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Elevated Serum Kininogen in Patients with Paget's Disease of Bone: A Role in Marrow Stromal/Preosteoblast Cell Proliferation

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Abstract Paget's disease (PD) of bone is a chronic focal skeletal disorder characterized by excessive bone resorption followed by abundant new bone formation. Enhanced levels of IL-6, RANKL, M-CSF, and endothelin-1 have been associated with PD. In the present study, we identified increased serum levels (2 to 5-fold) of inflammatory cytokine, kininogen (KNG) in patients with PD compared to normal subjects. Treatment of pagetic bone marrow derived stromal/preosteoblast cells with recombinant KNG (25 ng/ml) for 24 h period resulted in a 5-fold increase in the levels of phospho-HSP27 and a 3-fold increase in ERK1/2 phosphorylation in these cells. However, pagetic stromal cells stimulated with KNG in the presence of ERK activation inhibitor peptide did not significantly affect the levels of phospho-HSP27. KNG increased normal and pagetic marrow stromal cell proliferation at 1.4-fold and 2.5-fold, respectively. KNG in the presence of an ERK inhibitor peptide did not stimulate pagetic marrow stromal cell proliferation. Furthermore, siRNA suppression of HSP27 expression significantly decreased KNG inhibition of etoposide-induced caspase-3 activation and apoptosis in these cells. In summary, KNG modulate bone marrow derived stromal/preosteoblast cell proliferation and suppress etoposide-induced apoptosis through ERK and HSP27 activation, respectively. These results implicate a pathophysiologic role for KNG in patients with PD. J. Cell. Biochem. 98: 1681–1688, 2006.

Key words: kininogen (KNG); Paget's disease; stromal/preosteoblast cells; extracellular signal-regulated kinase (ERK); heat-shock protein

Paget's disease (PD) of bone is a chronic focal skeletal disorder that affects up to 2%–3% of the population over the age of 60 years. The pathologic abnormality in patients with PD involves increased bone resorption by the osteoclasts, followed by abundant new bone formation that is of poor quality [Roodman and Windle, 2005]. Genetic linkage analysis indicated that 40% of patients with PD have an affected first degree relative and 1% of patients develop osteosarcoma [Hansen et al., 1999]. PD is an autosomal dominant trait with genetic heterogeneity. Recurrent mutations in the ubiquitin-associated domain of Sequestosome 1 (SQSTM1/p62) are identified in patients with PD [Laurin et al., 2002; Johnson-Pais et al., 2003; Hocking et al., 2004]. Osteoclasts and osteoclast precursors from patients with PD contain paramyxoviral transcripts and appear hyperresponsive to 1,25-$(OH)_2D_3$ and RANK ligand (RANKL) [Neale et al., 2000; Roodman and Windle, 2005]. However, a cause and effect relationship for the paramyxoviral infection and SQSTM1/p62 gene mutations associated with this disease and osteoclast abnormalities are unclear.

The biochemical markers provide an integrated assessment of the cellular events occurring throughout the skeleton of patients with PD. Interleukin-6 (IL-6) levels were shown to increase in bone marrow plasma and peripheral blood of patients with PD. These studies further indicated that IL-6 is an autocrine/paracrine factor, which stimulates human osteoclast formation and increase the osteoclast precursor

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pool in patients with PD. In addition, RANKL, a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is also upregulated in areas involved with PD. The increased sensitivity of osteoclast precursors from Paget's patients to RANKL appears to be due to interactions of these precursors with IL-6. Addition of neutralizing antibodies to IL-6 decreased the RANKL sensitivity of osteoclast precursors to normal levels. Similarly, addition of IL-6 to cultures of normal osteoclast precursors enhanced the responsivity of these precursors to RANKL to the levels seen with pagetic osteoclast precursors. The enhanced expression of RANKL and IL-6 in pagetic lesions could contribute to the abnormal osteoclast development and highly localized nature of PD [Roodman and Windle, 2005]. In situ hybridization studies have further identified increased levels of IL-6, c-fos proto-oncogene, Bcl-2 anti-apoptotic gene mRNA expression in pagetic osteoclasts [Hoyland et al., 1994; Brandwood et al., 2003].

