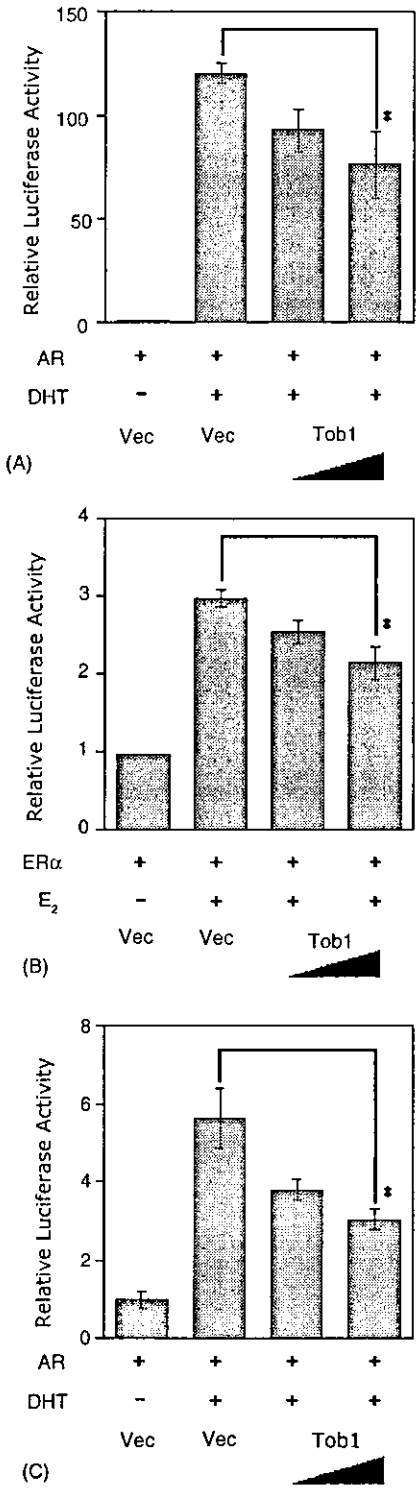
4. Discussion

In the present study, we demonstrated that expression of Tob protein in MC3T3-C1 osteoblastic cells suppressed the ligand-dependent transactivation function of steroid hormone



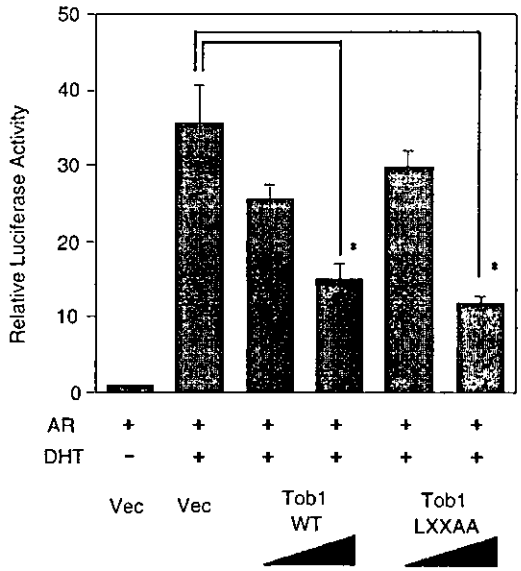
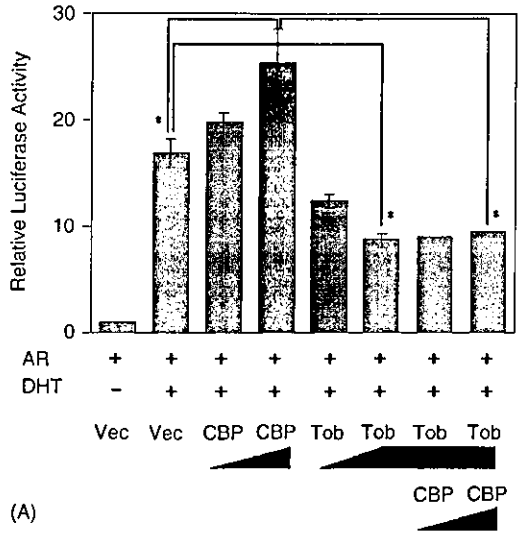


Fig. 4. Suppression of AR-mediated transcriptional activation by a Tob1 LXXAA mutant. The wild type (WT) and mutated (LXXAA) Tob1, carrying amino acid substitutions in its LXXLL motif (LXXLL to LXXAA), were expressed in MC3T3-E1 cells with the AR expression vector and reporter plasmids. The luciferase assay was performed as described in Section 2. The average of three independent experiments is shown with the standard deviation. $^{*}P<0.05$.

receptors, AR and ERα, and inhibited the ligand-dependent nuclear foci formation of AR.

Some of the Tob family proteins have an LXXLL motif that has been identified in many nuclear receptor coregulators. Through this motif, these coregulators can directly interact with nuclear receptors to modulate the transcriptional activation (Leo and Chen, 2000; Aranda and Pascual, 2001; Cheng et al., 2002; Heinlein and Chang, 2002). It has been reported that two Tob family proteins, BTG1 and BTG2, can either enhance or repress ligand-induced transactivation function of ERα, depending on the promoter context. BTG proteins do not directly interact with ERα, but BTG1 can

Fig. 3. Tob protein-induced suppression of AR- or ERα-mediated transactivation of MMTV (A), ERE2-tk109 (B) and PSA (C) promoter in PC3 (A and B) and COS-7 (C) cells. (A) Prostatic carcinoma cells, PC3, were cotransfected with AR expression vector (0.1 μg/well), 0.5 μg of pGL3-MMTV, 3 ng of pRL-CMV and 0.2 or 0.5 μg of the pYFP-Tob1 or the empty vector (Vec). Three hours after transfection, the ligand was added. Luciferase activity was measured after 24 h incubation. (B) PC3 cells were cotransfected with 0.1 μg/well of pSG5-ERα, 0.5 μg/well pERE2-tk109-luc, 3 ng of pRL-CMV and 0.2 or 0.5 μg of the pYFP-Tob1 or the empty vector (Vec). After the transfection, cells were treated with or without 10^{-6} M E_2 for 24 h and then luciferase assay was performed. (C) COS-7 cells were cotransfected with pCMV-hAR (0.1 μg/well), 0.3 μg of pGL3-PSA, 3 ng of pRL-CMV and 0.2 or 0.5 μg of the pYFP-Tob1 or the empty vector. After the transfection, cells were incubated in the absence or presence of 10^{-8} M DHT for 24 h and then luciferase assay was performed. Relative luciferase activity is shown and bars represent the fold change in the luciferase activity relative to the value without the ligand. The mean values and the standard deviation from three independent experiments are shown. $^{*}P<0.05$.



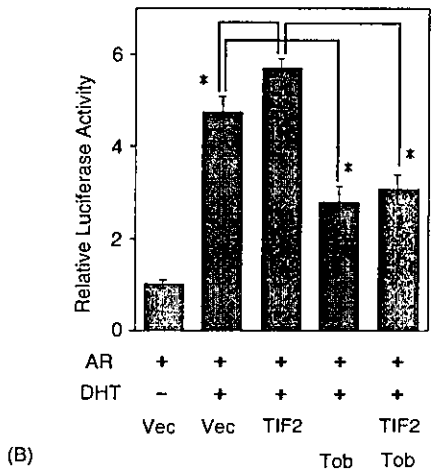


Fig. 5. Effects of coactivator expression on the Tob-mediated repression of the AR-mediated transactivation. (A) pcDNA/mCBP (0.1 or 0.3 μg/well) was cotransfected with pCMV-hAR (0.1 μg/well), pGL3-MMTV and pRL-CMV with or without the Tob expression vector (0.2 or 0.5 μg/well) into MC3T3-E1 cells. The cells were incubated in the absence or presence of DHT for 24 h, and then the luciferase activity was measured. (B) MC3T3-E1 cells were cotransfected with pCMV-hAR (0.1 μg/well), pGL3-MMTV, pRL-CMV, pYFP-Tob1 (0.5 μg/well) and pYFP-TIF2 (0.5 μg/well). After 24 h incubation with DHT, luciferase assay was performed. Bars show the fold change in the luciferase activity relative to the value by the wild type AR without DHT. $^{*}P < 0.05$.

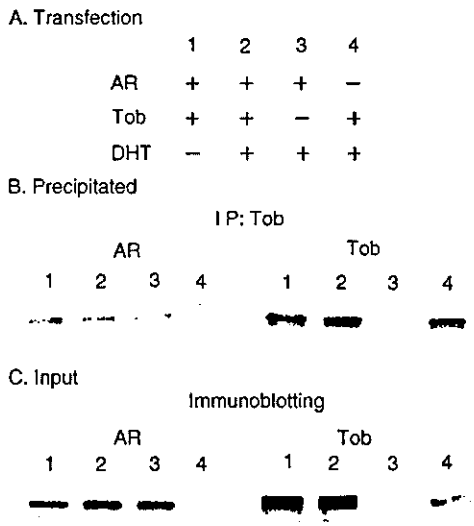


Fig. 6. Coimmunoprecipitation of AR with Tob1 protein. COS-7 cells were transfected with 2 μg of pCMV-hAR and pYFP-Tob1 and then incubated with or without 10^{-8} M DHT for 24 h. The whole cell extracts were subjected to an immunoprecipitation using anti-Tob antibody. Immunoprecipitated fractions were analyzed by immunoblotting as described in Section 2. (A) Transfected (+) and non-transfected (−) expression plasmids, and the presence (+) or absence (−) of ligand in each incubation (lanes 1, 2, 3 or 4) are indicated. (B) AR and Tob in the precipitated fractions were detected by immunoblotting. (C) Input levels of AR and Tob expressed by the transfection (before precipitation) were evaluated by immunoblotting.

regulate the ERα-mediated transcription through the interaction with CAF1, whose yeast homologue is a component of the CCR4-NOT transcription complex. The mutations of LXXLL motifs in BTG1 abolished both the effect of BTG1 on ERα-mediated transactivation and the interaction of BTG1 with CAF1 (Prevot et al., 2001). Tob1 and Tob2 proteins have also been shown to be associated with the CAF1 protein (Ikematsu et al., 1999), therefore, there is a possibility that the modulation of sex steroid hormone receptor-dependent transcription by Tob proteins would be also mediated by the CAF1 protein through an LXXLL motif. However, introduction of mutations into an LXXLL motif of the Tob protein failed to abolish the transcriptional repression. Furthermore PC3/BTG2, which carries two LXXLL motifs, failed to repress AR-mediated transcriptional activation in osteoblastic cells. In the amino acid sequence of the Tob1 protein, we also identified an LXXII motif, which was found in nuclear receptor binding domains of corepressors (Hu and Lazar, 1999; Privalsky, 2004). This LXXII motif was also mutated but repression by the mutant Tob was almost equal to that by the wild type protein. These results indicate that, in osteoblastic cells, the LXXLL and LXXII motifs in the Tob protein are not essential for suppression of nuclear receptor-mediated transactivation, nevertheless, the Tob-induced repression of the

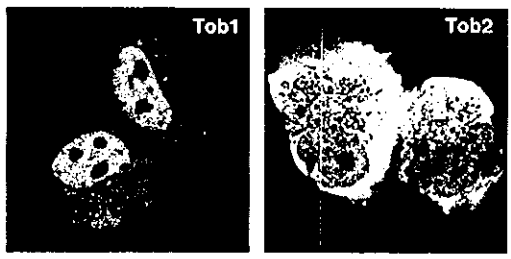


Fig. 7. Localization of wild type Tob1 and Tob2 proteins. YFP-Tob1 or YFP-Tob2 was expressed in MC3T3-C1 osteoblastic cells. Twenty-four hours after the transfection, fluorescent signals in the cells were observed by confocal laser scanning microscopy.

