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Serum Soluble Factors Induce the Proliferation, Alkaline Phosphatase Activity and Transforming Growth Factor-β Signal in Osteoblastic Cells in the Patient with Hepatitis C-associated Osteosclerosis

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Key words: Osteosclerosis TGF-β; Osteoblast; Smad3

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Summary

Hepatitis C-associated osteosclerosis (HCAO) is a rare syndrome characterized by severe, acquired, generalized osteosclerosis and hyperostosis in adults who are infected with the hepatitis C virus. However, the detail of the pathogenesis of HCAO is still unknown. We examined the effects of serum of the HCAO patient on the proliferation, ALP activity and transforming growth factor (TGF)-β-Smad signaling in mouse osteoblastic cells. The patient was compatible with HCAO, characterized by high bone mass, bone thickening and bone pain with normal lamelar bone. The serum from the HCAO patient increased the levels of TGF-β and Smad3 expression, compared with control subject, in osteoblastic MC3T3-E1 cells. Moreover, the serum from the HCAO patient significantly augmented TGF-β-induced transcriptional activity with luciferase assay using 3TP-Lux with a Smad3-specific responsive element. In addition, the serum from the HCAO patient significantly stimulated the MTT intensity, the level of proliferating cell nuclear antigen expression, a proliferation marker, and alkaline phosphatase activity, compared with that from control subject, in MC3T3-E1 cells. In conclusion, the present study indicated that the serum from HCAO patient stimulated TGF-β-Smad signaling as well as the proliferation and alkaline phosphatase activity in osteoblastic cells. Some soluble factors other than parathyroid hormone might be related to the pathogenesis of HCAO.
Introduction

Hepatitis C-associated osteosclerosis (HCAO) is a rare syndrome characterized by severe, acquired, generalized osteosclerosis and hyperostosis in adults who are infected with the hepatitis C virus (HCV) (Beyer et al., 1990, Villareal et al., 1992, Whyte et al., 1996, Hassoun et al., 1997, Whyte and Reasber 1997, Diamond and Depczynski, 1996, Khosla et al., 1998, Shaker et al., 1998, Manganelli et al., 2005). Bone mineral densities are elevated as much as two or three fold above mean values for age and gender (White et al., 1996). Radiographs show dense bones in the appendicular and axial skeleton, and bone formation markers, such as serum bone-type alkaline phosphatase (ALP) and osteocalcin, are elevated. However, bone from the patients appears histologically to be of good quality with intact lamellar patterns and is not usually complicated with fractures. These clinical features indicate that some systemic factors inducing bone formation exist in the blood of patients, suggesting some clue to develop the treatment of osteoporosis. However, the detail of the pathogenesis of HCAO is still unknown, although the previous study (Khosla et al., 1998) suggested abnormalities in the insulin-like growth factor (IGF) system.

Transforming growth factor (TGF)-β is most abundant in bone matrix compared with other tissues (Jennings and Mohan 1990, Janssens et al., 2005). TGF-β is stored in an inactive form, released from the bone matrix, and activated in the bone microenvironment (Janssens et al., 2005). It is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling (Janssens et al., 2005, Rodan 1998). TGF-β modulates the proliferation, differentiation, and production of bone matrix proteins of osteoblasts (Janssens et al., 2005). Several reports showed that TGF-β induced bone formation when it was locally administered into bone tissues in rats (Noda and Camilliere 1989, Joyce et al., 1990, Beck et al., 1993, Rosen et al., 1994). The Smad family proteins are critical components of the TGF-β signaling pathways (Massague and Chen 2000). TGF-β exerts
growth inhibitory and transcriptional response through the two receptor-
regulated Smads: Smad2 and Smad3 (Massague and Chen 2000). Receptor-
mediated phosphorylation of Smad2 or Smad3 induces their association with
the common partner Smad4, followed by translocation into the nucleus where
these complexes activate transcription of specific genes (Massague and Wotton
2000). Thus, the Smad pathway is the canonical TGF-β signaling pathway, but
not the sole pathway through which TGF-β exerts its effects. We recently
reported that Smad3 promotes the production of alkaline phosphatase (ALP)
activity, and mineralization in mouse osteoblastic MC3T3-E1 cells (Sowa et al.,
2002, 2003), and that PTH-Smad3-axis exerts anti-apoptotic action in
osteoblasts (Sowa et al., 2003). Moreover, the mice with the targeted disruption
of Smad3 exhibited osteopenia caused by decreased bone formation (Borton et
al., 2001). Based on these data, we have proposed that Smad3 is a molecule
promoting bone formation.