Urinary *N*-telopeptide, pyridinoline, and deoxypyridinoline have all been reported to be more specific indices of skeletal matrix resorption and are not influenced by dietary gelatin. Furthermore, serum calcium levels are typically normal in PD and also serum osteocalcin levels appear to be a poor index of the progression of the disease. The increased bone remodeling in unaffected bones has been ascribed to secondary hyperparathyroidism rather than to subclinical involvement of the bones with PD. However, less than 20% of patients with PD have elevated parathyroid hormone (PTH) levels [Siris, 1998]. Serum tartrate resistant acid phosphatase (TRAP), presumably released by osteoclasts, appears to be an index of bone resorption in PD but is not routinely used. The most useful markers for the increased osteoblast activity in PD are the total alkaline phosphatase and bone-specific alkaline phosphatase activity levels in serum [Reddy, 2004]. It has been reported that serum M-CSF levels are significantly elevated in patients with PD, however not significantly different in patients under treatment compared to normal subjects [Neale et al., 2002]. Patients also showed significantly higher endothelin-1 circulating levels than controls with a positive correlation with serum alkaline phosphatase, but not with urinary hydroxyproline [Tarquini et al., 1998]. In the present study, we identified high-level expression of an inflammatory cytokine, kininogen (KNG) in patient's sera compared to normal subjects, and further demonstrated potential role that KNG may play in marrow stromal cell proliferation. Our results further implicate a pathophysiologic role for KNG in PD.

MATERIALS AND METHODS

Materials

Recombinant human KNG was purchased from R&D systems, Inc. (Minneapolis, MN). Extracellular signal-regulated kinase (ERK) activation inhibitor peptide I was obtained from Calbiochem (Darmstadt, Germany).

Western Blot Analysis

Normal and pagetic bone marrow derived stromal/preosteoblast cells were isolated as described previously [Roccisana et al., 2004]. The cells were seeded in six-well plates at a density of 10^6 cells in 10 ml of α-minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and cultured for 24 h in the presence of human recombinant KNG. The cells were lysed in a buffer containing 20 mM Tris, pH 7.4, NaCl 150 mM, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 200 μM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (1 mg/ml). The protein content of the samples was measured using the BCA method as per the manufacturer's protocol (Pierce, Rockford, IL). Serum (4 μg total protein) or cell lysates (15 μg protein) samples were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE), using 12% Tris-HCl gels. The proteins were transferred from SDS gels onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) for immunoblot analysis. Blocking was performed with 5% non-fat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween-20 (TBST) buffer. The membrane was then incubated for 1 h with anti-KNG antibody (The Binding Site, Birmingham, UK), and anti-ERK1/2, phospho-ERK1/2, HSF-2 (heat-shock factor-2), RANKL mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-HSP27 (heat-shock protein-27), phospho-HSP27 (P-HSP27) antibodies (Cell signaling technology, Inc., Beverly, MA) diluted 1:500 in 5% non-fat dry milk-TBST. The blots then were incubated for 1 h with horseradish

peroxidase-conjugated goat anti-mouse IgG, diluted 1:2,500 in 5% non-fat dry milk-TBST, and developed using an ECL system (Amersham Biosciences, Little Chalfont, UK). NIH image program (National Institutes of Health, Bethesda, MD) was used for quantification analysis after digital scanning of the exposed X-ray films.