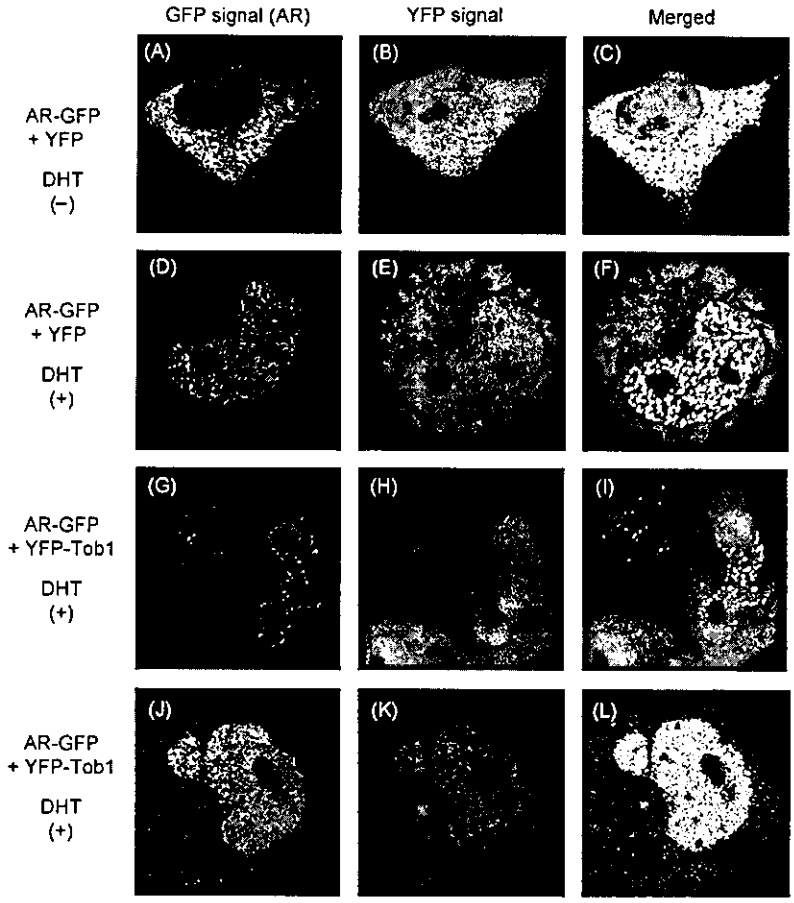


Fig. 8. Subnuclear foci formation of ligand-bound AR and inhibition of the foci formation by expression of the Tob1 protein. The YFP vector (YFP), carrying only a YFP cDNA, and pAR-GFP (AR-GFP) were cotransfected into MC3T3-E1 cells (A–F). Before adding DHT, fluorescent signals from AR-GFP (A) and YFP (B) were collected and the two signals were merged (C). Signals from AR-GFP (D) and YFP (E) in the DHT-treated cells were also collected and the two images were merged (F). MC3T3-E1 cells were cotransfected with pAR-GFP and pYFP-Tob1 (G–L). Fluorescent signals from AR-GFP (G and J) and YFP-Tob1 (H and K) were collected in the presence of DHT, and the two signals were merged (I–L). The experimental conditions were identical between G–I and J–K.

transactivation function of AR was suggested to be mediated by the direct interaction between Tob and AR as demonstrated in the present study by coimmunoprecipitation.

As we previously reported, ligand-dependent intranuclear foci formation of AR closely depends on the receptor being in a transcriptionally active conformation (Tomura et al., 2001; Saitoh et al., 2002). CBP, one of the coactivators for AR, is colocalized with AR at subnuclear foci after treatment with the ligand, and CBP is considered to be essential for the formation of nuclear foci of AR (Saitoh et al., 2002). It was shown that activation of AP-1 or NF-κB repressed AR-mediated transactivation. Extraction of endogenous CBP from the AR-mediated transactivation complex by these transcriptional factors is thought to be a cause of the repression because this repression is relieved by supplementation with exogenous CBP (Frønsdal et al., 1998; Aarnisalo et al., 1998). The dominant negative form of CBP also suppressed AR-mediated transactivation and destroyed AR subnuclear foci, however, cotransfection of a wild type CBP expression vector rescued AR foci formation (Saitoh et al., 2002). In the present paper, we demonstrated that expression of Tob protein inhibited the subnuclear foci formation of AR, being consistent with the results of the luciferase reporter assay. To examine whether CBP is involved in the Tob-induced repression of the transactivation function of AR, CBP was coexpressed with AR and Tob in osteoblastic cells. However, the CBP expression had no effect on the Tob-mediated repression (Fig. 5A). Similarly, the Tob-induced repression of transactivation was not recovered by supplementation of another common coactivator, TIF2 (Fig. 5B). CBP also failed to recover the AR foci formation destroyed by the expression of Tob (data not shown). According to these results, Tob-mediated repression of the AR transactivation function is not due to the sequestration of coactivators from AR transactivation complex. In the present experiments, Tob inhibited the subnuclear foci formation of AR. According to the previous studies including ours, these subnuclear foci are now considered to be the sites where nuclear receptors interact with

coactivators to form (pre)transactivation complexes before binding to DNA (Stenoien et al., 2000; Saitoh et al., 2002). Therefore, Tob seems to inhibit formation of the transcriptionally active complex of AR that should be an earlier step than AR-DNA binding. However, further study will be necessary to elucidate the precise mechanism of the Tob-induced repression of AR-mediated transactivation.

Tob1 knockout mice showed increased bone volume resulting from an increased number of osteoblasts, not due to reduced activity of osteoclasts (Yoshida et al., 2000). Sex steroid hormones are known to promote osteoblast proliferation and differentiation (Hofbauer and Khosla, 1999; Manolagas et al., 2002). Based on our findings that Tob protein represses AR and ERα-dependent transactivation in osteoblastic cells, Tob deficiency would result in upregulation of the transactivation functions of AR and ERα in bone formation. Such elevated function of steroid hormone receptors may also partially contribute to the enhancement of bone volume in Tob-deficient mice.

In conclusion, for the first time, to our knowledge, the present study revealed the repression effects of Tob proteins on sex steroid hormone receptor-mediated transactivation function, thus, Tob proteins would be one of the intracellular modulators for extracellular signals such as BMP-2 and sex steroids.

Acknowledgements

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References

Aarnisalo, P., Palvimo, J.J., Jänne, O.A., 1998. CREB-binding protein in androgen receptor-mediated signaling. Proc. Natl. Acad. Sci. U.S.A. 95, 2122–2127.

Adachi, M., Takayanagi, R., Tomura, A., Imasaki, K., Kato, S., Goto, K., Yanase, T., Ikuyama, S., Nawata, H., 2000. Androgen-insensitivity syndrome as a possible coactivator disease. N. Engl. J. Med. 343, 856–862.

Aranda, A., Pascual, A., 2001. Nuclear hormone receptors and gene expression. Physiol. Rev. 81, 1269–1304.

Cheng, S., Brzostek, S., Lee, S.R., Hollenberg, A.N., Balk, S.P., 2002. Inhibition of the dihydrotestosterone-activated androgen receptor by nuclear receptor corepressor. Mol. Endocrinol. 16, 1492–1501.

Frønsdal, K., Engedal, N., Slagsvold, T., Saatcioglu, F., 1998. CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J. Biol. Chem. 273, 31853–31859.

Heinlein, C.A., Chang, C., 2002. Androgen receptor (AR) coregulators: an overview. Endocr. Rev. 23, 175–200.

Hofbauer, L.C., Khosla, S., 1999. Androgen effects on bone metabolism: recent progress and controversies. Eur. J. Endocrinol. 140, 271–286.

Hu, X., Lazar, M.A., 1999. The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402, 93–96.

Ikematsu, N., Yoshida, Y., Kawamura-Tsuzuku, J., Ohsugi, M., Onda, M., Hirai, M., Fujimoto, J., Yamamoto, T., 1999. Tob2, a novel anti-proliferative Tob/BTG1 family member, associates with a component of the CCR4 transcriptional regulatory complex capable of binding cyclin-dependent kinases. Oncogene 18, 7432–7441.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K., Rosenfeld, M.G., 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85, 403–414.

Kawano, H., Sato, T., Yamada, T., Matsumoto, T., Sekine, K., Watanabe, T., Nakamura, T., Fukuda, T., Yoshimura, K., Yoshizawa, T., Aihara, K.I., Yamamoto, Y., Nakamichi, Y., Metzger, D., Chambon, P., Nakamura, K., Kawaguchi, H., Kato, S., 2003. Suppressive function of androgen receptor in bone resorption. Proc. Natl. Acad. Sci. U.S.A. 100, 9416–9421.

Leo, C., Chen, J.D., 2000. The SRC family of nuclear receptor coactivators. Gene 245, 1–11.

Maekawa, M., Nishida, E., Tanoue, T., 2002. Identification of the anti-proliferative protein Tob as a MAPK substrate. J. Biol. Chem. 277, 37783–37787.

Manolagas, S.C., Kousteni, S., Jilka, R.L., 2002. Sex steroids and bone. Recent Prog. Horm. Res. 57, 385–409.

Matsuda, S., Kawamura-Tsuzuku, J., Ohsugi, M., Yoshida, M., Emi, M., Nakamura, Y., Onda, M., Yoshida, Y., Nishiyama, A., Yamamoto, T., 1996. Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity. Oncogene 12, 705–713.

Matsuda, S., Rouault, J., Magaud, J., Berthet, C., 2001. In search of a function for the TIS21/PC3/BTG1/Tob family. FEBS Lett. 497, 67–72.

Miyagishi, M., Fujii, R., Hatta, M., Yoshida, E., Araya, N., Nagafuchi, A., Ishihara, S., Nakajima, T., Fukamizu, A., 2000. Regulation of Lef-mediated transcription and p53-dependent pathway by associating beta-catenin with CBP/p300. J. Biol. Chem. 275, 35170–35175.

Nakao, R., Haji, M., Yanase, T., Ogo, A., Takayanagi, R., Katsube, T., Fukumaki, Y., Nawata, H., 1992. A single amino acid substitution (Met786—Val) in the steroid-binding domain of human androgen receptor leads to complete androgen insensitivity syndrome. J. Clin. Endocrinol. Metab. 74, 1152–1157.

Ohnaka, K., Shimoda, S., Nawata, H., Shimokawa, H., Kaibuchi, K., Iwamoto, Y., Takayanagi, R., 2001. Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. Biochem. Biophys. Res. Commun. 287, 337–342.

Onoe, Y., Miyaura, C., Ito, M., Ohta, H., Nozawa, S., Suda, T., 2000. Comparative effects of estrogen and raloxifene on B lymphopoiesis and bone loss induced by sex steroid deficiency in mice. J. Bone Miner. Res. 15, 541–549.

Prevot, D., Morel, A.P., Voeltzel, T., Rostan, M.C., Rimokh, R., Magaud, J.P., Corbo, L., 2001. Relationships of the anti-proliferative proteins BTG1 and BTG2 with CAF1, the human homolog of a component of the yeast CCR4 transcriptional complex: involvement in estrogen receptor alpha signaling pathway. J. Biol. Chem. 276, 9640–9648.

Privalsky, M.L., 2004. The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu. Rev. Physiol. 66, 315–360.

Rouault, J.P., Prevot, D., Berthet, C., Birot, A.M., Billaud, M., Magaud, J.P., Corbo, L., 1998. Interaction of BTG1 and p53-regulated BTG2 gene products with mCaf1, the murine homolog of a component of the yeast CCR4 transcriptional regulatory complex. J. Biol. Chem. 273, 22563–22569.

Saitoh, M., Takayanagi, R., Goto, K., Fukamizu, A., Tomura, A., Yanase, T., Nawata, H., 2002. The presence of both the amino- and carboxyl-terminal domains in the AR is essential for the completion of a transcriptionally active form with coactivators and intranuclear compartmentalization common to the steroid hormone receptors: a three-dimensional imaging study. Mol. Endocrinol. 16, 694–706.

Stenoien, D.L., Mancini, M.G., Patel, K., Allegretto, E.A., Smith, C.L., Mancini, M.A., 2000. Subnuclear trafficking of estrogen receptor-alpha and steroid receptor coactivator-1. Mol. Endocrinol. 14, 518–534.

Suzuki, T., Kawamura-Tsuzuku, J., Ajima, R., Nakamura, T., Yoshida, Y., Yamamoto, T., 2002. Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. Genes Dev. 16, 1356–1370.

Tirone, F., 2001. The gene PC3 (TIS21/BTG2), prototype member of the PC3/BTG/Tob family: regulator in control of cell growth, differentiation, and DNA repair? J. Cell. Physiol. 187, 155–165.