In the present study, we examined the effects of serum of the HCAO patient
on proliferation, ALP activity and TGF-β -Smad signaling in mouse osteoblastic
cells.

Case report

Patient

A 48-year-old man with chronic hepatitis due to hepatitis C virus infection had
pain involving both legs since January, 2001. Since bone pain was sustained,
he was admitted to our hospital for the further examination in June, 2002. He
had tenderness and spontaneous pain in both lower legs. Neurological
examination was intact. No deformity was observed in limb, face, head and
body. The laboratory findings showed the increased levels of serum bone
metabolic indices [bone-type ALP 726 IU/L (normal range: 9.6-35.4);
osteocalcin 46 ng/ml (normal range: 2.5-13)] and the increased levels of urinary
bone resopotion markers [deoxy-pyridinoline 61.6 nM/mM.Cr (normal range: 2.8-
Renal function was not disturbed. Serum levels of calcium, phosphorus and intact parathyroid hormone were 9.1 mg/dl (normal range: 8.4-9.9), 4.3 mg/dl (normal range: 2.4-4.5), and 240 pg/ml (normal range: 10-65), respectively. High level of PTH was presumably due to secondary hyperparathyroidism, because serum level of PTH was reciprocally changed in response to serum calcium level and normalized by the improvement of bone pain after the administration of pamidronate. In X-ray examination, there were diffuse osteosclerotic changes, thickness of bone cortex, predominantly in lower legs (Figure 1A). There were no osteolytic lesions. The thickening of calvariae was not so severe. In bone scintigraphy, the diffusely increased radioisotope accumulation was observed in the whole bone, especially in the lower legs (Figure 1B). Bone biopsy was performed before the patient was admitted to our hospital to exclude the other bone diseases. Bone finding from the iliac bone showed no evidence of mosaic pattern, suggesting that the diagnosis of the patient was not Paget disease of bone. Normal lamellar bone pattern was observed. An increased thickness of bone trabeculae and number of osteoblasts were observed, although any fibrotic changes suggesting an excess of PTH were not detected. These findings were compatible with the bone biopsy findings in the patients with HCAO. Bone mineral density in lumbar spine, femoral neck and the distal radius (dual energy x-ray bone absorptiometry: Hologic QDR-2000) were 1.373 g/cm² (T-score +2.73 SD), 1.383 g/cm² (T-score + 4.09 SD), and 0.849 g/cm² (T-score + 0.60 SD), respectively. The administrations of pamidronate (60 mg and subsequent 30 mg administrations) partly reduced his symptom and decreased bone metabolic indices (Figure 2). After informed consent, overnight fasting serum sample was obtained from this patient. Serum was kept at -80°C until the use for the experiments. The serum from patient and controls were taken under similar conditions. The control of figure is the representative of controls. We used at least three normal controls for each experiment and the similar data
were obtained. Moreover, we also employed the serum taken from the patient on the other day for the experiments and similar results were obtained. The study was approved by the ethical review board of Kobe University Hospital. All subjects agreed to participate in the study and gave informed consent.

**Materials**

MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Ohu Dental College, Japan). Human recombinant TGF-β1 was purchased from Sigma (St. Louis, MO, USA). Anti-TGF-β and anti-proliferating cell nuclear antigen (PCNA) antibodies were purchased from Transduction Laboratories (Lexington, KY) and R & D systems, Inc. (Mineapolis, MN), respectively. The immunogen used to generate anti-TGF-β antibody was recombinant mouse TGF-β1. Anti-Smad3 and phosphorylated Smad3 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin antibody was from Sigma. All other chemicals used were of analytical grade.

**Cell culture**

MC3T3-E1 cells were cultured in α-minimal essential medium (α-MEM; containing 50 mg/ml of ascorbic acid) supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco BRL, Rockville, MD, USA). The medium was changed twice a week.

