Biochemical and Biophysical Research Communications **279,** 258–264 (2000)
doi:10.1006/bbrc.2000.3945, available online at http://www.idealibrary.com on IDEAL®

TNF-α-Dependent Activation of NF-κB in Human Osteoblastic HOS-TE85 Cells Is Repressed in Vector-Averaged Gravity Using Clinostat Rotation

Kenji Kobayashi,*,† Fukushi Kambe,*,[1] Kazutoshi Kurokouchi,† Tadahiro Sakai,*,† Naoki Ishiguro,† Hisashi Iwata,† Kazuo Koga,‡ Raphael Gruener,§ and Hisao Seo*

**Department of Endocrinology and Metabolism, Division of Molecular and Cellular Adaptation, and ‡Space Medicine Research Center, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan; †Department of Orthopedics, Nagoya University School of Medicine, Nagoya, Japan; and §Department of Physiology, University of Arizona College of Medicine, Tucson, Arizona*

Received November 7, 2000

Effects of vector-averaged gravity on tumor necrosis factor (TNF)-α-dependent activation of nuclear factor kappa B (NF-κB) in human osteoblastic HOS-TE85 cells were investigated by culturing the cells using clinostat rotation (clinorotation). Cell cultures were rotated for 72 h at 40 rpm in a clinostat. At the end of clinorotation, the cells were treated with TNF-α for 30 min under stationary conditions. Electrophoretic mobility shift assays revealed that TNF-α-dependent activation of NF-κB was markedly reduced in the clinorotated cells when compared with the cells in control stationary cultures or after horizontal rotation (motional controls). The NF-κB-dependent transactivation was also impaired in the clinorotated cells, as evidenced by a transient transfection assay with a reporter plasmid containing multimerized NF-κB sites. Consistent with these findings, the TNF-α-dependent induction of endogenous NF-κB-responsive genes p105, IκB-α, and IL-8, was significantly attenuated in clinorotated cells. These results demonstrate that vector-averaged gravity inhibits the responsiveness of osteoblasts to TNF-α by repressing NF-κB activation.

Key Words: **TNF-α; NF-κB; clinostat; vector-averaged gravity; osteoblasts; microgravity.**

A number of paracrine and autocrine factors regulate proliferation, differentiation and gene expression in osteoblasts. Among them, tumor necrosis factor (TNF)-α, a cytokine synthesized in the bone microenvironment, has been shown to exert pleiotropic effects on osteoblasts by affecting synthesis of DNA and collagen (1), osteocalcin (2), and proteolytic enzymes such as metalloproteinases (3). In altered gravity, however, either during space flights or after clinostat rotation (clinorotation; vector-averaged gravity conditions) the responses of osteoblasts to such hormones and cytokines are markedly affected. For example, human osteosarcoma MG-63 cells cultured for 9 days in space flight showed reduced induction of collagen, alkaline phosphatase and osteocalcin mRNA expression in response to 1,25-dihydroxyvitamin D3 or transforming growth factor β2 (4). Similarly, reduction in both basal alkaline phosphatase activity and its induction by treatment with 1,25-dihydroxyvitamin D3 was reported in human osteoblastic HuO9 cells which underwent clinorotation for 4 days (5). However, effects of microgravity on responses of osteoblasts to TNF-α have not been studied to date.

TNF-α has been shown to exert its action, in various cell types, by binding to a membrane receptor (TNF-R) with subsequent activation of a dimeric transcription factor NF-κB consisting of the Rel family proteins such as p65 (RelA), p50, p52, c-Rel and RelB. In unstimulated cells, NF-κB is sequestered in the cytoplasm bound to an inhibitory protein IκB. Several isoforms such as IκB-α and IκB-β have been identified (6, 7). TNF-α induces the activation of NF-κB by promoting the phosphorylation and degradation of IκB. NF-κB, which is released from IκB, translocates into the nucleus, binds to the distinct regulatory element of the target genes and controls their transcription (8, 9). Although the signaling pathway of TNF-α has not yet been fully defined in osteoblasts, we recently demonstrated that TNF-α increases expression of interleukin (IL)-6 and intercellular adhesion molecule-1 genes

[1] To whom correspondence should be addressed at Department of Endocrinology and Metabolism, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan. Fax: +81-52-789-3891. E-mail: kambe@riem.nagoya-u.ac.jp.

0006-291X/00 $35.00

through activation of NF-κB consisting of p50-p65 heterodimer in osteoblast-like cells (10).

The clinostat is an effective, ground-based tool to simulate microgravity (11–14). Clinorotation mimics certain aspects of the microgravity environment by nulling the gravitational vector through continuous averaging. In the present study, we utilized this system to investigate whether the vector-averaged gravity, generated by clinorotation, affects TNF-α-dependent activation of NF-κB in human osteoblastic HOS-TE85 cells.

