁰ A common finding in many of these studies is a strong association between NO and mineralization.^{19,23,26} Our findings are consistent with a role for NO as a promoter of mineralization, since addition of L-NAME resulted in a decrease of calcium deposition (Fig. 2A).

Even though the effects of NO on osteogenic phenotypic markers have been long established, the molecular mechanisms underlying these changes have only recently begun to unravel. Afzal *et al.* reported that osteoblasts from neonatal eNOS knockout mice show significantly reduced expression of RUNX2 and osteocalcin.²³ Furthermore, upregulation of NOS activity in mouse BMSC resulted in increased RUNX2 expression, mediated through cGMP.¹⁹ In our study, RUNX2 and osteocalcin expression also showed a dramatic downregulation after 4 weeks of L-NAME addition (Fig. 2C, D). The parallel pattern of expression for these two genes is expected and consistent with previous reports.²⁷ NO appears to regulate primarily RUNX2, which in turn modulates the expression of other important osteogenic genes, such as MMP-13.²⁸ L-NAME addition also decreased the expression of collagen type I at the end of the culture (Fig. 2B), an observation consistent with reports showing that NO donors stimulate its expression in fibroblasts.^{29,30} The exact mechanism for this regulation is still unclear.

One of the most intriguing findings was the upregulation of collagen type I, RUNX2, and osteocalcin after L-NAME addition at week 2 (Fig. 2B–D). This was associated with a small transient increase in eNOS activity at a stage when osteogenic markers were still not maximally expressed. It is therefore possible that NO has distinct functional roles depending on the stage of cell differentiation. A similar hypothesis was proposed by Afzal *et al.*, who reported that exogenously added NO restored RUNX2 and osteocalcin expression in osteoblasts from eNOS knockout mice to wild-type levels. However, this effect was evident only during the latter stages of osteoblast differentiation, associated with mineralizing activity and only when cells from younger animals were used.²³ Based on these findings, the authors proposed that NO is involved in coordinating specific phases of osteoblast differentiation and bone formation. Our results support this postulate and could even indicate a dual role for NO in osteogenic cell differentiation.

In conclusion, eNOS is expressed by human BMSC cultured in 3D silk scaffolds, but its enzymatic activity and concomitant NO production increases primarily at the later stages in culture. Increased NO production in the 3D environment is associated with upregulated expression of genes important for osteogenic differentiation and with calcium deposition. It appears that eNOS plays an important role in bone development and NO effects can be stage-specific. NO can therefore be a mediator with a distinct, multifaceted mode of action, which could also prove to be a significant tool for bone tissue engineering.

Acknowledgments

The research was supported by Institutional funds to Petros D. Damoulis and Eleni Gagari, as well as by grants from the NIH (R01EB003210, P41EB002520) to D. L. Kaplan.

References

1. Owen M. Marrow stromal stem cells. *J Cell Sci* 1988;10:63–76.
2. Caplan A. Mesenchymal stem cells. *J Orthop Res* 1991;9:641–650. [PubMed: 1870029]
3. Minguell J, Erices A, Conget P. Mesenchymal stem cells: minireview. *Exp Biol Med* 2001;226:507–520.
4. Cancedda R, et al. Bone marrow stromal cells and their use in regenerating bone. *Novartis Found Symp* 2003;249:133. [PubMed: 12708654]
5. Meinel L, et al. Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Ann Biomed Engng* 2004;32:112–122. [PubMed: 14964727]
6. Mauney JR, et al. *In vitro* and *in vivo* evaluation of differentially demineralized cancellous bone scaffolds combined with human bone marrow stromal cells for tissue engineering. *Biomaterials* 2005;26:3173–3185. [PubMed: 15603812]
7. Sofia S, et al. Functionalized silk-based biomaterials for bone formation. *J Biomed Mater Res* 2001;54:139–148. [PubMed: 11077413]

