Isolated osteoblasts from spinal cord-injured rats respond less to mechanical loading as compared with those from hindlimb-immobilized rats

Sheng-Dan Jiang, Yue-Hua Yang, Jiang-Wei Chen, Lei-Sheng Jiang

Department of Orthopaedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

Background: The pathogenesis of osteoporosis after spinal cord injury (SCI) may be different from disuse osteoporosis.

Objective: To investigate whether there is the differential anabolic response to mechanical loading between osteoblasts from SCI rats and those from hindlimb-immobilized rats.

Methods: Femoral bone-marrow was harvested for osteoblast culture from SCI rats, hindlimb-immobilized rats, and control rats 3 weeks after animal model creation. At the stage of differentiation, rat osteoblasts were plated in six-well plates for stretching. Cyclic strains were applied for 48 hours, and then alkaline phosphatase (ALPase) activity, procollagen, and osteocalcin production, and gene expression of osteocalcin, runt-related transcription factor 2 (Runx2), and osterix were measured in osteoblasts from SCI rats, hindlimb-immobilized rats, and control rats.

Results: ALPase activity, procollagen, and osteocalcin production, and gene expression of osteocalcin, Runx2, and osterix were significantly lower in osteoblasts after stretching from SCI rats compared with those from hindlimb-immobilized rats. However, there was no significant difference of these parameters between isolated osteoblasts from hindlimb-immobilized rats and those from control rats.

Conclusion: The activity of isolated osteoblasts from SCI rats was lower than control rats, and this suggested that osteoblasts from SCI rats responded less to mechanical loading as compared with those from control rats.

Keywords: Spinal cord injuries, Immobilization, Osteoblasts, Osteoporosis, Demineralization, Bone loss, Paraplegia

Introduction

Osteoporosis, a condition characterized by low bone mass and deterioration of the skeletal microarchitecture, is a known consequence of spinal cord injury (SCI).¹ Disuse is generally thought to be a major reason for bone loss in SCI.² However, osteoporosis after SCI is not just the same as disuse osteoporosis.^{3,4}

The pathogenesis of osteoporosis after SCI remains unclear. SCI resulted in rapid bone loss with more deteriorated trabecular microstructure in sublesional bones than immobilization,⁵ suggesting that osteoporosis

after SCI may be different from disuse osteoporosis. Additionally, some studies reported that intensive exercises did not retard the demineralization of the lower limbs in patients with SCI,⁶ whereas re-ambulation did reverse bone loss after bed rest.⁷ We postulate that the differential osteogenic response to mechanical loading after SCI and immobilization is intrinsic to bone cells and that comparative osteogenic response in osteoblasts derived from SCI rats and hindlimb-immobilized rats in response to mechanical strain could provide information concerning the mechanical stimulation of bone formation.

The objective of this study was to investigate whether there was a differential anabolic response to mechanical

Correspondence to: Lei-Sheng Jiang, Department of Orthopaedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, China. Email: jiangleisheng@126.com

loading between SCI rats and hindlimb-immobilized rats, using an *ex vivo* mechanical strain model as a surrogate of mechanical loading.

Materials and methods

Animals

The protocols for animal experimentation were previously approved by the Animal Research Committee of the university, and all subsequent animal experiments adhered to the “Guidelines for Animal Experimentation” of the university.

Eighteen male Sprague–Dawley rats, 6-week-old, were randomly assigned into three groups: SCI group, hindlimb cast-immobilized group, and control group, with six rats in each group. Rats in SCI group were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). The spinal cord was exposed by laminectomy at the T10–12 level, and then was completely transected with a sharp scalpel. The urinary bladders of SCI rats were emptied manually at least three times a day for the first 2 weeks until spontaneous micturition recovered. Rats in hindlimb cast-immobilized group were performed according to the previously described procedures,⁸ and these rats were lightly anesthetized in order to attach the plaster of a Paris cast. Briefly, bilateral hindlimbs were fixed from the waist down with the hips at extension, the knees at 110° flexion, and the ankles at 150° plantar flexion. The casts were extended to 1 cm below the feet, making weight bearing impossible.⁹ All rats were housed in a controlled environment at 22°C with a 12-hour light/dark cycle.