MATERIALS AND METHODS

Cell culture. Human osteoblastic HOS-TE85 cells [ATCC CRL-1543] which express osteoblast-like phenotypes (15, 16) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 mM Hepes (pH 7.4) and 10% fetal bovine serum. After the cells grew to about 50% confluency, in 12.5-cm^2 flasks, the medium was removed and the flasks were completely filled with fresh medium and sealed with paraffin to eliminate air bubbles thus reducing turbulence and shear forces during clinorotation. The cells were divided into three groups: (i) stationary (control condition), (ii) horizontal rotation (motional control for turbulence, shear forces and vibrations) in which the flasks were rotated around the vertical axis, and (iii) clinorotated cultures in which the flasks were rotated around the horizontal axis to produce a vector-averaged environment. Details of the clinostat apparatus were previously described (17). All cultures were placed in an incubator at 37°C. After 72-h rotation at 10 or 40 rpm, the cells were treated with 50 U/ml recombinant human TNF-α (2.5×10^3 U/μg; Asahi Chemical Industry Co. Ltd., Tokyo, Japan) for 30 min in stationary culture. The cells were then harvested, and nuclear extracts were prepared for electrophoretic mobility shift assay as described previously (18). For Northern blot analysis, the cells were treated with TNF-α and 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St. Louis, MO) for 6 h and 1 h, respectively, and total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform-extraction method (19). The cell proliferation was assessed by WST-1 assay (Cell Counting Kit; Dojindo Laboratories, Tokyo, Japan) after 24-h and 72-h rotation.

Electrophoretic mobility shift assay (EMSA). Details of the EMSA procedure were described previously (20). The nuclear extracts (10 μg protein) were subjected to EMSA. The κBwt oligonucleotide (5′-TCGAGCAGAGGGGACTTTCCGAGAGTCGA-3′) containing a canonical NF-κB binding site identified in mouse Igκ enhancer (underlined) (21) was labeled with [^{32}P]dCTP and used as a probe. The κBmu oligonucleotide (5′-TCGAGCAGAGCTCACTTTCCGAGAGTCGA-3′) in which a mutation was introduced in the NF-κB binding site (underlined) (22) and the unlabeled κBwt oligonucleotide were used as competitors for displacement analysis with 50-fold molar excess. The supershift analysis employed anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and preimmune rabbit serum.

Transfection assay. The construction of a luciferase reporter plasmid pGL3pro-3κB which is driven by a tandem repeat of three NF-κB-binding sites was described previously (18). The pGL3pro-3κB plasmid (1 μg/flask) together with a α-galactosidase-expressing plasmid (0.2 μg/flask; pSV-β-galactosidase control vector driven by simian virus 40 early promoter and enhancer segments, Promega Corporation, Madison, WI) were transfected into HOS-TE85 cells using the calcium-phosphate method as described previously (23). After a 16-h transfection, the flasks were filled with fresh medium and subjected to clinorotation for 48 h. The cells were then treated with 50 U/ml TNF-α for 4 h in stationary culture. The luciferase and β-galactosidase activities in the cell lysates were determined by a luminometer (LB9501, Berthold, Germany). The enzyme activities were normalized to the protein contents of cell lysates determined with a microassay kit (BIO-RAD Laboratories, Hercules, CA).

Northern blot analysis. The detailed procedure was described previously (24). After fractionation of 20 μg total RNA on 0.8% agarose gel, the RNA was transferred onto a Gene Screen Plus membrane (New England Nuclear, Boston, MA). The membrane was hybridized with human IκB-α, p105, IL-8, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and c-fos cDNA probes labeled with [^{32}P]dCTP. After hybridization, the membrane was subjected to quantitative analysis using BAS 2000 bioimage analyzing system (Fuji film Co., Tokyo, Japan) and then exposed to X-ray film. Human p105 and IκB-α cDNAs were synthesized by RT-PCR using mRNA prepared from TNF-α-treated FRTL-5 cells and HOS-TE85 cells, respectively. The primers for p105 were 5′-GATGGCCCATACCTTCAAATAT-3′ and 5′-GGCTTTACTGTCATAGATGGCG-3′ (25). The primers for IκB-α were 5′-ATGTTCCAGGCGGCCGAGCGCCCCCA-3′ and 5′-TCATAACGTCAGACGCTGGCCTCC-3′ (6). The amplified PCR product was ligated into pGEM-T-easy plasmid (TA cloning system; Promega). The authenticity of cDNA was verified by DNA sequencing. The preparations of IL-8, GAPDH and c-fos cDNAs were described in our previous reports (26, 27)

Enzyme-linked immunosorbent assay (ELISA) of IL-8. During 72-h clinorotation at 40 rpm, the cells were continuously exposed to 50 U/ml TNF-α. The concentrations of IL-8 in the media were determined by a human IL-8 ELISA kit (R&D systems Minneapolis, MN) using a microplate reader (BIO-RAD).