Tomura, A., Goto, K., Morinaga, H., Nomura, M., Okabe, T., Yanase, T., Takayanagi, R., Nawata, H., 2001. The subnuclear three-dimensional image analysis of androgen receptor fused to green fluorescence protein. J. Biol. Chem. 276, 28395–28401.

Yeh, S., Tsai, M.Y., Xu, Q., Mu, X.M., Lardy, H., Huang, K.E., Lin, H., Yeh, S.D., Altuwaijri, S., Zhou, X., Xing, L., Boyce, B.F., Hung, M.C., Zhang, S., Gan, L., Chang, C., Hung, M.C., 2002. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc. Natl. Acad. Sci. U.S.A. 99, 13498–13503.

Yoshida, Y., Tanaka, S., Umemori, H., Minowa, O., Usui, M., Ikematsu, N., Hosoda, E., Imamura, T., Kuno, J., Yamashita, T., Miyazono, K., Noda, M., Noda, T., Yamamoto, T., 2000. Negative regulation of BMP/Smad signaling by Tob in osteoblasts. Cell 103, 1085–1097.

Yoshida, Y., Nakamura, T., Komoda, M., Satoh, H., Suzuki, T., Tsuzuku, J.K., Miyasaka, T., Yoshida, E.H., Umemori, H., Kunisaki, R.K., Tani, K., Ishii, S., Mori, S., Suganuma, M., Noda, T., Yamamoto, T., 2003. Mice lacking a transcriptional corepressor Tob are predisposed to cancer. Genes Dev. 17, 1201–1206.

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Glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts

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Abstract

To explore the mechanism of glucocorticoid-induced osteoporosis, we investigated the effect of glucocorticoid on canonical Wnt signaling that emerged as a novel key pathway for promoting bone formation. Wnt3a increased the T-cell factor (Tcf)/lymphoid enhancer factor (Lef)-dependent transcriptional activity in primary cultured human osteoblasts. Dexamethasone suppressed this transcriptional activity in a dose-dependent manner, while 1,25-dihydroxyvitamin D3 increased this transcriptional activity. LiCl, an inhibitor of glycogen synthase kinase-3β, also enhanced the Tcf/Lef-dependent transcriptional activity, which was, however, not inhibited by dexamethasone. The addition of anti-dickkopf-1 antibody partially restored the transcriptional activity suppressed by dexamethasone. Dexamethasone decreased the cytosolic amount of β-catenin accumulated by Wnt3a and also inhibited the nuclear translocation of β-catenin induced by Wnt3a. These data suggest that glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts, partially through the enhancement of the dickkopf-1 production.

Keywords: Glucocorticoid; Wnt; Dickkopf-1; Osteoblast; Osteoporosis

Osteoporosis is one of the most frequent and serious side effects of long-term glucocorticoid therapy [1]. Glucocorticoids have profound effects on bone metabolism [2]. Glucocorticoid excess increases bone resorption and decreases bone formation; consequently rapid bone loss occurs. Nowadays, a direct inhibition of osteoblast activity by glucocorticoids is the most favored principal mechanism of glucocorticoid-induced osteoporosis [1–3]. However, detailed mechanism by which they inhibit osteoblast function remains to be fully elucidated.

Recent progress uncovers the importance of Wnt signaling in skeletal biology [4,5]. The loss-of-function mutations in human LDL receptor-related protein 5 (LRP5) gene cause osteoporosis-pseudoglioma syndrome (OPPG) characterized by low bone mass and abnormal eye development, while the gain-of-function mutations in this gene give rise to high bone mass syndrome [6–8]. Furthermore, the LRP5 gene knockout mice show the phenotype of low bone mass resembling that of human OPPG [9]. These findings highlighted Wnt signaling as another key pathway involved in the regulation of postnatal bone mass.

The Wnt signal transduction comprises three intracellular pathways: the canonical pathway, the Wnt/planar-cell-polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway [10,11]. Although which pathway is involved in bone formation remains to be fully elucidated, recent studies strongly suggest that the canonical pathway plays a central role in promoting bone formation [4,5,12]. Canonical Wnts bind to frizzled/LRP5 receptor complex, inactivate glycogen synthase kinase-3β

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(GSK-3β), and inhibit phosphorylation and consequential degradation of intracellular β-catenin [10,11]. Accumulated β-catenin translocates into the nucleus and activates target genes by a complex formed with transcription factors of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family [10,11].

Wnt signals are extracellularly regulated by several secreted antagonists including secreted frizzled-related protein (sFRP), Cerberus, Wnt inhibitory factor-1 (WIF-1), and dickkopf (Dkk) [13]. We have reported that glucocorticoid enhances the expression of dickkopf-1 (Dkk-1) in cultured human osteoblasts [14]. We extended our exploration of the effect of glucocorticoid on Wnt signaling, and found that glucocorticoid suppresses the canonical Wnt signal, in part mediated by the enhancement of the Dkk-1 production, in cultured human osteoblasts.

Materials and methods

Materials. Eagle's α-MEM, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was purchased from Sanko Junyaku (Tokyo, Japan). Dexamethasone, 17β-estradiol, dihydrotestosterone, 1,25-dihydroxyvitamin D3, LiCl, and goat immunoglobulin (IgG) were purchased from Sigma (St. Louis, MI). Anti-β-catenin monoclonal antibody and anti-Dkk-1 goat polyclonal antibody were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All other reagents were of analytical grade.

Cell culture. Human osteoblasts were prepared from the bone fragments of femur neck as described previously [15]. The cells were grown in Eagle's α-MEM with 10% FCS, 100 mU/ml penicillin, and 100 mU/ml streptomycin. The Wnt3a-expressing cell line (L Wnt-3A cell) and the control cell line (L cell) were obtained from American Type Culture Collection (Manassas, VA). Wnt3a-conditioned medium (Wnt3a-CM) and the control-conditioned medium (C-CM) were harvested according to the manufacturer's instructions. Wnt3a-CM was used in experiments at 10% final concentration, which gave the maximal effect on the Tcf/Lef-dependent transcriptional activity in preliminary studies (data not shown).

Plasmid constructs. The entire coding region of human β-catenin was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using KOD-plus DNA polymerase (Toyobo, Tokyo, Japan), confirmed by DNA sequencing, and subcloned into *ScaI*/*Bam*HI sites of pEGFP-C3 (Clontech, Palo Alto, CA) expression vector (designated as pEGFP-β-catenin). TOPflash, a Tcf-binding site reporter plasmid, was purchased from Upstate Biotechnology (Lake Placid, NY).

Transient transfection and reporter assay. Human osteoblasts were transiently transfected by means of calcium phosphate precipitation as described previously [14]. Reporter assay was performed by a dual luciferase assay kit (Promega, MI) according to the manufacturer's instructions.

Subcellular fractionation and immunoblot analysis. Subcellular fractionation and immunoblot analysis were performed essentially as described previously [15]. Soluble (cytosolic) proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and proteins in the gel were transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ) through electroblotting. For detection of β-catenin, blots were probed with an anti-β-catenin monoclonal antibody at a dilution of 1:1000. The protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL).

Confocal laser microscopic imaging. Human osteoblasts were cultured in 35-mm glass-bottomed dishes (Asahi Techno Glass, Tokyo, Japan) and transfected with pEGFP-β-catenin plasmid vector. The cells were maintained in α-MEM supplemented with 10% charcoal-treated FCS for 24 h and observed with a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany) as described previously [16].

Statistical analysis. Data are expressed as means ± SD. Statistical analyses were performed with ANOVA followed by Fisher's protected least significant difference test. Significance was accepted at $P < 0.05$.

Results

To investigate the effect of glucocorticoid on canonical Wnt signaling, we first examined whether glucocorticoid would affect the Tcf/Lef-dependent transcriptional activity by a Tcf-reporter gene (luciferase) assay in cultured osteoblasts (Fig. 1A). In primary cultured human osteoblasts, the addition of Wnt3a-conditioned medium (Wnt3a-CM) enhanced the Tcf/Lef-dependent transcriptional activity (approximately 3.5-fold). Dexamethasone suppressed the Wnt3a-induced Tcf/Lef-dependent transcriptional activity in a dose-dependent manner, and dexamethasone at 10^{-7} M suppressed the Wnt3a-stimulated transcriptional activity to the unstimulated basal level.

We also examined the effect of other steroid hormones on the Tcf/Lef-dependent transcriptional activity (Fig. 1B). The addition of 17β-estradiol or dihydrotestosterone did not affect the transcriptional activity stimulated by Wnt3a. The addition of 1,25-dihydroxyvitamin D3 (10^{-7} M) enhanced the Wnt3a-induced reporter activity in cultured human osteoblasts. Furthermore, 1,25-dihydroxyvitamin D3 partially restored the suppressed Wnt3a-induced transcriptional activity by dexamethasone.

Wnt proteins enhance Tcf/Lef-dependent transcription by the canonical signal cascade, namely, the inhibition of GSK-3β, its consequential accumulation of cytosolic β-catenin, translocation of the accumulated β-catenin into the nucleus, and its activation of Tcf/Lef. Therefore, we examined whether or not dexamethasone would affect the intracellular β-catenin level in cultured human osteoblasts. As shown in Fig. 2, the addition of Wnt3a-CM increased the amount of cytosolic β-catenin protein in human osteoblasts. Co-treatment with dexamethasone (10^{-9}–10^{-7} M) dose-dependently decreased the cytosolic level of β-catenin and dexamethasone at 10^{-7} M almost completely reduced to the unstimulated basal level, which is parallel to the suppressive effect of dexamethasone on the reporter luciferase activity. When pEGFP-β-catenin was transfected into human osteoblasts, the GFP-β-catenin was localized most abundantly in the cytosol (Fig. 3). The addition of Wnt3a-CM translocated the GFP-β-catenin into the nucleus, however, co-treatment with

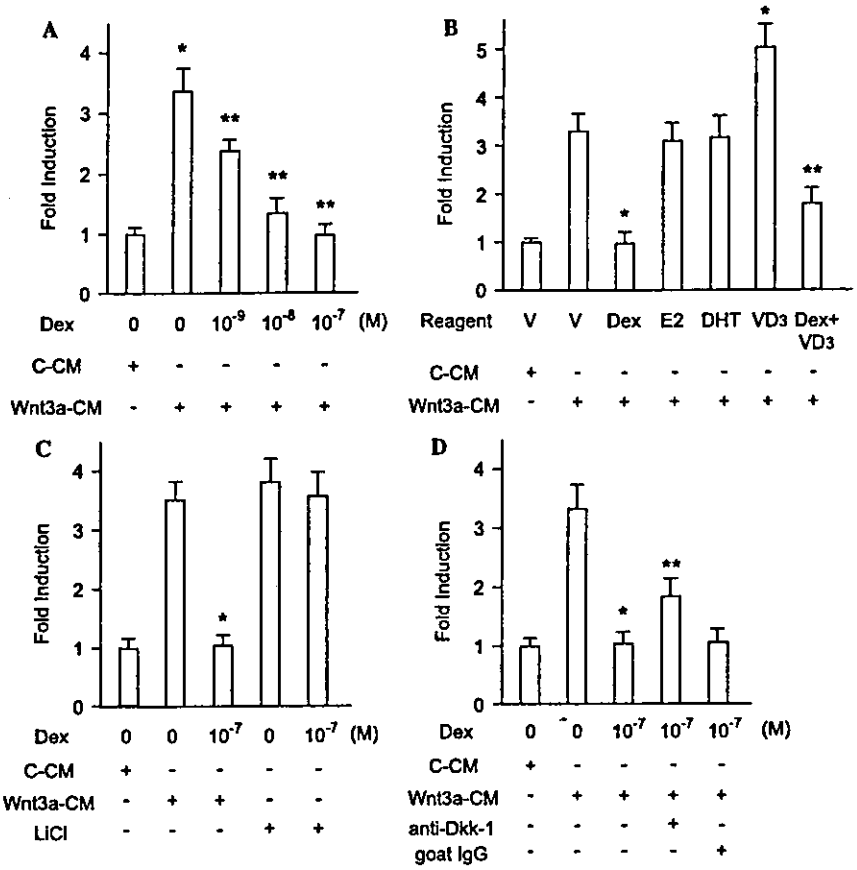

Fig. 1. Effects of Wnt3a, steroid hormones, LiCl, and anti-Dkk-1 antibody on the Tcf/Lef-dependent transcriptional activity in primary cultured human osteoblasts. Human osteoblasts were transfected with TOPflash plasmid vector, and incubated for 36 h with the control-conditioned medium (C-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of vehicle (ethanol) or various reagents indicated. The reporter luciferase activity was expressed as fold over the activity of TOPflash with C-CM and vehicle. Data are expressed as means ± SD ($n = 4$). One representative data of three independent experiments are shown. (A) 10^{-9}–10^{-7} M dexamethasone (Dex). $^{*}P < 0.01$ vs. C-CM with vehicle. $^{**}P < 0.01$ vs. Wnt3a-CM with vehicle. (B) Vehicle (V, ethanol), 10^{-7} M dexamethasone (Dex), 10^{-7} M 17β-estradiol (E2), 10^{-7} M dihydrotestosterone (DHT), or 10^{-7} M 1,25-dihydroxyvitamin D3 (VD3). $^{*}P < 0.01$ vs. Wnt3a-CM with vehicle. $^{**}P < 0.05$ vs. Wnt3a-CM with Dex. (C) Twenty-five millimolar of LiCl in the presence of vehicle (ethanol) or 10^{-7} M dexamethasone (Dex). $^{*}P < 0.01$ vs. Wnt3a-CM with vehicle. (D) 10^{-7} M dexamethasone (Dex) in combination with anti-Dkk-1 goat polyclonal antibody (anti-Dkk-1) or non-immune goat IgG (goat IgG). $^{*}P < 0.01$ vs. Wnt3a-CM with vehicle. $^{**}P < 0.05$ vs. Wnt3a-CM with Dex.