**Transient transfection and luciferase Assay**

MC3T3-E1 cells were seeded at a density of 2 x10^5/6-well plate. Twenty-four hr later, the cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux) and the pCH110 plasmid expressing β-galactosidase (1 μg) using Lipofectamine (Invitrogen). Fifteen hr later, the medium was changed to α-MEM containing 4% FBS, and the cells were incubated for an additional 9 hr. Thereafter, the cells were cultured for 24 hr in the absence or presence of TGF-β in α-MEM containing 0.2% FBS. As previously described (Kaji et al., 2001),
the cells were lysed, and the luciferase activity was measured and normalized to the relative β-galactosidase activity.

**Protein extraction and Western blot analysis**

Cells were lysed with radioimmunoprecipitation buffer with 0.5 mM of phenylmethylsulfonyl fluoride (PMSF), complete protease inhibitor mixture, 1% Triton X-100, and 1 mM of sodium orthovanadate. Cell lysates were centrifuged at 12,000g for 20 minutes at 4° C, and the supernatants were stored at -80° C. Protein quantitation was performed with BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of protein were denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gel. Proteins were transferred to polyvinylidene difluoride. Blots were blocked with Tris-buffered saline (TBS) plus 0.1% Tween 20 containing 3% dried milk powder. The antigen-antibody complexes were visualized using the appropriate secondary antibodies (Sigma) and the enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay**

MC3T3-E1 cells were cultured at 10^4/well for 48 hr before MTT-dye assay. Mitochondrial function was assayed by the ability of viable cells to convert soluble MTT-dye (Sigma) into an insoluble dark blue formazan reaction product, as previously described (Sowa et al., 2002), MTT was used at a concentration of 0.5 mg/ml to each well of a 96-well tissue culture plate and the plate was incubated at 37° C for 4 hr. Acid isopropanol (400 μl of 10 M HCl in 100 ml of isopropanol) was added to each well and mixed thoroughly, to ensure that all the crystals were dissolved. The plates were read on a microplate reader at a wavelength of 595 nm.
The DNA content and ALP activity
After reaching confluency, cells in 24-well plates were rinsed three times with PBS and 600 μl of distilled water was added to each well. The DNA assay and ALP activity were measured, as we previously described (Sowa et al., 2002). In brief, the assay mixtures contained 0.1 M of 2-amino-2-methyl-1-propanol, 1 mM of MgCl2, 8 mM of p-nitrophenyl phosphate disodium (Sigma), and cell homogenates. After 3 minutes of incubation, the reaction was stopped with 0.1N NaOH and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma). Each value was normalized with the value in DNA content.

Statistics
Data were expressed as mean±SEM. Statistical analysis was performed using an unpaired t-test.

Results
As shown in Materials and Methods, this case was compatible with HCAO. The clinical findings showed high bone mass, bone thickening and bone pain with normal lamellar bone. These findings raised the possibility that systemic factors in the serum might be responsible for the bone lesion of this disease. We, therefore, examined the effects of serum from the patient on the proliferation, alkaline phosphatase activity and TGFβ-Smad signaling in mouse osteoblastic MC3T3-E1 cells. We employed the serum from normal volunteers matched with age and gender, without any disease, as the control. We cultured MC3T3-E1 cells in the presence of 30 % serum from HCAO patient (CM) or 30 % serum from control (Control). As shown in Figure 3A, CM increased the expression of TGF-β, compared with Control medium in MC3T3-E1 cells. Moreover, CM induced the level of Smad3 as well as phosphorylated Smad3 in these cells.
(figure 3B). These findings indicate that soluble factors in the HCAO serum induce the levels of TGF-β and Smad3 expression in osteoblasts. We next examined the effects of CM on Smad3-induced transcriptional activity with luciferase assay using 3TP-Lux containing the promoter of plasminogen inhibitor 1 with a Smad3-specific responsive element. As shown in Figure 3C, CM significantly augmented TGF-β-induced transcriptional activity, compared with Control. The data were reproduced at least three times. The data of figure 3C were the representative data of these experiments. These results suggested that the serum from the HCAO patient augmented TGF-β-induced transcriptional activity of Smad3 in osteoblasts.