Western blot analysis. The preparation of whole cell lysates and procedure for Western blot analysis were described in our previous report (28). The protein contents in the whole cell lysates were measured by a microassay kit. Cell lysates containing 80 μg protein were denatured and subjected to SDS–12% polyacrylamide gel electrophoresis. The proteins in the gel were transferred onto Hybond-C membrane (Amersham Life Sciences, Arlington Heights, IL). The bands for IκB-α, IκB-β, tumor necrosis factor-receptor 1 (TNF-R1), and tumor necrosis factor-receptor 2 (TNF-R2) were visualized by using each specific antibody (Santa Cruz) diluted at 1:500 and the second antibody conjugated with alkaline phosphatase (Zeimed, San Francisco, CA) diluted at 1:1000.

Statistical analysis. Results from different treatment groups were subjected to one-way analysis of variance with Bonferroni test. A P value smaller than 0.05 was considered to demonstrate statistically significant differences.

RESULTS

Clinorotation Represses the Activation of NF-κB Induced by TNF-α

Figure 1A shows, by using the EMSA assay, that treatment of HOS-TE85 cells in stationary cultures with TNF-α induced a single protein/κBwt complex (lane 2). This complex was displaced with excess amount of unlabeled κBwt oligonucleotide (lane 4), but not with kBmu oligonucleotide in which the NF-κB-binding site was mutated (lane 5). Anti-p65 antibody supershifted the complex (lane 7). However, preimmune rabbit serum did not affect the mobility of the complex (lane 6). These results indicate that TNF-α induces the activation of NF-κB containing p65 in control HOS-TE85 cells.

Quantified DNA-binding activity of NF-κB was increased fivefold by TNF-α, over basal levels, in station-

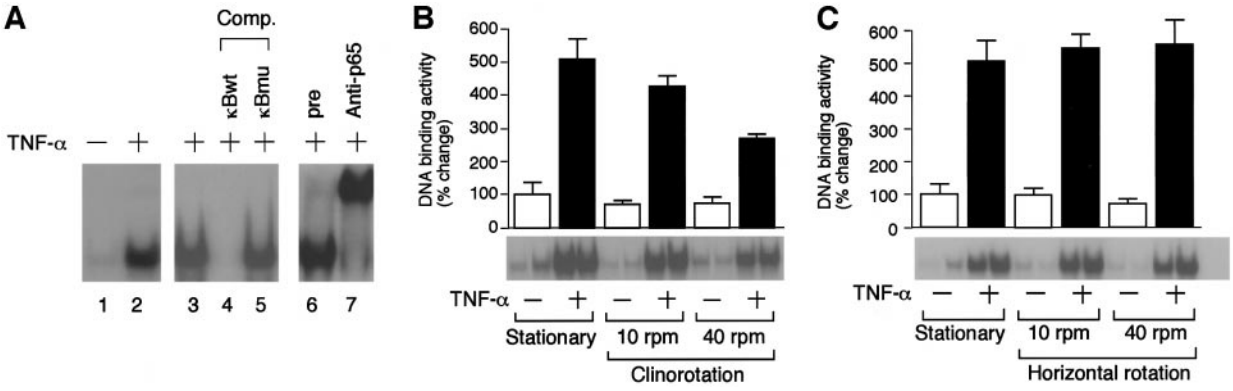

FIG. 1. Effects of clinorotation on TNF-α-dependent activation of NF-κB in HOS-TE85 cells. (A) Nuclear extracts prepared from HOS-TE85 cells treated with or without 50 U/ml TNF-α for 30 min in stationary culture were subjected to EMSA using a ^{32}P-labeled κBwt oligonucleotide as a probe. Displacement analysis was performed by using 50-fold molar excess of unlabeled κBwt (lane 4) and κBmu (lane 5) oligonucleotides as competitors. Subunits of NF-κB were characterized by supershift analysis employing antibody directed against p65 (lane 7) and preimmune rabbit serum (pre, lane 6). (B and C) The cells were cultured in (i) stationary conditions for 72 h, (ii) under clinorotation at 10 rpm and 40 rpm for 72 h, or (iii) under horizontal rotation at 10 rpm and 40 rpm for 72 h. They were then treated with or without TNF-α (50 U/ml). After a 30-min stationary incubation, the cells were harvested, and the nuclear extracts were subjected to EMSA using a κBwt oligonucleotide as a probe. The experiment was performed in duplicate. The radioactivity of the bands were measured by the BAS 2000 system. The data are presented as percentage of the levels of the untreated cells in stationary culture. The data are expressed as mean ± range. Similar results were obtained from two separate experiments.

ary cultures (Fig. 2B). However, after culturing cells for 72 h under clinorotation at 10 or 40 rpm, the magnitude of TNF-α-dependent increase in NF-κB binding activity was attenuated in a rotation speed-dependent manner. Approximately 50% reduction in the binding activity was measured in cells which underwent clinorotation at 40 rpm. In contrast, the extent of TNF-α-dependent increase in the binding activities in the cells after horizontal rotation (motional controls) at either 10 or 40 rpm were similar to that observed in the stationary control culture (Fig. 2C). These results demonstrate that clinorotation represses the TNF-α-dependent activation of NF-κB.