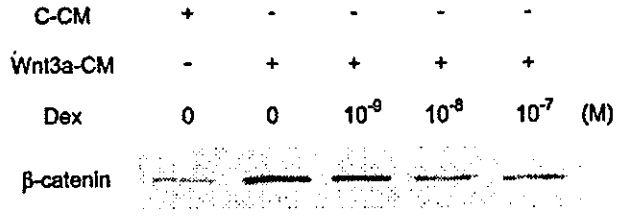

Fig. 2. Effect of dexamethasone on the level of the cytosolic β-catenin protein in primary cultured human osteoblasts. Human osteoblasts were incubated with the control-conditioned medium (control-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of vehicle (ethanol) or 10^{-9}–10^{-7} M dexamethasone (Dex) for 24 h, and fractionated to soluble and particulate fractions. Soluble proteins (20 μg) were loaded in each lane and subjected to SDS–PAGE (7.5% separating gel). Immunoblot analyses were performed using a specific anti-β-catenin monoclonal antibody. Results shown are representative of three independent experiments.

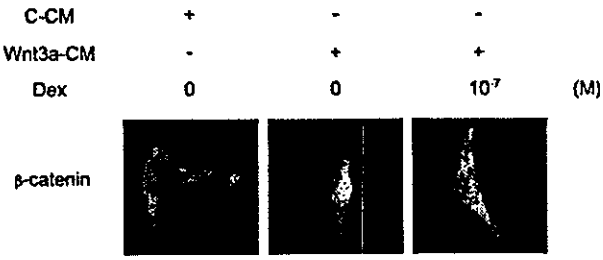

Fig. 3. Effect of dexamethasone on the localization of β-catenin in primary cultured human osteoblasts. Human osteoblasts were transfected with the pEGFP-β-catenin expression vector, and incubated for 24 h with the control-conditioned medium (control-CM) or Wnt3a-conditioned medium (Wnt3a-CM) in the presence of vehicle (ethanol) or 10^{-7} M dexamethasone (Dex). The cells were analyzed by laser confocal microscopy. A representative imaging of three independent experiments is shown (magnification, 100×).

dexamethasone at 10^{-7} M completely inhibited the nuclear translocation of cytosolic β-catenin (Fig. 3). These data suggest that dexamethasone suppressed the Tcf/Lef-dependent transcription through the canonical Wnt signaling cascade rather than by affecting via other signaling pathway(s) or inhibiting directly the Tcf/Lef expression in human osteoblasts.

To further assess the effect of dexamethasone on canonical Wnt signaling, we observed the effect of dexamethasone on the Tcf reporter activity in the presence of LiCl, an inhibitor of GSK-3β, in human osteoblasts (Fig. 1C). The addition of 25 mM LiCl increased the Tcf/Lef-dependent transcriptional activity comparable to that by Wnt3a-CM. However, this increase by LiCl was not suppressed by the treatment with dexamethasone at 10^{-7} M. Since glucocorticoid enhances the expression of Dkk-1, an antagonist of Wnt signaling in human osteoblast [14], we examined the involvement of Dkk-1 in suppressive effect of dexamethasone on canonical Wnt signaling. The addition of anti-Dkk-1-specific goat polyclonal antibody in part (35–45%) restored the suppression of Wnt3a-induced Tcf/Lef-dependent transcriptional activity by dexamethasone, while non-specific goat IgG had no effect on it (Fig. 1D).

Discussion

In the present study, we demonstrated that dexamethasone suppressed the Tcf/Lef-dependent canonical Wnt signaling pathway in primary cultured human osteoblasts. This effect was in part attributed to the increase of Dkk-1 expression by dexamethasone.

Glucocorticoid suppresses osteoblastic differentiation and proliferation by affecting multiple aspects of osteoblast function [1–3]. One well-known effect of glucocorticoid on osteoblast is the inhibition of the expression for Runx2/Cbfa1 [17], a crucial transcriptional factor for differentiation of osteoblast lineage [18]. Runx2/Cbfa1 promotes early osteoblast differentiation from undifferentiated mesenchymal cells, but rather inhibits late osteoblast maturation [18,19]. On the other hand, the Wnt signal plays an essential role in postnatal bone accrual in a Runx2/Cbfa1-independent manner [9]. Our data suggest that glucocorticoid at a therapeutic pharmacological dose may almost completely suppress the canonical Wnt signaling pathway promoting postnatal bone formation in human osteoblasts.

Dexamethasone did not affect the enhancement of Tcf/Lef-dependent transcriptional activity by LiCl, a GSK-3β inhibitor. Therefore, it is presumed that the dexamethasone affects canonical Wnt signaling through GSK-3β itself or upstream of GSK-3β. Indeed, it was reported that glucocorticoid activates GSK-3β and inhibits cell cycle progression in murine preosteoblastic MC3T3-E1 cells [20]. We have previously shown that glucocorticoid enhances the expression of Dkk-1, a secreted antagonist of Wnt signaling, in cultured human osteoblasts [14]. Treatment with anti-Dkk-1-specific antibody partially (approximately 40%) restored the suppression by dexamethasone of Wnt3a-induced Tcf/Lef-dependent transcriptional activity. Although there are several secreted antagonists of Wnt signaling [13] and we did not examine the involvement of other antagonists than Dkk-1 proteins, our data suggest that the inhibition of the canonical Wnt signal by glucocorticoid may be in part through the antagonistic effect of the enhanced Dkk-1 production in cultured human osteoblasts.

Interestingly, 1,25-dihydroxyvitamin D3 enhanced the Tcf/Lef-dependent transcriptional activity induced by Wnt3a in cultured human osteoblasts. Furthermore, 1,25-dihydroxyvitamin D3 restored the suppressed Wnt3a-induced transcriptional activity by dexamethasone. Previous study reported that the vitamin D receptor with its ligand inhibits β-catenin-Tcf/Lef-dependent gene transcription in colon carcinoma cells [21]. The vitamin D effect on Wnt signaling may be different by the types of cell and target genes, which will require further investigations. Active vitamin D metabolites are used for prevention and treatment of glucocorticoid-induced osteoporosis [22,23]. Besides the known effects of vitamin D3 on bone and mineral metabolism [23], the effect on Wnt signaling may contribute to the clinical effect of vitamin D3 for the treatment of osteoporosis.

LRP5 knockout mice show low bone mass due to decreased osteoblast proliferation [9], but the precise mechanism whereby the Wnt/LRP5 signal promotes bone formation remains to be fully clarified. However, given the significance of the canonical Wnt signaling pathway in postnatal control of bone formation, strong inhibition of this pathway by glucocorticoid may in part explain the impairment of osteoblastic function and bone formation induced by glucocorticoid excess. Our findings in this study and further investigations will provide clues to new strategies for the treatment of glucocorticoid-induced osteoporosis.

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References

[1] K.G. Saag, Glucocorticoid-induced osteoporosis, Endocrinol. Metab. Clin. North Am. 32 (2003) 135–157.

[2] E. Canalis, Clinical review 83: Mechanisms of glucocorticoid action in bone: Implications to glucocorticoid-induced osteoporosis, J. Clin. Endocrinol. Metab. 81 (1996) 3441–3447.
[3] S.C. Manolagas, R.S. Weinstein, New developments in the pathogenesis and treatment of steroid-induced osteoporosis, J. Bone Miner. Res. 14 (1999) 1061–1066.
[4] M.L. Johnson, K. Harnish, R. Nusse, W. Van Hul, LRP5 and Wnt signaling: A union made for bone, J. Bone Miner. Res. 19 (2004) 1749–1757.
[5] J.J. Westendorf, R.A. Kahler, T.M. Schroeder, Wnt signaling in osteoblasts and bone diseases, Gene 341 (2004) 19–39.
[6] Y. Gong, R.B. Slee, N. Fukai, G. Rawadi, S. Roman-Roman, A.M. Reginato, H. Wang, T. Cundy, F.H. Glorieux, D. Lev, M. Zacharin, K. Oexle, J. Marcelino, W. Suwairi, S. Heeger, G. Sabatakos, S. Apte, W.N. Adkins, J. Allgrove, M. Arslan-Kirchner, J.A. Batch, P. Beighton, G.C. Black, R.G. Boles, L.M. Boon, C. Borrone, H.G. Brunner, G.F. Carle, B. Dallapiccola, A. De Paepe, B. Floege, M.L. Halfhide, B. Hall, R.C. Hennekam, T. Hirose, A. Jans, H. Juppner, C.A. Kim, K. Keppler-Noreuil, A. Kohlschuetter, D. LaCombe, M. Lambert, E. Lemyre, T. Letteboer, L. Peltonen, R.S. Ramesar, M. Romanengo, H. Somer, E. Steichen-Gersdorf, B. Steinmann, B. Sullivan, A. Superti-Furga, W. Swoboda, M.J. van den Boogaard, W. Van Hul, M. Vikkula, M. Votruba, B. Zabel, T. Garcia, R. Baron, B.R. Olsen, M.L. Warman, LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development, Cell 107 (2001) 513–523.
[7] L.M. Boyden, J. Mao, J. Belsky, L. Mitzner, A. Farhi, M.A. Mitnick, D. Wu, K. Insogna, R.P. Lifton, High bone density due to a mutation in LDL-receptor-related protein 5, N. Engl. J. Med. 346 (2002) 1513–1521.
[8] R.D. Little, J.P. Carulli, R.G. Del Mastro, J. Dupuis, M. Osborne, C. Folz, S.P. Manning, P.M. Swain, S.C. Zhao, B. Eustace, M.M. Lappe, L. Spitzer, S. Zweier, K. Braunschweiger, Y. Benchekroun, X. Hu, R. Adair, L. Chee, M.G. FitzGerald, C. Tulig, A. Caruso, N. Tzellas, A. Bawa, B. Franklin, S. McGuire, X. Nogues, G. Gong, K.M. Allen, A. Anisowicz, A.J. Morales, P.T. Lomedico, S.M. Recker, P. Van Eerdewegh, R.R. Recker, M.L. Johnson, A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait, Am. J. Hum. Genet. 70 (2002) 11–19.
[9] M. Kato, M.S. Patel, R. Levasseur, I. Lobov, B.H. Chang, D.A. Glass, C. Hartmann, L. Li, T.H. Hwang, C.F. Brayton, R.A. Lang, G. Karsenty, L. Chan, Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor, J. Cell Biol. 157 (2002) 303–314.
[10] A. Wodarz, R. Nusse, Mechanisms of Wnt signaling in development, Annu. Rev. Cell Dev. Biol. 14 (1998) 59–88.
[11] A. Bejsovec, Wnt signaling: An embarrassment of receptors, Curr. Biol. 10 (2000) R919–R922.
[12] H. Hu, M.J. Hilton, X. Tu, K. Yu, D.M. Ornitz, F. Long, Sequential roles of Hedgehog and Wnt signaling in osteoblast development, Development 132 (2005) 49–60.
[13] Y. Kawano, R. Kypta, Secreted antagonists of the Wnt signalling pathway, J. Cell Sci. 116 (2003) 2627–2634.
[14] K. Ohnaka, H. Taniguchi, H. Kawate, H. Nawata, R. Takayanagi, Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: Novel mechanism of glucocorticoid-induced osteoporosis, Biochem. Biophys. Res. Commun. 318 (2004) 259–264.
[15] K. Ohnaka, S. Shimoda, H. Nawata, H. Shimokawa, K. Kaibuchi, Y. Iwamoto, R. Takayanagi, Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts, Biochem. Biophys. Res. Commun. 287 (2001) 337–342.
[16] H. Kawate, Y. Wu, K. Ohnaka, H. Nawata, R. Takayanagi, Tob proteins suppress steroid hormone receptor-mediated transcriptional activation, Mol. Cell. Endocrinol. 230 (2005) 77–86.
[17] D.J. Chang, C. Ji, K.K. Kim, S. Casinghino, T.L. McCarthy, M. Centrella, Reduction in transforming growth factor β receptor I expression and transcription factor CBFa1 on bone cells by glucocorticoid, J. Biol. Chem. 273 (1998) 4892–4896.
[18] T. Komori, H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R.T. Bronson, Y.H. Gao, M. Inada, M. Sato, R. Okamoto, Y. Kitamura, S. Yoshiki, T. Kishimoto, Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts, Cell 89 (1997) 755–764.
[19] W. Liu, S. Toyosawa, T. Furuichi, N. Kanatani, C. Yoshida, Y. Liu, M. Himeno, S. Narai, A. Yamaguchi, T. Komori, Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures, J. Cell Biol. 155 (2001) 157–166.
[20] E. Smith, G.A. Coetzee, B. Frenkel, Glucocorticoids inhibit cell cycle progression in differentiating osteoblasts via glycogen synthase kinase-3 β, J. Biol. Chem. 277 (2002) 18191–18197.
[21] H.G. Palmer, J.M. Gonzalez-Sancho, J. Espada, M.T. Berciano, I. Puig, J. Baulida, M. Quintanilla, A. Cano, A.G. de Herreros, M. Lafarga, A. Munoz, Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling, J. Cell Biol. 154 (2001) 369–387.
[22] J.Y. Reginster, C. de Froidmont, M.P. Lecart, N. Sarlet, J.O. Defraigne, Alphacalcidol in prevention of glucocorticoid-induced osteoporosis, Calcif. Tissue Int. 65 (1999) 328–331.
[23] R.N. de Nijs, J.W. Jacobs, A. Algra, W.F. Lems, J.W. Bijlsma, Prevention and treatment of glucocorticoid-induced osteoporosis with active vitamin D3 analogues: A review with meta-analysis of randomized controlled trials including organ transplantation studies, Osteoporos. Int. 15 (2004) 589–602.