Experimental design

All rats were sacrificed 3 weeks later by exsanguination from the abdominal aorta. To harvest the bone-marrow for cell culture, the femora were quickly excised and soft tissues were removed following sacrifice of the rats. The femora were immersed in 70% ethanol for 3 seconds, and rinsed four times for 2 minutes each in a phosphate-buffered saline (PBS) solution containing antibiotics (penicillin and streptomycin) and fungizone under sterile conditions. The epiphyses of each bone were removed with a razor blade. The marrow cells were flushed from the diaphyses using a syringe and 26.5-gauge needle, and collected in culture medium (alpha modified essential medium containing l-glutamine and nucleosides (Mediatech, Herndon, VA, USA)), supplemented with 15% fetal bovine serum (Gibco BRL, Rockville, MD, USA), 1% antibiotics (penicillin and streptomycin; Wako Pure Chemical Co., Osaka, Japan), 0.1% fungizone, 50 µg/ml ascorbic

acid (Wako), 10 mmol/l sodium beta-glycerophosphate (Sigma, St. Louis, MO, USA), and 10 nmol/l dexamethasone (Sigma). The marrow cell suspension was gently drawn through an 18-gauge needle to mechanically dissociate the mixture into a homogeneous single-cell suspension. Cells were grown in six-well plates. The medium was changed every 48 hours to remove non-adherent cells. At the stage of differentiation, rat osteoblasts ($1 \times 10^4/\text{cm}^2$) were plated in six-well plates for stretching.

Stretching

The plates were placed on a Loading Station (Flexcell International, Hillsborough, NC, USA) to produce equibiaxial strain. Stretching experiments were performed inside a 37°C, 5% CO₂ incubator, and vacuum is applied to the area under six-well plates, causing the flexible silicon bottom to stretch over loading posts underneath. The unit produces equibiaxial stretch¹⁰ and was adapted to produce physiologically relevant strain levels with minimal vertical displacement of the silicon bottom to minimize build-up of fluid flow.¹¹ Wells were subjected to a vacuum at 650 mbar, 1.0 Hz, using an Ø32 mm diameter loading post. This generated cyclic strains of 2.0% (20 000 µstrain). The computer system controlled the frequency of deformation and the negative pressure applied to the culture plates. Cyclic strains were applied for 48 hours. Following stretching, the medium were collected for analysis of the production of procollagen I C-terminal propeptide (PICP) and osteocalcin. Then cells were immediately placed on ice and washed once with ice-cold PBS, and were collected in appropriate lysis buffer for either real-time polymerase chain reaction (PCR) analysis or alkaline phosphatase (ALP) activity assays.

Assay of ALPase activity

After exposure to stretch, cells were washed twice with Tris-buffered saline, scraped into extraction buffer solution (10 mM Tris (pH 7.4), 0.9% NaCl, 0.1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany)), sonicated, and centrifuged at 10 000g for 15 minutes at 4°C. The supernatants were then used for ALPase assay and protein determination. ALPase activity was measured using the ALP B-test Wako kit (Wako), and the absorbance at 405 nm was read on a spectrophotometer. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin as the standard. The enzyme activity was expressed as

nanomoles of p-nitrophenol produced per minute per milligram of protein.

Procollagen and osteocalcin production

Production of type I procollagen was determined through measuring the levels of C-terminal PICP in medium. Medium levels of osteocalcin and PICP were measured using a rat sandwich enzyme-linked immunosorbent assay kit from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA) and Metra Biosystems Inc. (Mountain View, CA, USA) following the manufacturer's protocols and all samples were assayed in duplicate.