Clinorotation Decreases NF-κB-Dependent Transactivation

We next examined whether NF-κB-dependent gene expression is altered by clinorotation. We performed a transient transfection assay using a reporter plasmid containing a luciferase gene driven by multimerized NF-κB sites (pGL3pro-3κB). As shown Fig. 2A, the luciferase activity in the cells transfected with pGL3pro-3κB was markedly increased by TNF-α by more than threefold in stationary cultures. A similar induction was also observed in cultures subjected to horizontal rotation. In contrast, after 40-rpm clinoro-

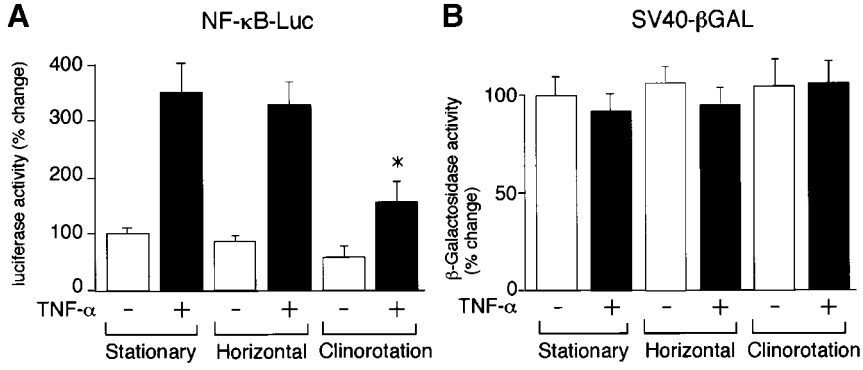

FIG. 2. Effects of clinorotation on TNF-α-dependent transactivation of NF-κB in HOS-TE85 cells. Cells were transiently transfected with pGL3pro-3κB containing a luciferase reporter gene driven by multimerized NF-κB sites, together with β-galactosidase-expressing plasmid (pSV-β-gal). After a 16-h transfection, the cells underwent the clinorotation (40 rpm), horizontal rotation (40 rpm) or stationary culture for 48 h, and then the cells were treated with or without TNF-α (50 U/ml) for 4 h in stationary culture. The luciferase and β-galactosidase activities in cell lysates were determined, and normalized by the protein contents of the lysates. The data are presented as percentage of the levels of the untreated cells in stationary culture, and expressed as mean ± SD (n = 3). Similar results were obtained from a separate experiment. *$P < 0.05$ vs horizontal rotation with TNF-α.