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SPECIAL REPORT

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The Subcommittee to Study Diagnostic Criteria for
Glucocorticoid-Induced Osteoporosis**

Guidelines on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research (2004)

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Key words Steroid (glucocorticoid) · Osteoporosis · Guideline

Introduction

Osteoporosis is the most frequent adverse effect of glucocorticoids. Management guidelines were developed [1] in the United States in 1996 when the seriousness of glucocorticoid-induced osteoporosis as a complication of glucocorticoid therapy was recognized, and they have since been revised [2–6]. In Japan, the Japanese Society for Bone and Mineral Research established a study group on osteoporosis diagnostic criteria in 1999 and then the Subcommittee to Study Diagnostic Criteria for Glucocorticoid-Induced Osteoporosis in 2001 to examine diagnostic criteria for glucocorticoid-induced osteoporosis, and the present guidelines were developed for clinical practice (Fig. 1).

Guidelines on the management and treatment of glucocorticoid-induced osteoporosis

Drafting policy

The Subcommittee to Study Diagnostic Criteria for Glucocorticoid-Induced Osteoporosis was initially organized to determine diagnostic criteria. However, guidelines on glucocorticoid-induced osteoporosis in various countries

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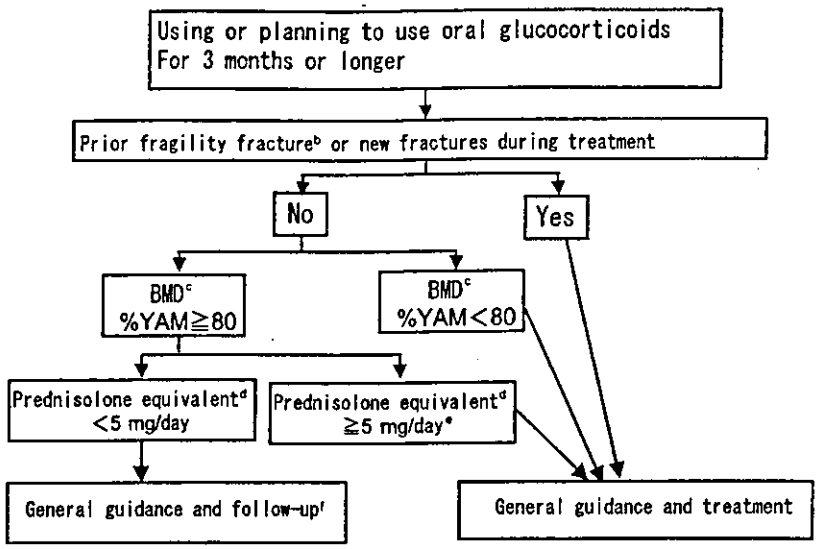
Fig. 1. Guidelines on the management and treatment of corticosteroid-induced osteoporosis (2004 edition)*



- General guidance
  Lifestyle guidance, nutritional guidance, and exercise therapy are based on those for primary osteoporosis
- Follow-up observation
  Bone mineral density measurements and thoracic and lumbar vertebra X-rays are performed on a regular basis (every 6 months or 1 year)
- Drug treatment
  1. Bisphosphonates are first-line drugs
  2. Active vitamin D_3 and vitamin K_2 are second-line drugs

YAM. young adult mean (20–44 years old); BMD, bone mineral density
*These Guidelines cover patients 18 years of age and older
[b]Definition of fragility fractures is the same as that for primary osteoporosis
[c]Bone mineral density (BMD) measurements are based on those for primary osteoporosis (2000 revised edition)
[d]Mean daily dose
*Patients administered 10 mg or more per day are at risk of fractures even when bone mineral density is high (cut-off value, %YAM90)
[f]Risk of fractures is higher in the elderly

are guidelines for primary and secondary prevention and do not specify diagnostic criteria. The subcommittee discussed this point, and a draft of management and therapeutic guidelines in Japan based on evidence available at present was prepared.

Patients studied by the Subcommittee to Study Diagnostic Criteria for Glucocorticoid-Induced Osteoporosis

The survey items were sex, age, height, body weight, underlying disease, bone mineral density (type of instrument used, region, measured value), glucocorticoid treatment history (dose, mean dose for 6 months until measurement of bone mineral density, maximum dose throughout administration period, total administration period, total dose, pulse therapy within 1 year, use of glucocorticoids for inhalation), osteoporosis treatment history, and fracture history. The facilities connected with the Subcommittee, that is, Osaka City University, Kawasaki Medicine University, Kyushu University, Kyushu University Hospital at Beppu, Kinki University, Sapporo Yamanoue Hospital, Research Institute and Practice for Involutional Diseases, Tokyo Metropolitan Tama Geriatric Hospital, Tokyo Metropolitan Geriatric Medical Center, Hamamatsu University School of Medicine, Fujita Health University, and Radiation Effects Research Foundation, were requested to conduct the survey. As a result, a total of 692 patients were recruited up to 2002 in addition to the 299 patients recruited in 1999 and 2000, including 627 women and 65 men. The most common underlying disease was rheumatoid arthritis (RA) in 319 patients, and 373 patients had diseases other than RA; these included 162 cases of systemic lupus erythematosus (SLE), 27 of progressive systemic sclerosis (PSS), 26 with mixed connective tissue disease (MCTD), 20 with polymyositis/dermatomyositis (PM/DM), 16 with polymyalgia rheumatica (PMR), 12 with nephrosis, 10 with asthma, 10 with idiopathic thrombocytopenic purpura

(ITP), and 90 with other diseases. The results of an analysis of a 2-year follow-up survey on 220 patients administered glucocorticoids by Tanaka and Oshima [7] were added to these analytical results, and work on establishment of the guidelines proceeded.

Evidence for drafting the guidelines

Subjects

These guidelines cover men and women 18 years of age or older. Growth disorders caused by glucocorticoids are a serious problem in children, but at present no evidence that can be used has been reported in Japan or overseas, and children were excluded. Because of the same lack of evidence concerning glucocorticoids injected intravenously, only patients using oral glucocorticoids for which evidence is available in Japan and overseas were subjects. There is no evidence concerning the administration period in Japan. The most recent guidelines of the United States, UK, and Canada cover treatment with administration for 3 months or longer [3–6]. In a meta-analysis of the risk of bone fractures after starting treatment with oral glucocorticoids overseas, it was reported that the incidence of new vertebral bone fractures reaches a maximum at 3–6 months after administration and forms a plateau thereafter [8], suggesting that treatment simultaneously with or in the very early stage of glucocorticoid administration is important. Therefore, the subjects were patients with planned administration for 3 months or longer (see Fig. 1).

Prior fragility fractures

The results of an analysis in a 2-year longitudinal study by Tanaka and Oshima showed that the risk of new bone fractures in patients with prior fragility fractures showed the highest value compared with other fractures at an odds ratio of 7.92 [7]. Among the patients collected by the Subcommittee to Study Diagnostic Criteria, the 154 cases (103 cases of RA, 51 cases of collagen disease) that could be analyzed longitudinally for 2 years had a high odds ratio of 5.22. Therefore, the first evaluation criterion for starting treatment was patients with prior fragility fractures and patients with new bone factures during treatment. The definition of a fragility fracture is the same as that for primary osteoporosis [9].

Bone mineral density

Table 1 shows the cut-off values of bone mineral density (BMD), which can efficiently separate fracture and nonfracture cases, estimated from the receiver-operating characteristic (ROC) curve based on an analysis of cases collected by the Subcommittee to Study Diagnostic Criteria. The cut-off value for all patients was 0.776 g/cm^2. When the patients were divided into those with RA, the most common underlying disease, and those with diseases other than RA, the cut-off values were 0.744 g/cm^2 and 0.820 g/cm^2, respectively. In cases with SLE, the most common underlying disease other than RA, the cut-off value was 0.841 g/cm^2. Judging from these results, it appeared necessary to set different cut-off values for RA and for other underlying diseases. Table 2 shows the results of an investigation of this point with respect to age. In Table 2, all patients were grouped by age from those less than 40 to those 70 years of age and older. The cut-off values of bone mineral density in fracture and nonfracture cases were obtained by age and expressed as percent (%) young adult mean (YAM). When the level of differences in cut-off values (%YAM) was examined for age differences of 10 years, the values were 6.5% for patients in their forties and in their fifties and 7.1% for patients in their fifties and in their sixties. The mean age of RA patients was 60.4 years and that for diseases other than RA 48.8 years, an age difference of 11.6 years. The cut-off values were 73.6% for RA and 81.1% for diseases other than RA, a difference of 7.5%; i.e., this difference was almost the same as that for age, and the difference in cut-off values of RA and diseases other than RA is not considered as a difference caused by differences in disease but a difference due to age.