8. Wang Y, et al. Stem cell-based tissue engineering with silk biomaterials. *Biomaterials* 2006;27:6064–6082. [PubMed: 16890988]
9. Koyama A, et al. Nitric oxide accelerates the ascorbic acid-induced osteoblastic differentiation of mouse stromal ST2 cells by stimulating the production of prostaglandin E(2). *Eur J Pharmacol* 2000;391:225–231. [PubMed: 10729362]
10. Aguirre I, et al. Endothelial nitric oxide synthase gene-deficient mice demonstrate marked retardation in postnatal bone formation, reduced bone volume, defects in osteoblast maturation activity. *Am J Pathol* 2001;158:247–257. [PubMed: 11141498]
11. Moncada S, Palmer RM, Higgs EA. Biosynthesis of nitric oxide from L-arginine: a pathway for the regulation of cell function and communication. *Biochem Pharmacol* 1989;38:1709–1715. [PubMed: 2567594]
12. Feelisch, M.; Stamler, JS. *Methods in nitric oxide research*. John Wiley & Sons, Inc.; New York: 1996.
13. Alderton W, Cooper C, Knowles G. Nitric oxide synthases: structure, function and inhibition. *Biochemistry* 2001;357:593–615.
14. Marletta M. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* 1994;78:927–930. [PubMed: 7522970]
15. Klinz FJ, et al. Phospho-eNOS Ser-114 in human mesenchymal stem cells: constitutive phosphorylation, nuclear localization and upregulation during mitosis. *Eur J Cell Biol* 2005;84:809–818. [PubMed: 16270749]
16. Kim HJ, et al. Influence of macroporous protein scaffolds on bone tissue engineering from bone marrow stem cells. *Biomaterials* 2005;26:4442–4452. [PubMed: 15701373]
17. Meinel L, et al. Engineering bone-like tissue *in vitro* using human bone marrow stem cells and silk scaffolds. *J Biomed Mater Res* 2004;71A:25–34.
18. Misco TP, et al. A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 1993;214:11–16. [PubMed: 7504409]
19. Pan W, et al. Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. *J Cell Biochem* 2005;94:307–316. [PubMed: 15526288]
20. Ortiz P, Garvin J. Trafficking and activation of eNOS in epithelial cells. *Acta Physiol Scand* 2003;179:107–114. [PubMed: 14510773]
21. Murphy MP. Nitric oxide and cell death. *Biochimica et Biophysica Acta* 1999;1411:401–414. [PubMed: 10320672]
22. Turner CH, et al. Nitric oxide inhibitor L-NAME suppresses mechanically induced bone formation in rats. *Am J Physiol* 1996;270:E634–E639. [PubMed: 8928770]
23. Afzal F, Polak J, Buttery L. Endothelial nitric oxide synthase in the control of osteoblastic mineralizing activity and bone integrity. *J Pathol* 2004;202:503–510. [PubMed: 15095278]
24. Chae H, et al. Nitric oxide is a regulator of bone remodelling. *J Pharmacy Pharmacol* 1997;49:897–902.
25. Danziger R, Zuckerbraun B, Pensler J. Role of nitric oxide in the regulation of osteoblast metabolism. *Plastic Reconstr Surg* 1997;100:670–673.
26. Yasuhara R, et al. Nitric oxide in pulp cell growth, differentiation, and mineralization. *J Dent Res* 2007;86:163–168. [PubMed: 17251517]
27. Mundlos S, et al. Differential regulation of Cbfa-1/Runx2 and osteocalcin gene expression by vitamin-D3, Dexamethasone, and local growth factors in primary human osteoblasts. *J Cell Biochem* 2002;86:348–356. [PubMed: 12112004]
28. Zaragoza C, et al. Cbfa-1 mediates nitric oxide regulation of MMP-13 in osteoblasts. *J Cell Sci* 2006;119:1896–1902. [PubMed: 16636074]
29. Hsu YC, et al. Exogenous nitric oxide stimulated collagen type I expression and TGF-beta1 production in keloid fibroblasts by a cGMP-dependent manner. *Nitric Oxide* 2007;16:258–265. [PubMed: 17052928]
30. Obayashi K, et al. Exogenous nitric oxide enhances the synthesis of type I collagen and heat shock protein 47 by normal human dermal fibroblasts. *J Dermatol Sci* 2006;41:121–126. [PubMed: 16171977]

FIGURE 1.

(A) Western Analysis of BMSC lysates for eNOS and β -actin (as a housekeeping gene) at 5 weekly time points, as described in "Materials and Methods." Human endothelial cell lysate was used as a positive control (PC). **(B)** Nitrite and nitrate ions (NO_x^-) measured in conditioned media at the same weekly time points, as described in "Materials and Methods." NO_x^- levels reflect eNOS activity over a period of the preceding 3 days. Values represent the mean $\pm$ SEM from three independent experiments.

FIGURE 2.

Effect of eNOS inhibition on BMSC mineralization and gene expression. L-NAME was used as a competitive eNOS inhibitor at a concentration of 250 μM , starting on day 4 of the experiment. Assays were performed weekly for 4 (gene expression) or 5 (calcium content) weeks. Week 0 reflects the day of cell seeding. Continuous lines with closed triangles represent control cultures whereas dotted lines with open diamonds represent the L-NAME group. Values represent the mean $\pm$ SD from three independent experiments. Asterisks denote statistical significance based on the Student's *t*-test (* $P < 0.05$; *** $P < 0.001$). (A) Calcium content; (B) collagen type I normalized to GAPDH; (C) RUNX2 normalized to GAPDH; and (D) osteocalcin normalized to GAPDH.