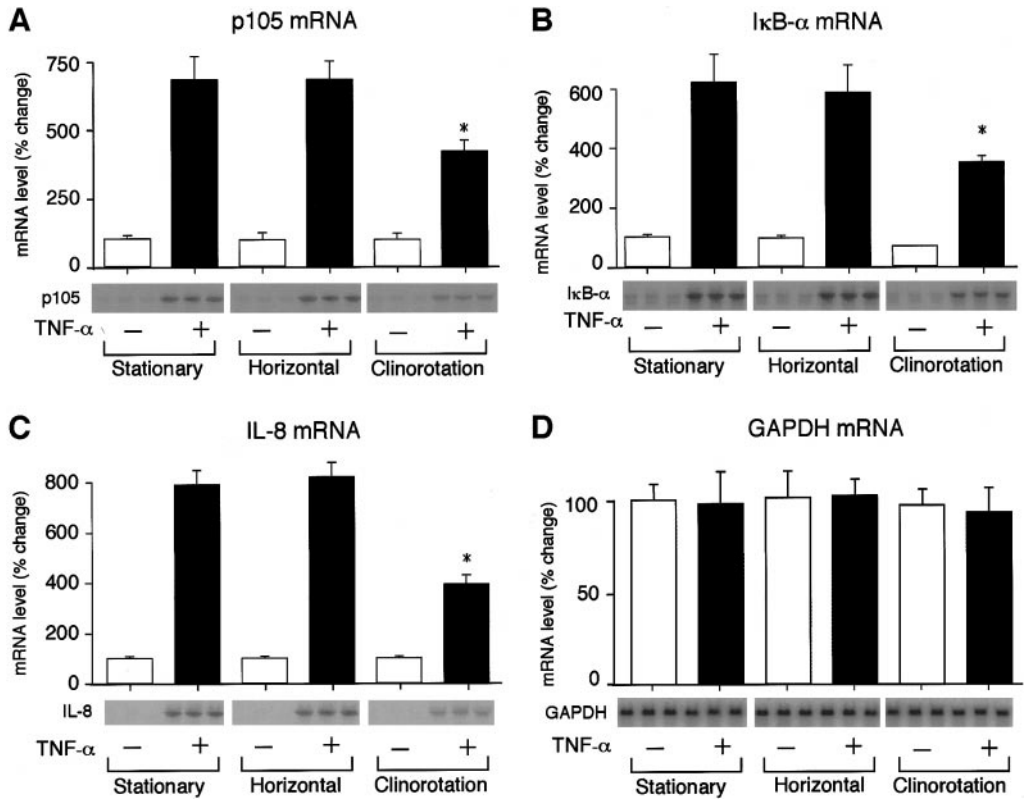

FIG. 3. Clinorotation suppresses the induction of endogenous NF-κB target genes by TNF-α. After 72-h clinorotation (40 rpm), horizontal rotation (40 rpm) or stationary culture, the cells were treated with or without TNF-α (50 U/ml) for 6 h. Total RNA was subjected to Northern blot analysis using p105 (A), IκB-α (B), IL-8 (C) and GAPDH (D) cDNAs as probes. Experiment was performed in triplicate. After the radioactivity of the bands were measured by the BAS 2000 system, the mRNA levels were normalized by the GAPDH mRNA levels, and then expressed as a percentage of the level of the stationary untreated cells. The data are expressed as mean ± SD ($n = 3$). *$P < 0.05$ vs horizontal rotation with TNF-α. Similar results were obtained from a separate experiment.

tation, a significant decrease in the induction was found in comparison to that of stationary or horizontal rotation controls. While the basal level under clinorotation was somewhat lower than stationary or horizontal controls, this difference was not statistically significant. These results demonstrate that the reduction in DNA-binding activities of NF-κB consequent to clinorotation leads to the reduced expression of the reporter gene. This effect appears to be specific, because the expression of β-galactosidase reporter gene (which is not driven by NF-κB sites) did not differ among the three culture conditions (Fig. 2B).

Effects of Clinorotation on Expression of Endogenous Target Genes for NF-κB

It has been reported that the IκB-α, p105 (a precursor of p50), and IL-8 genes are targets of NF-κB. Indeed, as shown in Fig. 3, Northern blot analysis revealed that TNF-α markedly increased these three mRNA levels in stationary cultures (Figs. 3A–3C). In the cells under horizontal rotation, the basal levels and the magnitude of their increase by TNF-α were similar to those observed in stationary cultures. However, in cultures subjected to clinorotation, the magnitude of the increase in these mRNAs was significantly decreased. In contrast, GAPDH mRNA levels were not affected by TNF-α after clinorotation (Fig. 3D).

Consistent with the change in the mRNA levels, concentrations of IL-8 in the medium were markedly increased by TNF-α in stationary cultures, and this increase was significantly attenuated in cells which underwent clinorotation (Fig. 4). These results clearly show that down-regulation of NF-κB also resulted in the decreased expression of its endogenous target genes.

Effects of Clinorotation on c-fos Expression, Degradation of IκB-α, and Cell Growth

We next examined the effects of clinorotation on PMA-dependent induction of c-fos gene. HOS-TE85 cells that underwent clinorotation at 40 rpm for 72 h were treated with 100 nM PMA for 1 h, and Northern blot analysis was performed. As shown in Fig. 5A, PMA induced a marked increase in c-fos mRNA levels in the cells under stationary and horizontal cultures. Similar induction was also observed in the cells under clinoro-

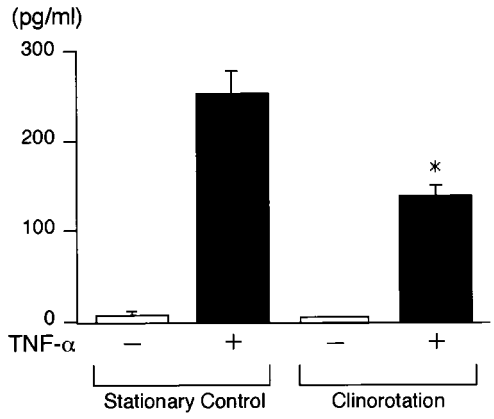

FIG. 4. Clinorotation represses TNF-α-dependent IL-8 secretion in HOS-TE85 cells. At the beginning of 72-h clinorotation (40 rpm), TNF-α (50 U/ml) was added to the culture medium. The concentrations of IL-8 in the media were determined by ELISA. The data are expressed as mean ± SD ($n = 4$). *$P < 0.05$ vs stationary control with TNF-α. Similar results were obtained from a separate experiment.

tation. These results again suggest that the effect of clinorotation is specific to TNF-α-NF-κB pathway.