Inhibition of ERK Activation

Inhibition of ERK activation was performed as described previously [Kelemen et al., 2002]. Briefly, serum-starved pagetic bone marrow stromal/preosteoblast cells were treated with the ERK activation inhibitor peptide (25 μM) for 4 h at 37°C and then stimulated with KNG (25 ng/ml) for an additional 24 h in α-MEM containing 10% FCS. Cells were washed with ice-cold phosphate buffered saline, suspended in 0.5 ml of icecold lysis buffer, and scraped from flasks. Cell lysate was sonicated, centrifuged to remove remaining insoluble material, and measured protein concentration. The protein concentration in the cell lysate was determined by BCA protein assay system (Pierce Chemical Co.).

Cell Proliferation Assay

Normal and pagetic human bone marrow stromal/preosteoblast cell proliferation was determined using a CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI). The cells were seeded at 5×10^3 cells/well on 96-well plates, and incubated in the presence or absence of KNG (0–50 ng/ml) for 48 h at 37°C in humidified, 5% CO_2 atmosphere. After 48 h period, cell proliferation rate was assayed following the manufacturer's protocol.

To determine the role of HSP27 in cell proliferation, we have used siRNA to suppress HSP27 expression in pagetic stromal cells. The cells were seeded at 5×10^3 cells/well in a 96-well plate and transiently transfected with HSP27 siRNA or control siRNA (10 nM) (Santa Cruz Biotechnology, Inc.) by Lipofectamine method. Briefly, 1 μl of 10 μM siRNA, 100 μl of α-MEM media, and 6 μl of Lipofect AMINE Plus reagent (Invitrogen, Grand Island, NY) were premixed for 15 min at room temperature. During this time, 5 μl of Lipofect AMINE transfection reagent was mixed with 100 μl of α-MEM media. The two mixtures were then combined and incubated for 15 min at room temperature to form a complex. The reaction mixture was diluted with 800 μl of α-MEM medium and 100 μl aliquot of the entire mixture was added to each well. After 12 h, the cells were treated with α-MEM containing 10% FCS with KNG (0.5, 25 ng/ml) for additional 48 h, and subjected to the proliferation assay.

Inhibition of ERK phosphorylation was performed by seeding the pagetic stromal cells at 5×10^3 cells/well in 96-well plates, and treated with the ERK activation inhibitor peptide (25 μM) for 4 h. The cells were then stimulated with KNG (0–25 ng/ml) for an additional 48 h in α-MEM containing 10% FCS, and subjected to the proliferation assay. Each treatment was analyzed in triplicate and the results represent mean values of three independent experiments ($P < 0.05$).

Apoptosis Assay

To determine the effect of KNG and role of HSP27 in stromal/preosteoblast cell apoptosis, Paget's bone marrow stromal cells were transiently transfected with HSP27 siRNA or control siRNA as described above. The cells were stimulated with KNG (25 μM) for 24 h and cultured in the presence of 25 μM etoposide (Sigma-Aldrich, St. Louis, MO) at 37°C for additional 24 h period. Total cell lysates were prepared with a lysis buffer containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The supernatant of the cell lysate (100 μg of protein) was then assayed for caspase-3 activation to detect cell apoptosis using the CaspACE assay system (Promega) following the manufacturer's protocol.

Etoposide-induced apoptosis in pagetic stromal/preosteoblast cells was confirmed by immuno-fluorescence method. Briefly, paget's bone marrow stromal cells were cultured on glass coverslips in the presence of KNG (25 ng/ml) or etoposide (25 μM) alone, and in combination for 4 h. The cells were fixed in 4% paraformaldehydephosphate buffered saline buffer and permeabilized with 0.1% Triton X-100 in PBS. The cells were incubated overnight with a mouse anti-cytochrome c antibody (PharMingen, San Diego, CA) diluted 1:1,000 in PBS containing 1% bovine serum albumin. We used an Alexa ($\lambda_{ex} = 488$ nm and $\lambda_{em} = 519$ nm)-coupled anti-mouse IgG (1:600 dilution) to detect cytochrome c release using a confocal microscope.