Real-time quantitative PCR

TRIzol was used to isolate total RNA of osteoblasts from SCI rats, hindlimb-immobilized rats, and control rats. cDNA was synthesized with the Omniscript Reverse Transcription kit using 2 µg of total RNA in a 20 µl reaction volume. For real-time PCR, the cDNA was amplified using an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer QuantiTech SYBR PCR to allow for the quantitative detection of the PCR product in a 25 µl reaction volume. The temperature profile of the reaction was 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 15 seconds, annealing, and extension at 58°C for 1 minute. The amount of target gene in the sample was determined from the standard curves. Six samples were run in triplicate. An internal housekeeping control gene, β -actin, was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. The size of the PCR product was verified on a 1.5% agarose gel followed by melting-curve analysis. The primer pairs used for PCR were osteocalcin (5'-GGAGGGCAGTAAGGTGGTGAA-3'/5'-GCCATAGATGCGCTTGTAGGC-3'), runt-related transcription factor 2 (Runx2) (5'-CGACAGCCCCAACTTCCT-3'/5'-ACGGTAACCACGGTCCCAT-3'), osterix (5'-TGAGGAAGAAGCCCGATTAC-3'/5'-ACTTCTTCTCCCGGGTGTG-3'), and β -actin (5'-CCTGTACGCCAACACAGTGC-3'/5'-ATACTCCTGCTTGCTGATCC-3').

Statistical analysis

Data are presented as means $\pm$ standard deviation (SD). Data comparisons among the groups were performed with analysis of variance test and the *post hoc* test using SPSS 15.0 software (SPSS Inc. Chicago, IL, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

General observations

The initial body weights did not significantly differ among three groups (SCI rats: 189.6 ± 19.3 g; hindlimb-immobilized rats: 194.1 ± 18.2 g; control rats: 192.7 ± 17.9 g). The final body weights in SCI rats (241.5 ± 23.7 g) were lower than those in hindlimb-immobilized rats (252.1 ± 26.3 g), but not significantly.

Assay of ALPase activity

ALPase activity (0.18 ± 0.03 IU/mg) in osteoblasts from SCI rats was significantly lower than that (0.26 ± 0.04 IU/mg) from hindlimb-immobilized rats after stretching ($P < 0.05$). ALPase activity in osteoblasts from hindlimb-immobilized rats was lower than that (0.28 ± 0.04 IU/mg) from control rats after stretching, but not significantly ($P > 0.05$).

Procollagen and osteocalcin production

Medium PICP and osteocalcin levels (18.7 ± 2.3 pg/ml; 211.4 ± 22.6 pg/ml) in osteoblasts from SCI rats were significantly lower than those from hindlimb-immobilized rats (27.1 ± 2.9 pg/ml; 283.2 ± 33.1 pg/ml) after stretching ($P < 0.05$). Medium PICP and osteocalcin levels in osteoblasts from hindlimb-immobilized rats were lower than those from control rats (29.4 ± 3.7 pg/ml; 294.7 ± 37.8 pg/ml) after stretching, but not significantly ($P > 0.05$).

Real-time PCR

The expression of gene coding for osteocalcin, Runx2, and osterix in osteoblasts from SCI rats was significantly lower compared with that from hindlimb-immobilized rats after stretching. There was no significant difference of osteocalcin, Runx2, and osterix expression in

Figure 1 Expression of osteocalcin, Runx2, and osterix mRNA in isolated osteoblasts from SCI rats, hindlimb-immobilized rats, and control rats after stretching. *Indicates $P < 0.05$. SCI, spinal cord injury; HCl, hindlimb cast-immobilized; CON, control.

osteoblasts from hindlimb-immobilized rats and those from control rats after stretching (Fig. 1).

Discussion

The current study demonstrated, for the first time, that osteoblasts from SCI rats responded less to mechanical loading than those from hindlimb-immobilized rats. It is well known that mechanical stress is one of the fundamental physiological factors for regulating structure and function in bones. To date, many papers have been published on the effects of mechanical stress affecting osteoblasts.^{12,13} Physiological strains recorded in human long bones during strenuous activity are of the order of 2000–4000 μ strain,¹⁴ and 1500 μ strain is the minimum effective strain under which bones will undergo modeling.¹⁵ It has been reported that strains on the order of 1–3% elongation (10 000–30 000 μ strain) are needed to obtain a cellular response *ex vivo*.¹⁶ Thus, we used an *ex vivo* cell strain system that allowed the quantification of mechanical stimulus at the magnitude of mechanical stress, with cycle strains of 2% elongation (20 000 μ strain).