To elucidate possible mechanisms involved in the reduced activation of NF-κB by TNF-α under clinostat conditions, we studied the expression of TNF receptor 1 (TNF-R1), TNF-R2, IκB-α and IκB-β. Western blot analysis showed that the cellular levels of these proteins were not changed by 72-h clinorotation (data not shown). Also, degradation of IκB-α in response to the treatment with TNF-α for 30 min after clinorotation was not altered when compared to those after stationary and horizontal cultures (Fig. 5B). Cell proliferation rates were assessed after 24-h and 72-h clinorotation by WST-1 assay. Percent changes in cell number were as follows: 24 h after stationary culture 100 ± 16.8% (mean ± SD), 24 h after clinorotation 95.69 ± 19.1; 72 h after stationary culture 100 ± 6.26%, 72 h after clinorotation 95.74 ± 11.9%. These results indicated that cell proliferation rates were not altered by clinorotation.

DISCUSSION

The present study demonstrates for the first time that cells subjected to vector-averaged gravity (simulated microgravity) conditions using clinostat rotation represses the TNF-α-dependent activation of NF-κB in human osteoblastic HOS-TE85 cells. Consistent with the impairment of NF-κB activation, the NF-κB-dependent transactivation of a luciferase reporter gene and the endogenous genes, IκB-α, p105, and IL-8, were also significantly suppressed by clinorotation, but not by horizontal rotation which serves as a motional control. The effect of clinorotation can be attributed to the vector-averaged gravity condition, because cells under horizontal rotation, which controls for turbulence, shear forces, and vibrations, responded to TNF-α to a similar extent found in stationary control cultures.

The clinostat simulates the microgravity of space by doing two things: (i) it produces a vector-averaged gravity environment. When the "g" vector is averaged, its value becomes theoretically zero, and (ii) the clinostat, working as a low level centrifuge, produces a small centrifugal force. This force (which could cancel out the vector-averaged environment, if it were large compared to "g" the gravity force) can be calculated using an estimation of the cell mass (in the $10^{-12} \times$ g range), the distance from the axis of rotation (less than 15 mm) and the speed of rotation (less than 100 rpm). The calculated value of the centrifugal force, under these conditions, is about 10^{-4} to $10^{-3}g$ (29–31). Thus, the expected "g" force value during clinorotation becomes less than $10^{-3}g$ (note that the "g" force value itself while still existing on earth has a vectorial value of zero due to the continuous averaging produced in the clinostat by the rotation of the cultured cells). This value is within the definition of the "microgravity" of space where the gravity vectorial force is reduced to about $10^{-6}g$ (32).

It should be noted that vector-averaged gravity did not affect the β-galactosidase activity in the transfection experiments. The β-galactosidase gene in pSV-β-gal plasmid is driven by simian virus 40 early promoter, which was shown to be activated by a ubiquitous transcription factor Sp-1 (33). It is thus considered that Sp-1 activity is not affected by vector-

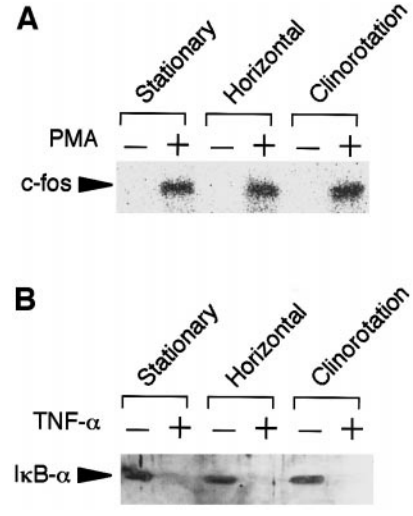

FIG. 5. Effects of clinorotation on c-fos expression and degradation of IκB-α. After 72-h clinorotation (40 rpm), horizontal rotation (40 rpm) or stationary culture, HOS-TE85 cells were treated with 100 nM PMA for 1 h (A) or 50 U/ml TNF-α for 30 min (B). Total RNA extracted from the PMA-treated cells was subjected to Northern blot analysis using human c-fos cDNA as a probe. Whole cell lysates from TNF-α-treated cells were subjected to Western blot analysis using anti-IκB-α antibody.

averaged gravity. Similarly, GAPDH mRNA levels were also unaffected by clinorotation. In addition, the expression of c-fos, another inducible transcription factor, was not affected by clinorotation. These results indicate that vector-averaged gravity exerts a specific effect on TNF-α-dependent activation of NF-κB.