Bone formation is accelerated by osteoblast proliferation as well as osteoblast differentiation. Therefore, we examined the effects of the serum from the HCAO patient on the proliferation and differentiation in mouse osteoblastic cells. We used MTT-dye assay to assess viability and PCNA expression in immunoblot, which is the marker of the proliferation. Because PCNA is expressed mainly in the synthesis phase during the cell cycle, it is thought to be a marker for proliferating cells. As shown in figure 4A, CM significantly stimulated the MTT intensity, compared with Control in MC3T3-E1 cells. Moreover, it increased the level of PCNA expression in these cells (Figure 4B). These findings indicated that the serum from the HCAO patient induces osteoblast proliferation. Next, we examined the effects of CM on ALP activity as a differentiation marker. CM significantly induced ALP activity, compared with Control in MC3T3-E1 (Figure 4C). The data in Figure 3 and 4 were confirmed in the other control serum. Moreover, these data were reproduced and confirmed at least three times.

Discussion
The pathogenesis of the increased bone formation of the whole body is unknown in HCAO, although the infection with hepatitis C virus is related to HCAO. Several studies suggest that the pathogenesis of Paget’s disease of
bone is associated with viral infection of osteoclasts (Jeach et al., 2001, Roodman and Windle, 2005). Thus, the HCV virus may infect bone cells in HCAO. However, viral infection into bone cells does not seem to be the main pathogenesis, because the bone lesions of HCAO are very diffuse, in contrast with the focal lesions of Paget’s disease of bone. Taking the systemic lesions with the diffuse increased thickness of cortical bone and increased bone mineral density into account, the soluble factors in the serum presumably induced from the liver or the other tissues affected by HCV infection may be responsible for the systemic bone lesions in this disease. Kohsla et al (Kohsla et al., 1998) reported that HCAO patients have a specific increase in circulating big IGF-II precursor and IGFBP-2 levels. They speculated that IGFBP-2 might be targeting IGF-II precursor to the skeleton in these patients, resulting in the stimulation of bone formation. However, some other soluble factors, which stimulate bone formation, might exist in the serum of the patient.

Several reports showed that TGF-β induced bone formation when it was locally administered into bone tissues in rats (Noda and Camilliere 1989, Joyce et al., 1990, Beck et al., 1993, Rosen et al., 1994). It is disputable whether TGF-β would possess bone anabolic effects in vitro (Janssens et al., 2005). On the other hand, Borton et al (Borton et al., 2001) recently reported that mice with targeted deletion of Smad3 are osteopenic compared with wild-type littermates, because of a lower rate of bone formation. Moreover, our previous studies indicated that Smad3 plays an important role in osteoblastic bone formation, and PTH, a strong bone forming agent, exerts anti-apoptotic action through Smad3 and augments bone anabolic action of TGF-β in osteoblasts (Sowa et al., 2003). In addition, Camurati-Engelmann disease is a rare bone disease by the mutation of TGFβ1, which might lead to the induction of TGF-β-Smad signaling (Kinoshita et al., 2000, Janssens et al., 2000, 2003, 2005). The characteristic feature is hyperostosis that occurs on both periosteal and endosteal surfaces of long bones. In severe cases, osteosclerosis is
widespread in the whole bone (Sparkes and Graham, 1972). The phenotype is partly similar with the bone lesions of HCAO. These findings suggested that the soluble factors in the serum from HCAO patient might affect TGF-β-Smad signaling in bone cells. Actually, the present study indicated that the serum from HCAO patient stimulated TGF-β expression and Smad3 expression in osteoblastic cells. Moreover, it augmented the transcriptional activity induced by TGF-β. These findings indicated that there are some soluble factors stimulating TGF-β-Smad3 signaling in the serum of HCAO patients. The factors, which stimulate TGF-β-Smad3 signal, may be an important clue in the development of bone forming agents for treatment of osteoporosis.

In the present data, the serum from HCAO patient increased both the proliferation and ALP activity in MC3T3-E1 cells. Since our previous studies indicated that TGF-β inhibited both the proliferation and ALP activity in these cells, the soluble factors from HCAO patient do not appear to stimulate this phenotype by activating TGF-β pathways. Our previous study revealed that Smad3 promotes ALP activity and mineralization in MC3T3-E1 cells. Thus, the effects of TGF-β and Smad3 on ALP activity and mineralization are different (Sowa et al., 2003). Therefore, as for ALP activity, the soluble factors from HCAO patients might induce a Smad3-induced and TGF-β-independent pathway. As for the induction of proliferation, the serum from HCAO patient stimulated osteoblast proliferation in a manner independently of TGF-β-Smad pathways, since both TGF-β and Smad3 suppress the proliferation in these cells (Sowa et al., 2002).