In this study, we compared the mechano-responsiveness measured as ALPase activity, release of PICP and osteocalcin, and gene expression of osteocalcin, Runx2, and osterix in isolated osteoblasts from SCI rats versus hindlimb-immobilized rats. We found that ALPase activity, PICP and osteocalcin release, and osteogenic gene expression were significantly lower in isolated osteoblasts from SCI rats than control rats after stretching. However, there was no significant difference of ALPase activity, PICP and osteocalcin release, and osteogenic gene expression between isolated osteoblasts from hindlimb-immobilized rats and those from control rats. Isolated osteoblasts from SCI rats responded to mechanical strain with a different response pattern as compared with those from hindlimb-immobilized rats. This suggested that the differential osteogenic response to mechanical loading was intrinsic to bone cells.

Many authors claim that SCI-induced bone loss is attributable to disuse.^{2,17,18} However, this may not necessarily be the case. In patients with SCI, demineralization predominates in the long bones of the lower limbs, and the most affected sites are the trabecular metaphysical-epiphyseal areas of the distal femora and proximal tibiae.¹⁹ However, maximal bone loss occurs in the calcaneus and the hip after continuous bed rest, with less involvement of the tibiae, and bone loss after SCI is much more severe than that after bed rest.⁷ In addition, the levels of serum bone resorption markers after SCI were extremely high,²⁰ compared with those

reported in short- and long-duration bed-rest studies.²¹ Furthermore, some studies reported that intensive exercises did not retard the demineralization of the lower limbs in patients with SCI,⁶ while re-ambulation could restore the bone loss after bed rest.⁷ Combined with our finding that ALPase activity, PICP, and osteocalcin release and osteogenic gene expression was significantly lower in osteoblasts after stretching from SCI rats than hindlimb-immobilized rats, taken together, from the pathophysiological point of view, these findings demonstrated that the pathogenesis of osteoporosis after SCI may differ from disuse osteoporosis.

The differences in the response to mechanical loading between the two groups, as found in the present study, might therefore lead to differences in the balance between the bone-forming activity of osteoblasts and the bone-resorbing activity of osteoclasts. This may explain the phenomenon that intensive exercises do not retard the demineralization of the lower limbs in patients with SCI, while re-ambulation can restore the bone loss after bed rest. However, many factors contribute to osteoporosis after SCI, and it remains unresolved whether the differences as found in the present study were the cause, or rather the result of the osteoporosis after SCI and disuse osteoporosis.

It is theoretically possible to link the present *ex vivo* findings with the difference in bone mass and microstructure between SCI and hindlimb-immobilized bones, based on current knowledge of the effects of ALPase activity, PICP, and osteocalcin release and osteogenic gene expression on bone remodeling. Osteocalcin, synthesized by osteoblasts, is one of the most abundant non-collagenous proteins of bone matrix, and seems to influence mineralization. The skeletal isoform of ALP is a membrane-bound protein with enzymatic activity, and plays an important role in bone mineralization. PICP reflects the synthesis of new collagen. Lower ALPase activity, PICP, and osteocalcin release of osteoblasts from SCI rats compared with hindlimb-immobilized rats may indicate less mechanical loading-induced bone formation in SCI rats than that in hindlimb-immobilized rats. Systemic hormones changes such as parathyroid hormone, vitamin D3, sex steroids, thyroid hormone, and leptin and low nerve fiber innervation density in the paralyzed bones may explain these differences.

This study may be limited in the design. One is that none of the parameters relevant to influence of nerve function and nerve released growth factors on these osteoblasts and osteoclasts were examined. The second is that it is an *ex vivo* study, and responsiveness of osteoblasts to mechanical loading *ex vivo* cannot fully reflect

responsiveness of the paralyzed limbs to mechanical loading *in vivo*. Third, there are some differences in the anesthetics, surgical protocols, and physiological stress such as weight loss between SCI rats and hindlimb-immobilized rats, and to some extent, these differences may influence osteoblast activity.

Acknowledgments

This study was supported by National Natural Science Foundation of China (81000793; 81171757), the Science and Technology Commission of Shanghai Municipality (08411950100), Shanghai Rising-Star Program (08QA1405000), and Shanghai Educational Development Foundation (2008CG21).