Repression of TNF-α-dependent activation of NF-κB by clinorotation could be caused by reduction in TNF-R number or by an increase in IκB content. We thus examined the amounts of TNF-R1, TNF-R2, IκB-α and IκB-β, and the degradation of IκB-α in response to TNF-α after clinorotation. However, these parameters were not altered. Previous reports have demonstrated that actual microgravity (space flight) and its simulation via clinorotation are associated with alterations to the cytoskeleton of osteoblasts (17, 34). Such an alteration may provide a clue to the mechanism involved in the findings we report here. Thus, the altered cytoskeletal network may impair the translocation of NF-κB to the nucleus or the trafficking of NF-κB/IκB kinases in these cells.

In summary, we show here that the TNF-α-dependent induction of several endogenous NF-κB target genes was suppressed in osteoblastic cells cultured under vector-averaged gravity conditions. Considering the wide variety of target genes of NF-κB (8, 9), impairment of the NF-κB activation would affect various functions of osteoblasts such as cell growth and differentiation, as well as responses to hormones and cytokines, which in turn might disturb normal bone remodeling and lead to the development of bone atrophy which has been reported to take place in humans and animals after space flight (35–37).

ACKNOWLEDGMENTS

This study was funded as a part of the "Ground Research for Space Utilization", promoted by Japan Space Forum, and was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan and by Research Grants for Longevity Sciences from the Ministry of Health and Welfare of Japan.

REFERENCES

1. Nanes, M. S., McKoy, W. M., and Marx, S. J. (1989) Inhibitory effects of tumor necrosis factor-alpha and interferon-gamma on deoxyribonucleic acid and collagen synthesis by rat osteosarcoma cells (ROS 17/2.8). *Endocrinology* **124,** 339–345.
2. Nanes, M. S., Rubin, J., Titus, L., Hendy, G. N., and Catherwood, B. (1991) Tumor necrosis factor-alpha inhibits 1,25-dihydroxyvitamin D3-stimulated bone Gla protein synthesis in rat osteosarcoma cells (ROS 17/2.8) by a pretranslational mechanism. *Endocrinology* **128,** 2577–2582.
3. Panagakos, F. S., and Kumar, S. (1994) Modulation of proteases and their inhibitors in immortal human osteoblast-like cells by tumor necrosis factor-alpha in vitro. *Inflammation* **18,** 243–265.
4. Carmeliet, G., Nys, G., and Bouillon, R. (1997) Microgravity reduces the differentiation of human osteoblastic MG-63 cells. *J. Bone Miner. Res.* **12,** 786–794.
5. Kunisada, T., Kawai, A., Inoue, H., and Namba, M. (1997) Effects of simulated microgravity on human osteoblast-like cells in culture. *Acta Med. Okayama.* **51,** 135–140.
6. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S. Jr. (1991) Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell* **65,** 1281–1289.
7. Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995) I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell* **80,** 573–582.
8. Baeuerle, P. A., and Henkel, T. (1994) Function and activation of NF-kappa B in the immune system. *Ann. Rev. Immunol.* **12,** 141–179.
9. Baldwin, A. J. (1996) The NF-kappa B and I kappa B proteins: New discoveries and insights. *Ann. Rev. Immunol.* **14,** 649–683.
10. Kurokouchi, K., Kambe, F., Yasukawa, K., Izumi, R., Ishiguro, N., Iwata, H., and Seo, H. (1998) TNF-alpha increases expression of IL-6 and ICAM-1 genes through activation of NF-kappaB in osteoblast-like ROS17/2.8 cells. *J. Bone Miner. Res.* **13,** 1290–1299.
11. Cogoli, A., and Cogoli, G. M. (1997) Activation and proliferation of lymphocytes and other mammalian cells in microgravity. *Adv. Space Biol. Med.* **6,** 33–79.
12. Duke, P. J., and Montufar-Solis, D. (1998) Rotating systems used as microgravity simulators for studies of cartilage differentiation. *J. J. Aerospace Env. Med.* **35,** 41–54.
13. Koga, K. (1998) Day dreaming about human colonization of space should not substitute for basic research on the role of gravity on earth. *J. J. Aerospace Env. Med.* **35,** 23–26.
14. Gruener, R. (1998) Neuronal responses to vector-averaged gravity: A search for gravisensing and adaptation mechanisms—a preliminary report. *J. J. Aerospace Env. Med.* **35,** 63–83.
15. McAllister, R. M., Gardner, M. B., Greene, A. E., Bradt, C., Nichols, W. W., and Landing, B. H. (1971) Cultivation in vitro of cells derived from a human osteosarcoma. *Cancer* **27,** 397–402.
16. Purohit, A., Flanagan, A. M., and Reed, M. J. (1992) Estrogen synthesis by osteoblast cell lines. *Endocrinology* **131,** 2027–2029.
17. Sarkar, D., Nagaya, T., Koga, K., Nomura, Y., Gruener, R., and Seo, H. (2000) Culture in vector-averaged gravity under clinostat rotation results in apoptosis of osteoblastic ROS 17/2.8 cells. *J. Bone Miner. Res.* **15,** 489–498.
18. Kikumori, T., Kambe, F., Nagaya, T., Imai, T., Funahashi, H., and Seo, H. (1998) Activation of transcriptionally active nuclear factor-kappaB by tumor necrosis factor-alpha and its inhibition by antioxidants in rat thyroid FRTL-5 cells. *Endocrinology* **139,** 1715–1722.
19. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
20. Kambe, F., Nomura, Y., Okamoto, T., and Seo, H. (1996) Redox regulation of thyroid-transcription factors, Pax-8 and TTF-1, is involved in their increased DNA-binding activities by thyrotropin in rat thyroid FRTL-5 cells. *Mol. Endocrinol.* **10,** 801–812.
21. Fujita, T., Nolan, G. P., Ghosh, S., and Baltimore, D. (1992) Independent modes of transcriptional activation by the p50 and p65 subunits of NF-kappa B. *Genes Dev.* **6,** 775–787.
22. Edbrooke, M. R., Foldi, J., Cheshire, J. K., Li, F., Faulkes, D. J., and Woo, P. (1991) Constitutive and NF-kappa B-like proteins in the regulation of the serum amyloid A gene by interleukin 1. *Cytokine* **3,** 380–388.
23. Sarkar, D., Kambe, F., Hayashi, Y., Ohmori, S., Funahashi, H., and Seo, H. (2000) Involvement of AP-1 and steroidogenic factor (SF)-1 in the cAMP-dependent induction of human adrenocorti-