Table 1. Cut-off values of bone mineral density (BMD) to efficiently separate fracture and nonfracture cases

		BMD (g/cm^2)	T score	% YAM
Primary osteoporosis		0.708	−2.60	70%
Osteopenia		0.809	−1.70	80%
Glucocorticoid-treated patients	All patients	0.776	−1.97	76.8%
	RA	0.744	−2.24	73.6%
	Non-RA	0.820	−1.60	81.1%
	SLE	0.841	−1.43	83.2%

YAM, young adult mean; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

Table 2. Cut-off values of bone mineral density by age and underlying disease of patients administered glucocorticoid

Age (all patients)	%YAM		Mean age (years)	Underlying disease
Less than 40 years old	86.9			
Forties	85.9			
	6.5			
Fifties	79.4	81.1	48.8	Non-RA
	7.1	7.5	11.6	
Sixties	72.3			
		73.6	60.4	RA
Seventies and older	69.3			

Table 3. Cut-off values of bone mineral density by dose of glucocorticoids

	Daily dose (prednisolone equivalent) (mg)	%YAM (T score)
All patients	≧5	77.7 (−1.90)
	≧7.5	80.3 (−1.67)
	≧10	82.1 (−1.52)
RA	≧7.5	75.1 (−2.12)
Non-RA	≧5	81.8 (−1.55)
	≧7.5	82.6 (−1.48)

Table 3 shows the relationship between glucocorticoid dose and the cut-off values. In all patients, the cut-off value (%YAM) in the group with a prednisolone equivalent dose of 5 mg/day or higher was 77.7%, that in the group with a dose of 7.5 mg/day or higher was 80.3%, and that in the group with a dose of 10 mg/day or higher was 82.1%. These results showed that as the daily dose increased, factures occurred at higher bone mineral densities. For RA, the cut-off value was 75.1% in the group with a prednisolone equivalent dose of 7.5 mg/day or higher, and for diseases other than RA, it was 81.8% in the group with a dose of 5 mg/day or higher and 82.6% in the group with a dose of 7.5 mg/day or higher. The results of a cross-sectional analysis of all patients collected by the Subcommittee to Study Diagnostic Criteria showed a cut-off value of %YAM 77%, and the cut-off value in the group with a prednisolone equivalent dose of 5 mg/day or higher, at which it was reported based on a meta-analysis that the bone mineral density rapidly decreased and bone fractures increased, was 78%. It was clear that it is not necessary to consider differences in cut-off values due to differences in underlying diseases. In the longitudinal analysis by Tanaka and Oshima, the cut-off value in the group with a prednisolone equivalent dose of 5 mg/day or higher was %YAM 80% [7]. Based on these results, bone mineral density of less than %YAM 80% was taken as the second evaluation criterion for starting treatment.

Dose of glucocorticoids

The number of patients collected by the Subcommittee was not enough to clarify the relationship between the fracture risk and the total glucocorticoid dose, and the analysis could not be performed. Almost no difference was found in the relationship between the fracture risk and the administration period, but this was because of insufficient data on individual patients, and the relationship will have to be clarified in the future. Sufficient evidence on the relationship between the glucocorticoid dose and fracture risk rate or its cut-off value of bone mineral density has still not been obtained in Japan, and overseas reports had to be used for reference. The results of an overseas meta-analysis showed a reverse correlation between the bone mineral density of the lumbar vertebra and the total dose of glucocorticoid (daily dose × period), and the risk of a spinal fracture even at a daily dose of less than 2.5 mg of prednisolone equivalent was more than 1.0, i.e., 1.55. The fracture rate increased dose dependently and was 5.18 at 7.5 mg or higher doses [8]. It has been reported that a dose of 5 mg or higher is the threshold value for increased fracture risk. Therefore, the third evaluation criterion for the start of treatment was proposed as a dose of 5 mg/day or higher (mean daily dose) as prednisolone equivalent (see Fig. 1). However, in a longitudinal study, the cut-off value of bone mineral density was %YAM 90% in the group administered a 10 mg/day or higher dose of prednisolone equivalent, and even at a %YAM close to 100%, the risk rate of fractures was clearly higher in the patients given glucocorticoid than in patients not administered glucocorticoids [7].

Old age

In the study by Tanaka and Oshima [7], the incidence of bone fractures increased significantly with increase in age, and age was identified as a risk factor of new spinal fractures in patients administered glucocorticoids; however, a cut-off value of age to clearly separate fracture and nonfracture cases could not be determined.

Treatment of glucocorticoid-induced osteoporosis

General guidance. In the same way as with primary osteoporosis, it is necessary to provide guidance on improvements in lifestyle and on nutrition, as well as exercise therapy. This guidance is based on that given for primary osteoporosis [10].

Follow-up observation. The risk of bone fractures is higher in patients administered glucocorticoid than in those who were not treated with glucocorticoid. Therefore, in patients evaluated as the follow-up observation group based on the present guidelines, it is essential to conduct follow-up observation by measuring bone mineral density and taking X-rays of the thoracic and lumbar vertebra on a regular basis.

Drug treatment. In prospective randomized control trials (RCT) overseas [11–15] and in Japan [16,17], evidence that the bisphosphonate products etidronate, alendronate, and risedronate significantly prevent bone fractures caused by glucocorticoid-induced osteoporosis has been reported. Therefore, these drugs have been recommended as first-line drugs at present. Active vitamin D_3 has been reported to have fracture-preventing effects, although these are inferior to those of the bisphosphonates [18], and vitamin K_2 has also been found to have fracture-preventing effects from a longitudinal study in Japan [7]. These two vitamins have been recommended as second-line drugs. Although the parameter was bone mineral density, it was reported based on a meta-analysis that vitamin D and bisphosphonates administered concomitantly are more effective than bisphosphonates alone in the treatment of glucocorticoid-induced osteoporosis [19]. Concomitant administration of active vitamin D_3 and bisphosphonates should be considered in patients with serious or high-risk osteoporosis. When bisphosphonates are difficult to administer to postmeno-

pausal women because of problems such as side effects, selection of raloxifene, a selective estrogen receptor modulator (SERM) [4,20,21] may be considered; however, therapeutic evidence of SERM for glucocorticoid-induced osteoporosis is still insufficient and further study will be necessary.

Conclusion

The 2004 edition of the guidelines on the management and treatment of glucocorticoid-induced osteoporosis has been developed based on the results of a longitudinal study by subcommittee members and the results of an analysis of patients collected by the Subcommittee to Study Diagnostic Criteria for Corticosteroid-Induced Osteoporosis, together with evidence obtained overseas and in Japan at present. It will be necessary to verify and revise the present guidelines based on newly collected evidence in the future.

References

1. American College of Rheumatology Task Force on Osteoporosis Guidelines (1996) Recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis. Arthritis Rheum 39:1791–1801
2. Eastell R, Reid DM, Compston J, Cooper C, Fogelman I, Francis RM, Hosking DJ, Purdie DW, Ralston SH, Reeve J, Russell RG, Stevenson JC, Torgerson DJ (1998) A UK Consensus Group on management of glucocorticoid-induced osteoporosis: an update. J Intern Med 244:271–292
3. Adachi JD, Olszynski WP, Hanley DA, Hodsman AB, Kendler DL, Siminoski KG, Brown J, Cowden EA, Goltzman D, Ioannidis G, Josse RG, Ste-Marie LG, Tenenhouse AM, Davison KS, Blocka KL, Pollock AP, Sibley J (2000) Management of corticosteroid-induced osteoporosis. Semin Arthritis Rheum 29:228–251
4. American College of Rheumatology Ad Hoc Committee on Glucocorticoid-Induced Osteoporosis (2001) Recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis. Update. Arthritis Rheum 44:1496–1503
5. Brown JP, Josse RG (2002) Clinical practice guidelines for the diagnosis and management of osteoporosis in Canada. Can Med Assoc J 167:1S–34S
6. Bone and Tooth Society, National Osteoporosis Society, Royal College of Physicians (2002) Glucocorticoid-induced osteoporosis: guidelines for prevention and treatment. Royal College of Physicians, London
7. Tanaka I, Oshima H (2003) A longitudinal study of diagnosis and treatment for glucocorticoid-induced osteoporosis. Osteoporosis Jpn 11:11–14
8. Van Staa TP, Leufkens HG, Cooper C (2002) The epidemiology of corticosteroid-induced osteoporosis: a meta-analysis. Osteoporos Int 13:777–787
9. Orimo H, Hayashi Y, Fukunaga H, et al (2001) Diagnostic criteria of primary osteoporosis (2000 revision). Jpn J Bone Miner Res 18:76–82
10. Working Group on Preparation of Guidelines on Treatment of Osteoporosis (Drug Treatment) (representative, Orimo H) (2002) Guidelines on treatment of osteoporosis (drug treatment). 2002 revision. Osteoporosis Jpn 10:637–709
11. Adachi JD, Bensen WG, Brown J, Hanley D, Hodsman A, Josse R, Kendler DL, Lentle B, Olszynski W, Ste-Marie LG, Tenenhouse A, Chines AA (1997) Intermittent etidronate therapy to prevent corticosteroid-induced osteoporosis. N Engl J Med 337:382–387
12. Saag KG, Emkey R, Schnitzer TJ, Brown JP, Hawkins F, Goemaere S, Thamsborg G, Liberman UA, Delmas PD, Malice MP, Czachur M, Daifotis AG (1998) Alendronate for the prevention and treatment of glucocorticoid-induced osteoporosis. Glucocorticoid-Induced Osteoporosis Intervention Study Group. N Engl J Med 339:292–299
13. Cohen S, Levy RM, Keller M, Boling E, Emkey RD, Greenwald M, Zizic TM, Wallach S, Sewell KL, Lukert BP, Axelrod DW, Chines AA (1999) Risedronate therapy prevents corticosteroid-induced bone loss: a twelve-month, multicenter, randomized, double-blind, placebo-controlled, parallel-group study. Arthritis Rheum 42:2309–2318
14. Reid DM, Hughes RA, Laan RF, Sacco-Gibson NA, Wenderoth DH, Adami S, Eusebio RA, Devogelaer JP (2000) Efficacy and safety of daily risedronate in the treatment of corticosteroid-induced osteoporosis in men and women: a randomized trial. European Corticosteroid-Induced Osteoporosis Treatment Study. J Bone Miner Res 15:1006–1013
15. Adachi JD, Saag KG, Delmas PD, Liberman UA, Emkey RE, et al (2001) Two-year effects of alendronate on bone mineral density and vertebral fracture in patients receiving glucocorticoids: a randomized, double-blind, placebo-controlled extension trial. Arthritis Rheum 44:202–211
16. Sato S, Ohosone Y, Suwa A, Yasuoka H, Nojima T, Fujii T, Kuwana M, Nakamura K, Mimori T, Hirakata M (2003) Effect of intermittent cyclical etidronate therapy on corticosteroid induced osteoporosis in Japanese patients with connective tissue disease: 3 year followup. J Rheumatol 30:2673–2679
17. Nakayamada S, Okada Y, Saito K, Tanaka Y (2004) Etidronate prevents high dose glucocorticoid induced bone loss in premenopausal individuals with systemic autoimmune diseases. J Rheumatol 31:163–166
18. De Nijs RN, Jacobs JW, Algra A, Lems WF, Bijlsma JW (2004) Prevention and treatment of glucocorticoid-induced osteoporosis with active vitamin D(3) analogues: a review with meta-analysis of randomized controlled trials including organ transplantation studies. Osteoporos Int 16:625–632
19. Amin S, Lavalley MP, Simms RW, Felson DT (2002) The comparative efficacy of drug therapies used for the management of corticosteroid-induced osteoporosis: a meta-regression. J Bone Miner Res 17:1512–1526
20. Sambrook PN, Diamond T, Ferris L, Flatarone-Singh M, Flicker L, MacLennan A, Nowson C, O'Neill S, Greville H (2001) Corticosteroid-induced osteoporosis. Guidelines for treatment. Aust Fam Physician 30:793–796
21. Sewell K, Schein JR (2001) Osteoporosis therapies for rheumatoid arthritis patients: minimizing gastrointestinal side effects. Semin Arthritis Rheum 30:288–297

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Dehydroepiandrosterone negatively regulates the p38 mitogen-activated protein kinase pathway by a novel mitogen-activated protein kinase phosphatase

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Abstract

Dehydroepiandrosterone-sulfate, the sulfated form of dehydroepiandrosterone, is the most abundant steroid in young adults, but gradually declines with aging. In humans, the clinical application of dehydroepiandrosterone targeting some collagen diseases, such as systemic lupus erythematosus, as an adjunctive treatment has been applied in clinical trial. Here, we report that dehydroepiandrosterone may negatively regulate the mitogen-activated protein kinase pathway in humans via a novel dual specificity protein phosphatase, DDSP (dehydroepiandrosterone-enhanced dual specificity protein phosphatase). DDSP is highly homologous to LCPTP/HePTP, a tissue-specific protein tyrosine phosphatase (PTP) which negatively regulates both ERK and p38-mitogen-activated protein kinase, and is transcribed from the PTPN7 locus by alternative splicing. Although previous reports have shown that the mRNA expression of the LCPTP/HePTP gene was inducible by extracellular signals such as T-cell antigen receptor stimulation, reverse transcribed (RT)-PCR experiments using specific sets of primers suggested that the expression of LCPTP/HePTP was constitutive while the actual inducible sequence was that of DDSP. Furthermore DDSP was widely distributed among different types of human tissues and specifically interacted with p38-mitogen-activated protein kinase. This inducible negative regulation of the p38-mitogen-activated protein kinase-dependent pathway may help to clarify the broad range of dehydroepiandrosterone actions, thereby aiding the development of new preventive or adjunctive applications for human diseases.