The previous study revealed that IGFBP-2 and a IGF-II precursor were elevated in the serum of HCAO patients without the changes of IGF-I and IGF-II (Khosla et al., 1998). Unfortunately, we did not have the data about IGFBP2/IGF-II as well as TGF-β1 in the present study. Therefore, those IGF systems might play some role in the pathogenesis of the present case. Alternatively, the elevation of PTH was observed in the patient, presumably due to secondary
hyperparathyroidism. Our previous study revealed that PTH induced the levels of TGF-β and Smad3 in MC3T3-E1 cells (Sowa et al., 2003), although PTH did not induce proliferation and ALP activity of these cells (data not shown). Therefore, it does not seem probable that PTH is responsible for the pathogenesis of HCAO in the patient, although PTH might enhance the bone anabolic activity of some soluble factors of the patient. Moreover, Manganelli et al [8] reported a pathogenic role of the OPG/RANKL system imbalance in HCAO. Further studies will be necessary for the identification of the soluble factors in the serum from HCAO patients. It might be some potential factor, which markedly stimulates bone formation.

In conclusion, we examined the case of HCAO. The serum from this patient stimulated TGF-β-Smad signal as well as the proliferation and ALP activity in osteoblastic cells.

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Figure Legend

Fig. 1  Clinical features of a patient with HCAO

A X-ray of lower leg; There was diffuse thickness of bone cortex in lower legs B

Bone scintigram: The diffusely increased radioisotope accumulation was observed in the whole bone, especially in the lower legs.

Fig. 2  The effect of pamidronate on serum alkaline phosphatase and urinary N-telopeptide

The administrations of pamidronate (60 mg and subsequent 30 mg administrations) partly reduced his symptom and decreased bone metabolic indices.

Fig. 3  The serum from the HCAO patient stimulates TGF-β -Smad3 pathway.

A, B After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were treated with 30 % serum from the HCAO patient and normal control for 24 hr. Then, protein extraction and Western blot analysis were performed, as described under "Patients and Methods." C MC3T3-E1 cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux), the pCH110 plasmid expressing β -galactosidase (1 μg) per well in 6-well plate. Twenty-four hr later, cells were treated with 30 % of serum from the HCAO patient and normal control for 24 hr. Then, cells were harvested and relative luciferase activity was measured. Values of relative luciferase activity represent the mean±SEM . *, p < 0.01 from TGF-β -treated control group.

Fig. 4  The serum from the HCAO patient stimulates cell proliferation and ALP activity. A, Confluent MC3T3-E1 cells were cultured with 30 % serum from the HCAO patient and normal control for 24 hr, MTT dye assay was performed as described in "Patients and Methods." Each value is the mean±SEM of triplicate determinations. *, P < 0.01 compared with control group. B. After
confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were treated with 30 % serum from the HCAO patient and normal control for 24 hr. Then, protein extraction and Western blot analysis were performed, as described under "Patients and Methods." C. Confluent MC3T3-E1 cells were cultured with 30 % of serum from the HCAO patient and normal control for 24 hr, and ALP activity was measured, as described under "Patients and Methods." Each value represents as absolute values (nmol/min/μg protein). Each value is the mean ± SEM of four determinations. *, p < 0.01, compared with the control group.
Figure 1

A

B

ANTERIOR
Figure 2

- ALP (IU/L)
- U-NTX (nMBCE/mM.Cr)

Pamidronate doses:
- 60mg
- 30mg
- 30mg

Time periods:
- April
- May
- June
- July
- August
- September
- October
Figure 3

(A) TGF-β and β-actin expression levels in Cont1, Cont2, HCAO groups.

(B) Smad3 and p-Smad3 expression levels in Cont1, HCAO, Cont2, HCAO groups.

(C) Luciferase activity (fold induction) in Cont1, Cont2, HCAO groups.