RESULTS

Identification of KNG Overexpression in the Serum from Patients with PD

Enhanced levels of KNG (63 kDa) are associated with inflammatory conditions [Colman and Schmaier, 1997; Carretero, 2005]. In the present study, we examined KNG expression in serum samples obtained from normal subjects and patients with PD by Western blot analysis. As shown in Figure 1, Western blot analysis of serum samples (4 μg total protein) of five representative individuals from a total of nine PD patients and normals analyzed further indicated 2 to 5-fold increases in levels of KNG (63 kDa) in patients with PD compared to normal subjects. These data are consistent with a potential pathologic role for KNG in PD.

KNG Enhances ERK and HSP27 Phosphorylation in Pagetic Marrow Stromal/Preosteoblast Cells

RANKL, a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is upregulated in PD. We have recently demonstrated that HSF-2 is a downstream target of b-FGF-induced RANKL expression in SAKA-T normal human bone marrow derived stromal cells [Roccisana et al., 2004]. We therefore, further examined the effects of KNG on HSP27 and ERK phosphorylation in pagetic marrow stromal cells. As shown in Figure 2, Western blot analysis of total cell lysates obtained from the pagetic stromal cells treated with KNG (25 ng/ml) for a period of 24 h demonstrated a significant increase (5-fold) in the levels of HSP27 phosphorylation compared to untreated cells. However, there was no significant change in the levels of HSF-2 expression in these cells. In addition, KNG enhanced a 3-fold increase in ERK1/2 phosphorylation compared with untreated cells (Fig. 3A). To further delineate if ERK signaling is involved in HSP27 phosphorylation, we used ERK1/2 activation inhibitor. Pagetic stromal cells were stimulated with

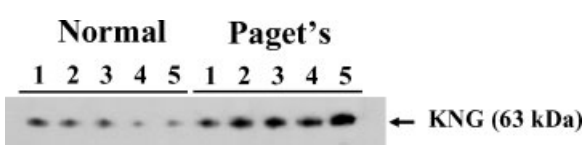

Fig. 1. Western blot analysis for KNG expression in normal and Paget's patient's serum. Serum samples (4 μg) from normal and Paget's patients shown are representative of total nine subjects of each analyzed. Sample loading was normalized for protein concentration.

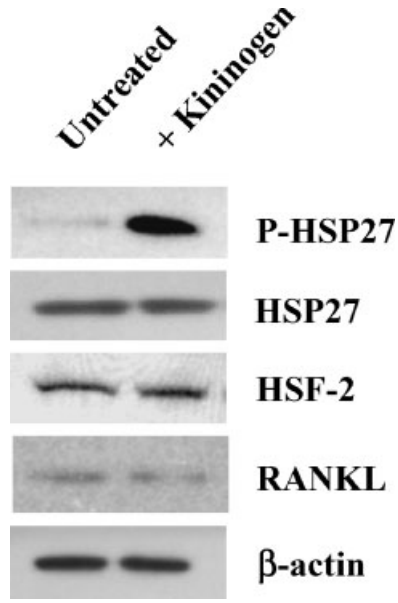

Fig. 2. KNG induces HSP27 phosphorylation. Pagetic marrow stromal/preosteoblast cells were treated with KNG (25 ng/ml) for 24 h, and total cell lysates (15 μg) were subjected to Western blot analysis using anti-human HSP27, P-HSP27, HSF-2, and RANKL antibodies as described in methods.