cotropic hormone receptor (ACTHR) promotor. *Endocrine J.* **47,** 63–75.

24. Kambe, F., Seo, H., Murata, Y., and Matsui, N. (1988) Cloning of a complementary deoxyribonucleic acid coding for human thyroxine-binding globulin (TBG): Existence of two TBG messenger ribonucleic acid species possessing different 3′-untranslated regions. *Mol. Endocrinol.* **2,** 181–185.
25. Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israel, A. (1990) The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. *Cell* **62,** 1007–1018.
26. Sato, M., Miyazaki, T., Nagaya, T., Murata, Y., Ida, N., Maeda, K., and Seo, H. (1996) Antioxidants inhibit tumor necrosis factor-alpha mediated stimulation of interleukin-8, monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. *J. Rheumatol.* **23,** 432–438.
27. Miyamoto, N., Seo, H., Kanda, K., Hidaka, H., and Matsui, N. (1992) A 3′,5′-cyclic adenosine monophosphate-dependent pathway is responsible for a rapid increase in c-fos messenger ribonucleic acid by adrenocorticotropin. *Endocrinology* **130,** 3231–3236.
28. Asai, T., Kambe, F., Kikumori, T., and Seo, H. (1997) Increase in Ref-1 mRNA and protein by thyrotropin in rat thyroid FRTL-5 cells. *Biochem. Biophys. Res. Commun.* **236,** 71–74.
29. Todd, P. (1992) Physical effects on the cellular level under altered gravity conditions. *Adv. Space Res.* **12,** 43–49.
30. Briegleb, W. (1992) Some qualitative and quantitative aspects of the fast-rotating clinostat as a research tool. *ASGSB Bulletin* **5,** 23–30.
31. Brown, A. (1992) Centrifuges: Evolution of their uses in plant gravitational biology and new directions for research on the ground and in spaceflight. *ASGSB Bulletin* **5,** 43–57.
32. Albrecht-Buehler, G. (1992) The simulation of microgravity conditions on the ground. *ASGSB Bulletin* **5,** 3–10.
33. Dynan, W. S., and Tjian, R. (1983) The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* **35,** 79–87.
34. Hughes, F. M., and Lewis, M. L. (1996) Effects of microgravity on osteoblast growth activation. *Exp. Cell Res.* **224,** 103–109.
35. Morey, E. R., and Baylink, D. J. (1978) Inhibition of bone formation during space flight. *Science* **201,** 1138–1141.
36. Tavassoli, M. (1986) Medical problems of space flight. *Am. J. Med.* **81,** 850–854.
37. Smith, S. M., Nillen, J. L., Leblanc, A., Lipton, A., Demers, L. M., Lane, H. W., and Leach, C. S. (1998) Collagen cross-link excretion during space flight and bed rest. *J. Clin. Endocrinol. Metab.* **83,** 3584–3591.