References

- Jiang SD, Dai LY, Jiang LS. Osteoporosis after spinal cord injury. *Osteoporos Int* 2006;17(2):180–92.
- Uebelhart D, Demiaux-Domenech B, Roth M, Chantaine A. Bone metabolism in spinal cord injured individuals and in others who have prolonged immobilisation. A review. *Paraplegia* 1995; 33(11):669–73.
- Jiang SD, Jiang LS, Dai LY. Mechanisms of osteoporosis in spinal cord injury. *Clin Endocrinol (Oxf)*. 2006;65(5):555–65.
- Jiang SD, Jiang LS, Dai LY. Effects of spinal cord injury on osteoblastogenesis, osteoclastogenesis and gene expression profiling in osteoblasts in young rats. *Osteoporos Int* 2007;18(3):339–49.
- Liu D, Zhao CQ, Li H, Jiang SD, Jiang LS, Dai LY. Effects of spinal cord injury and hindlimb immobilization on sublesional and suprallesional bones in young growing rats. *Bone* 2008;43(1): 119–25.
- Jones LM, Legge M, Goulding A. Intensive exercise may preserve bone mass of the upper limbs in spinal cord injured males but does not retard demineralisation of the lower body. *Spinal Cord* 2002; 40(5):230–5.
- Leblanc AD, Schneider VS, Evans HJ, Engelbretson DA, Krebs JM. Bone mineral loss and recovery after 17 weeks of bed rest. *J Bone Miner Res* 1990;5(8):843–50.
- Booth FW. Time course of muscular atrophy during immobilization of hindlimbs in rats. *J Appl Physiol* 1977;43(4):656–61.
- Tuukkanen J, Peng Z, Vaananen HK. The effect of training on the recovery from immobilization-induced bone loss in rats. *Acta Physiol Scand* 1992;145:407–11.
- Brown TD. Techniques for mechanical stimulation of cells in vitro: a review. *J Biomech* 2000;33(1):3–14.
- Weyts FA, Bosmans B, Niesing R, van Leeuwen JP, Weinans H. Mechanical control of human osteoblast apoptosis and proliferation in relation to differentiation. *Calcif Tissue Int* 2003;72(4): 505–12.
- Katz S, Boland R, Santillan G. Modulation of ERK 1/2 and p38 MAPK signaling pathways by ATP in osteoblasts: involvement of mechanical stress-activated calcium influx, PKC and Src activation. *Int J Biochem Cell Biol* 2006;38(12):2082–91.
- Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. Mechanical stress-mediated Runx2 activation is dependent on Ras/ERK1/2 MAPK signaling in osteoblasts. *J Cell Biochem* 2007;101(5):1266–77.
- Burr DB, Milgrom C, Fyhrie D, Forwood M, Nyska M, Finestone A, *et al*. In vivo measurement of human tibial strains during vigorous activity. *Bone* 1996;18(5):405–10.
- Frost HM. Perspectives: bone's mechanical usage windows. *Bone Miner* 1992;19(3):257–71.
- Burger EH, Klein-Nulend J. Mechanotransduction in bone: role of the lacuno-canalicular network. *FASEB J* 1999;13:S101–12.
- Kiratli BJ, Smith AE, Nauenberg T, Kallfelz CF, Perikash I. Bone mineral and geometric changes through the femur with immobilization due to spinal cord injury. *J Rehabil Res Dev* 2000;37(2): 225–33.
- Takata S, Yasui N. Disuse osteoporosis. *J Med Invest* 2001; 48(3–4):147–56.
- Finsen V, Indredavik B, Fougner KJ. Bone mineral and hormone status in paraplegics. *Paraplegia* 1992;30(5):343–7.
- Roberts D, Lee W, Cuneo RC, Wittmann J, Ward G, Flatman R, *et al*. Longitudinal study of bone turnover after acute spinal cord injury. *J Clin Endocrinol Metab* 1998;83(2):415–22.
- Lueken SA, Arnaud SB, Taylor AK, Baylink DJ. Changes in markers of bone formation and resorption in a bed rest model of weightlessness. *J Bone Miner Res* 1993;8(12):1433–8.