Keywords: Dehydroepiandrosterone; Dual specificity protein phosphatase; Protein tyrosine phosphatase; p38-mitogen-activated protein kinase; Mitogen-activated protein kinase; Mitogen-activated protein kinase phosphatase

1. Introduction

The mitogen-activated protein kinase (MAPK) family plays a central role in signaling pathways stimulated by extracellular stimuli such as growth factors, cytokines and physical stress. In higher organisms, this kinase family includes the extracellular stimulus-regulated kinases (ERKs) and two stress-stimulated kinase groups, the stress-activated protein kinase/c-JUN N-terminus kinase (SAPK/JNK) and p38-MAPK/p38 High Osmolarity Glycerol response (HOG) 1 [1–5]. The activation of MAPKs requires phosphorylation of conserved tyrosine and threonine residues within the catalytic domain. This phosphorylation is mediated by dual

Abbreviations: RT-PCR, reverse transcribed PCR; MAPK, mitogen-activated protein kinase; PTP, protein tyrosine phosphatase; DSP, protein dual specificity phosphatase; LCPTP, leukocyte-specific protein tyrosine phosphatase; HePTP, hematopoietic tissue-specific protein tyrosine phosphatase; DHEA, dehydroepiandrosterone; DDSP, dehydroepiandrosterone-induced protein dual specificity phosphatase; ERK, extracellular stimulus-regulated kinase; JNK, c-*Jun* N-terminus kinase; TcR, T-cell antigen receptor; GFP, green fluorescence protein; PMA, phorbol-12-myristate-13-acetate; aa, amino acid; SSH, suppression subtraction hybridization

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specificity protein kinases, members of the MAPK kinase family. In contrast, in the absence of a signal the constituents of the MAPK cascade return to their inactive dephosphorylated state, suggesting an essential role for protein phosphatases in the negative regulation of the MAPK cascade. Protein phosphatases are classified into three groups, protein serine/threonine phosphatases, protein tyrosine phosphatases (PTPs) and protein dual specificity serine/threonine/tyrosine phosphatases (DSPs), depending on their phosphoamino acid specificity [6].

Dehydroepiandrosterone-sulfate (DHEA-S), the sulfated form of DHEA, is the most abundant steroids in young adults, but gradually decline with aging. Although the molecular basis of DHEA action still remains to be elucidated, recent findings have suggested modulatory actions of DHEA on the MAPK signal transduction pathway [7–9]. To date, much data have been accumulated on the biological action of DHEA, although some of these were carried out using rodents in which the P450 C17 activities are extremely low, thus leading to trace or nearly undetectable levels of serum DHEA or DHEAS concentrations. In humans, the clinical application of DHEA targeting hormone replacement therapy [10–13] has been tested in clinical trials.

We have been interested in the actions of DHEA using both in vitro and in vivo experiments [14] (for review). We have previously reported that a human clonal T lymphocyte, the PEER cell [15], which is stimulated with phorbol-12-myristate-13-acetate (PMA) and calcium ionophore A23187 to mimic the activation of the T-cell antigen receptor (TcR), revealed the specific binding of [3H]-DHEA to its putative receptor. This specific binding was further increased when treated with 100 nM of DHEA itself in addition to the PMA and A23187 treatment [16], while the subcellular localization of the DHEA-bound molecule(s) was not determined. In the experiment, the MAPK cascade was activated by PMA, which bypasses all receptor-induced proximal tyrosine phosphorylation events by directly activating the Raf kinase through protein kinase C [17,18]. Thus, PEER cells are likely to provide a good model for investigating the cellular phenotypes altered by the DHEA action on the MAPK cascade, or for identifying the putative receptor for DHEA. Here, we report that DHEA negatively regulates the MAPK pathway in humans via a novel MAPK phosphatase, tentatively named DDSP (DHEA-enhanced DSP), which is highly homologous to LCPTP/HePTP [19,20] not only controlling the activity of MAPKs but also mediating crosstalk between the cAMP system and the MAPK cascade [21].

2. Materials and methods

2.1. Cells

Human T lymphoblastic leukemic cells, PEER, were maintained in RPMI 1640 (Gibco) supplemented with 10% FBS, 60 μg/ml of benzylpenicillin, 100 μg/ml of streptomycin, 2 mmol/L of L-glutamine, and 50 μmol/L of 2-mercaptoethanol. The cells were plated at a concentration of 1×10^5/ml and then treated with 5 nM PMA and 500 ng/ml of calcium ionophore A23187 in the presence or absence of 50 to 100 nM of DHEA (PEER(+) and PEER(−), respectively) for 28 h. NIH3T3 mouse fibroblasts were maintained in DMEM (Gibco) supplemented with 10% FBS, 60 μg/ml of benzylpenicillin, 100 μg/ml of streptomycin, and 2 mmol/L of L-glutamine.

2.2. Suppression subtractive hybridization screening and reverse transcribed-PCR (RT-PCR)

Total RNA was isolated using ISOGEN (Nippon Gene Co.). Suppression subtraction hybridization (SSH) was performed to construct a subtraction cDNA library using a commercially available kit (PCR-Select cDNA Subtraction Kit, Clonetech Laboratories Inc.). Briefly, double-stranded cDNAs were synthesized using the poly(A)$^+$RNAs from PEER(+) or PEER(−) cells. After the completion of two rounds of hybridization, the suppression PCR products were amplified using the GeneAmp 9600 PCR System (Perkin Elmer Applied Biosystems Division), size-fractionated using Chroma Spin+TE-200 Columns (Clontech Laboratories Inc.) and then blunt ligated into pBluescript II SK—which was cleaved with *Sma*I. DH5α was transformed with the ligation mixtures to construct the plasmid library without amplification.

The nucleotide sequences of 400 randomly chosen clones were determined using a DSQ-1000 DNA Sequencer (Shimadzu Co) and subjected to a homology search. PCR primers of 16-mers specific for the nucleotide sequence of each clone were synthesized and used for RT-PCR to confirm the effect of the DHEA treatment. Semiquantitative RT-PCR was performed using total RNAs from untreated PEER cells, PEER(+) and PEER(−) cells. The PCR fragments were electrophoresed in a 4% polyacrylamide gel. The gels were stained with Cyber Green (Amersham Life Science) and the intensities of the fluorescent signals were analyzed directly using a STORM 860 Image Analyzer (Molecular Dynamics Inc.). A phage cDNA library using the mRNAs from PEER (+) cells was constructed using a ZAP-cDNA Synthesis Kit (Stratagene) and then probed with a clone 1–20 cDNA insert. Thereafter, 1×10^6 plaques were subjected to screening.

2.3. Phosphatase assay

The full-length cDNA sequence of DDSP was ligated into the pAcGHLT baculovirus expression vector (Pharmingen), containing a 6× histidine tag and a glutathione *S*-transferase (GST) tag upstream of the multiple cloning sites, and then transfected into SF-9 (*Spodoptera frugiperda* pupal ovary) insect cells using the calcium phosphate precipitation method. Cells were grown at 25 °C and the viruses were enriched according to the manufacturer's protocol. The expressed GST-DDSP protein was purified using a glutathione sepharose affinity column. Phosphatase assays were performed using the Tyrosine Phosphatase Assay System and the Serine/Threonine Phosphatase Assay System (Promega Co.).

2.4. Tissue distribution and hormonal regulation of DDSP mRNA

The tissue distribution and hormonal regulation of DDSP or LCPTP/HePTP mRNA were examined by semiquantitative RT-

PCR. To compare the tissue distribution and steroid hormone-specific mRNA induction between DDSP and LCPTP/HePTP, 2 sets of primers were designed (Fig. 1), namely one for amplifying the sequences specific to DDSP (5′-GGA-TATTGTGTGCCAACTGC-3′ for the forward and 5′-GAGA-CAGGGTTTACACCATG-3′ for the reverse) and the other for amplifying the sequences specific to LCPTP/HePTP (5′-CAGCTGCTTCAGCAGACCTC-3′ for the forward and 5′-

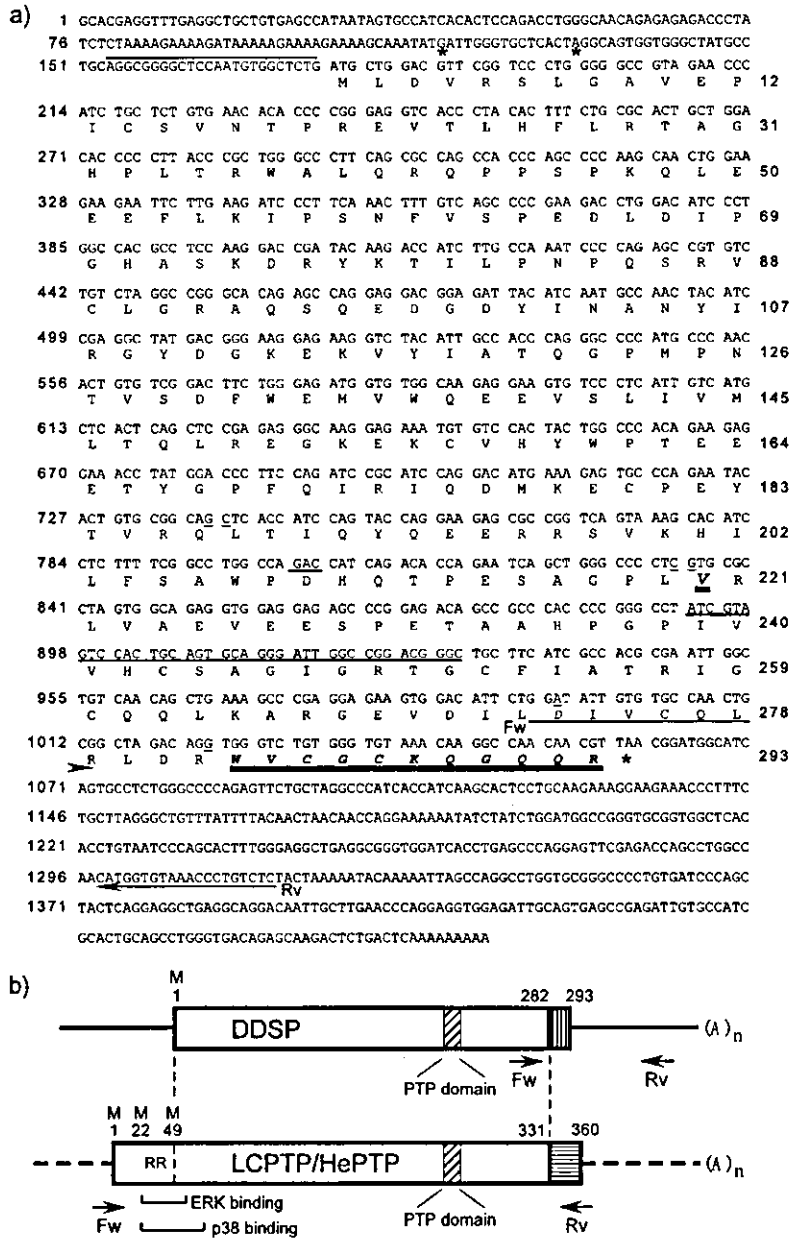

Fig. 1. The structure of DDSP. (a) The nucleotide and aa sequence of DDSP. The nucleotide number and the aa residue number are shown on the left side and right side, respectively. The substitution of the LCPTP/HePTP amino acid residues with the DDSP specific residues are highlighted by italic letters and bold lines, and the 2 termination codons are shown by asterisks. The putative PTP/DSP catalytic domain and Asp209, which is thought to be critical for PTP/DSP activity, are underlined. Arrows labeled with Fw and Rv are forward (Fw) and reverse (Rv) primers, respectively. (b) Schematic comparison of the DDSP structure with the LCPTP/HePTP structure. In DDSP, the aa residues required for ERK binding (including Arg41 and Arg42 shown as "RR" in the figure) are lacking (see text). The C-terminal 11 aa residues of DDSP (vertical bars) are different from those of LCPTP/HePTP (horizontal bars). The dashed box represents the PTP/DSP central catalytic domain. Arrows labeled with Fw and Rv are forward (Fw) and reverse (Rv) primers, respectively.

GGGGCTGGGTTCCTCAGGCA-3′ for the reverse). Tissue cDNA panels (Clontech Laboratories Inc.) were used for the RT-PCR.

2.5. Subcellular localization of DDSP

The full length DDSP cDNA sequence was ligated in-frame into pEGFP (Clontech), thus generating pDDSP-GFP in which the C′-terminus of the DDSP sequence was fused to the N′-terminus of green fluorescence protein (GFP). pDDSP-GFP was transfected into COS-7 cells and the cells were observed using a Leica TCS-SP System confocal laser microscope (Leica Microsystems). The cells were imaged for green fluorescence by excitation with the 488 nm line from an argon laser and the emission was viewed through a 496 to 505 nm band pass filter.

2.6. Immunocoprecipitation and Western blot analysis

For the immunocoprecipitation experiments, NIH3T3 mouse fibroblasts were transfected with a plasmid expressing flag-tagged DDSP using Superfect (Qiagen). At 24 h posttransfection, the cells were stimulated with 0.5 M NaCl for 20 min (for p38- and JNK-MAPK) or 50 ng/ml of PMA for 15 min after incubation in a serum free medium for 15 h (for ERK). Whole cell lysates were prepared by lysing the cells in a buffer (1.0% Nonidet P-40, 50 mM Tris–HCl pH 7.8, 150 mM NaCl, 1 mM DTT, 1 tablet of a protease inhibitor cocktail (Roche)). The lysates were incubated at 4 °C for 1 h with antibodies against ERK, JNK- or p38-MAPK (Cell Signaling) in an immunoprecipitation buffer (0.5% Nonidet P-40, 1 mM EDTA, 50 mM Tris–HCl pH 7.8, 200 mM NaCl, 1 mM DTT, 1 tablet of the protease inhibitor cocktail), and then further incubated with protein-A sepharose beads (Pharmacia) at 4 °C for 2 h. For Western blotting, the samples were separated by SDS-PAGE, transferred to a nitrocellulose filter, and then probed with an antibody against flag according to the manufacturer's protocol. To test the inactivation of the MAPK pathway by DDSP, two sets of transfection experiments were performed. In one experiment, the flag-tagged DDSP cDNA was transfected into NIH3T3 cells, and the transfected cells were then treated to activate ERK, p38 or JNK-MAPKs as in the immunocoprecipitation experiments. The transfection efficiency was monitored by the transfection of pEGFP in a separate dish and resulted in 30 to 50% efficiency. Western blotting was performed to observe the dephosphorylation of endogenous MAPK in the whole cell lysates of transfected cells using anti-phospho-MAPK antibodies (Cell Signaling). To observe the dephosphorylation of the endogenous activated MAPKs, the MACSelect system (Miltenyi Biotech) was used to enrich the transfected cells. The flag-tagged DDSP cDNA was ligated into the pMACSK^{k}II vector plasmid to generate pMACS-DDSP and the NIH3T3 cells were transfected in a 10 cm dish with 20 μg of pMACS-DDSP, or pMACSK^{k}II as a control. The transfected cells were subjected to affinity-column separation and then treated with 0.4 M sorbitol for 20 min to activate p38-MAPK, or 50 ng/ml of PMA for 15 min after incubation in a serum free medium for 20 h for ERK. Nearly 80% of the recovered cells were revealed to be transfected when the efficiency was preliminarily monitored by the cotransfection of pMACSK^{k}II and pEGFP. Western blotting was performed using anti-phospho-p38 or -ERK antibodies (Cell Signaling).

3. Results

3.1. Isolation of a cDNA sequence homologous to LCPTP/HePTP

Human T-cell leukemia cells (PEER) were treated with PMA and calcium ionophore A23187 to mimic TcR activation with (PEER(+)) or without (PEER(−)) 50 nM of DHEA. We performed the SSH screening by constructing a cDNA library in which the cDNAs from PEER(−) cells were subtracted from those from PEER(+) cells. After the SSH subtraction, the cDNAs for the MAPK phosphatases were enriched. One of these clones (named 1–20) contained 600 bases of sequence highly homologous to a leukocyte-specific PTP (LCPTP), also known as hematopoietic tissue-specific PTP (HePTP), a sequence which was originally isolated as a cytoplasmic PTP [19,20].

However, 1–20 contained another 150 bases of unique sequence and thus a phage cDNA library from the activated PEER cells was screened to obtain the full-length cDNA. The translation of the full length 1–20 (DDSP) cDNA sequence revealed one long open reading frame consisting of 293 amino acid (aa) residues, and also a striking homology (96% homology at the aa level) to LCPTP/HePTP (Fig. 1a). In the 50 aa residues of the LCPTP/HePTP N-terminus, there were 3 methionine (Met) residues: translation initiation Met 1 for LCPTP, translation initiation Met 22 for HePTP and Met 49 (Fig. 1b). A putative translation initiation Met for 1–20 corresponded to Met 49 of the LCPTP. And the preceding 24 nt of the 5′ noncoding sequence were identical to those of LCPTP/HePTP (sequence encoding aa residues 41 to 48 of LCPTP, as shown by the thin line above the nucleotide sequence preceding the translation initiation Met for 1–20 in Fig. 1a). However, about 150 bases of the sequence further upstream, including 2 in-frame termination codons, were unique. Although the aa sequence known as the PTP/DSP central catalytic domain was highly conserved, the striking homology to LCPTP/HePTP was disrupted at the C-terminal end, resulting in 11 novel aa sequences (Fig. 1a). Interestingly, the first 25 nt sequences encoding these 11 aa residues were identical to the partial sequence reported for the exon 9/intron 9 junction of LCPTP/HePTP [22].

A BLAST search of the human genome sequence using the 5′- and 3′-noncoding regions of 1–20 assigned each sequence to within the PTPN7 locus on chromosome 1q31 (the 5′-noncoding sequence was the intron 2/exon 3 junction, and the 3′-noncoding sequence was the read-through of the exon 9/intron 9 junction downstream to intron 9) that encodes LCPTP/HePTP, thus strongly suggesting that the 1–20 sequence was a novel alternatively spliced variant of the PTPN7 gene. Another RT-PCR experiment using RNA from human peripheral blood lymphocytes with or without reverse transcription confirmed that the full-length cDNA derived from the mRNA (data not shown).

3.2. Phosphatase activity

To test whether or not clone 1–20 possessed phosphatase activities, a GST fusion product with 1–20 was expressed in SF-9 insect cells since bacterially expressed fusion proteins always formed inclusion bodies. SDS-PAGE showed a stably expressed fusion protein with the expected molecular weight (data not shown). The expressed protein showed a rapid loss of phosphate from the phosphotyrosine in a time dependent manner. The activity was strongest at pH 6.0, and was suppressed by the PTP specific inhibitor sodium orthovanadate (Na_3VO_4) (Fig. 2a). In contrast to LCPTP/HePTP, the expressed 1–20 protein also caused a rapid loss of the phosphate from the phosphothreonine. Although threonine phosphatase activity was weaker (about 50% at the optimal pH 8.0) compared to tyrosine phosphatase activity at the optimal pH, the activity was suppressed by sodium fluoride (NaF), a serine/threonine phosphatase specific inhibitor (Fig. 2b).

3.3. Subcellular localization

The DDSP sequence was in-frame ligated into pEGFP generating pEGFP-DDSP which was transfected into COS-7 cells to observe the subcellular localization of GFP fluorescence. The GFP fluorescence was detected solely in the cytoplasm as in the case of LCPTP/HePTP. Furthermore, the distribution of the GFP-fluorescence was homogeneous, suggesting that this protein was not associated with the membrane structure (Fig. 3a). The treatment of the trans-

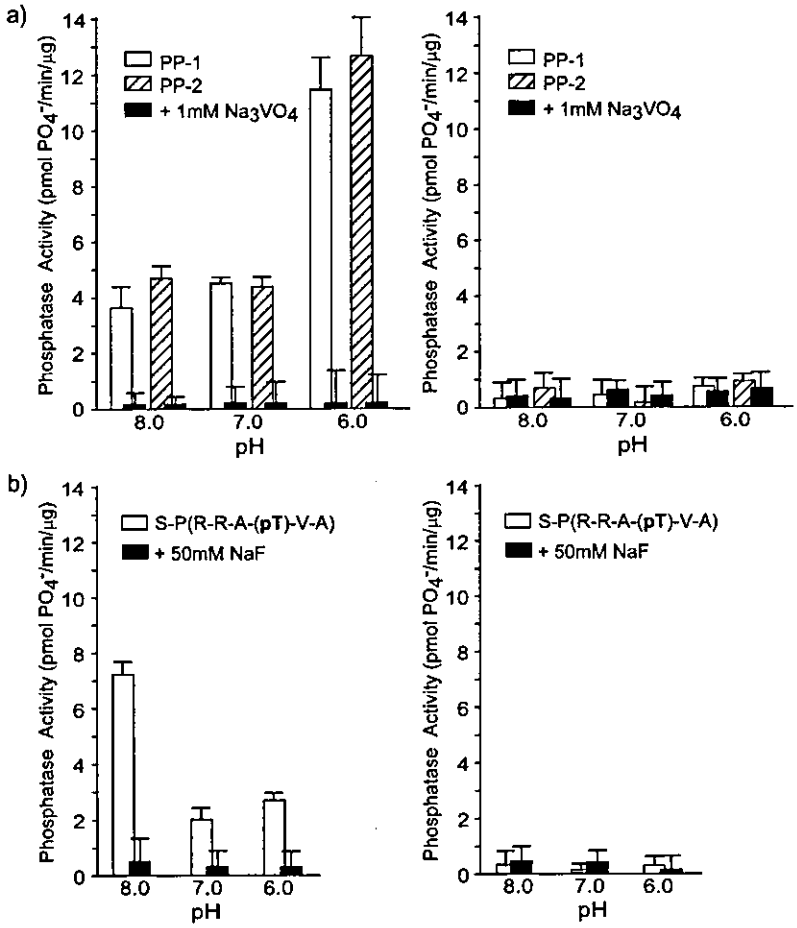

Fig. 2. Phosphatase activities of GST-DDSP. The sequence encoding GST-fused DDSP was ligated into the baculovirus expression vector and expressed in SF-9 cells. (a) Phosphotyrosine phosphatase activity of DDSP (left panel). PP-1 (open column) and PP-2 (dashed column) are 2 different kinds of phosphotyrosine substrate supplied by the manufacturer. Sodium vanadate (Na_3VO_4) is a PTP-specific inhibitor. The phosphatase activity was measured in the absence (open or dashed column) or presence (filled column) of 1 mM Na_3VO_4 and is shown as pmol phosphate released/min/μg of enriched cytosol after passage through a GST-affinity column. The right panel represents the activity of the sample from cells transfected with an empty vector plasmid. (b) Phosphothreonine phosphatase activity of DDSP (left panel). S-P is a phosphothreonine substrate. Sodium fluoride (NaF) is a serine/threonine phosphatase-specific inhibitor. The phosphatase activity was measured in the absence (open) or presence (filled column) of 50 mM NaF. The right panel represents the activity of the sample from cells transfected with an empty vector plasmid.