KNG in the presence of ERK activation inhibitor peptide-1 (25 μM), which binds to ERK2 and prevents interaction with MEK (mitogen-activated protein kinase kinase). Western blot analysis of total cell lysates obtained from

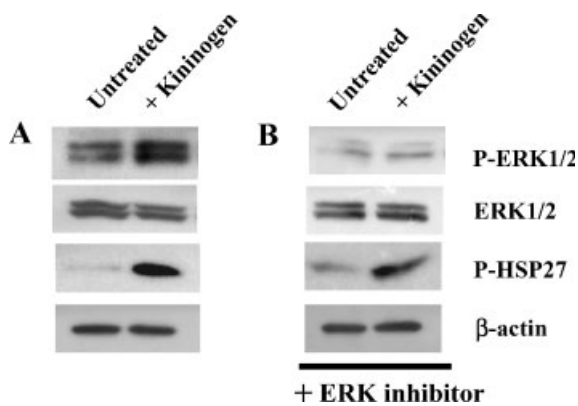

Fig. 3. KNG promotes ERK phosphorylation and ERK activation inhibitor did not affect HSP27 phosphorylation. **A**: KNG enhanced ERK and HSP27 phosphorylation. Pagetic stromal cells were stimulated with KNG (25 ng/ml) for 24 h, and total cell lysates (15 μg) prepared were subjected to Western blot analysis using anti-human ERK1/2, phospho-ERK1/2, and phospho-HSP27 as described. **B**: HSP27 phosphorylation is independent of ERK1/2 signaling pathway. Serum-starved pagetic stromal cells were treated with the ERK activation inhibitor peptide (25 μM) for 4 h at 37°C and then stimulated with KNG (25 ng/ml) for an additional 24 h. Total cell lysates (15 μg) obtained were subjected to Western blot analysis.

KNG stimulated cells did not demonstrate a significant change in the levels of phospho-HSP27 (Fig. 3B), suggesting that HSP27 phosphorylation is independent of ERK signaling pathway. We have observed similar effect of KNG using normal human bone marrow derived stromal cells (data not shown).

KNG Enhances Proliferation of Paget's Bone Marrow Stromal Cells Through ERK Activation

Since ERK signaling is associated with cellular proliferation, we have examined the potential of KNG to stimulate normal and pagetic bone marrow stromal/preosteoblastic cell growth. As shown in Figure 4, KNG treatment significantly increased normal and pagetic bone marrow stromal cells proliferation in a dose-dependent manner. KNG (25 ng/ml) increased normal and pagetic marrow derived stromal cell proliferation at 1.4-fold and 2.5-fold compared to untreated control cells, respectively. Since KNG significantly enhanced HSP27 and ERK phosphorylation in pagetic bone marrow derived stromal cells, we further examined if HSP27 and ERK signaling is involved in KNG stimulation of cell proliferation. We used siRNA and ERK inhibitor peptide

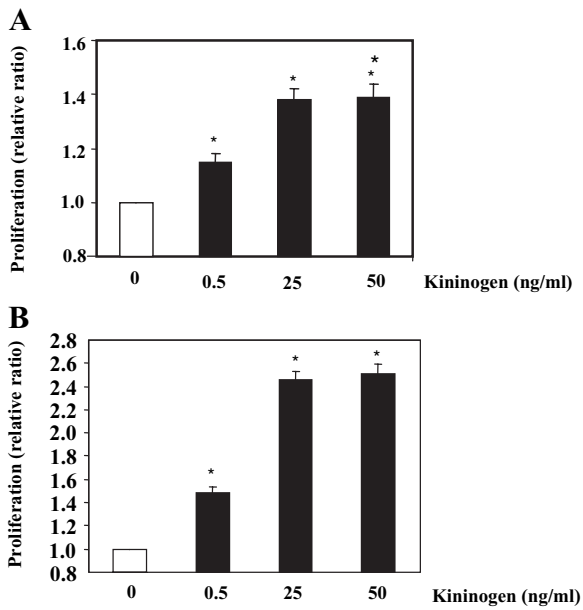

Fig. 4. Effect of KNG on proliferation of bone marrow stromal/preosteoblast cells. **A**: Normal human bone marrow derived stromal cells and (**B**) Pagetic marrow derived stromal/preosteoblastic cells were seeded at 5×10^3 cells/well in 96-well plates, and incubated in the presence or absence of KNG (0.5–50 ng/ml) for 48 h, and proliferation assay was performed as described in methods.

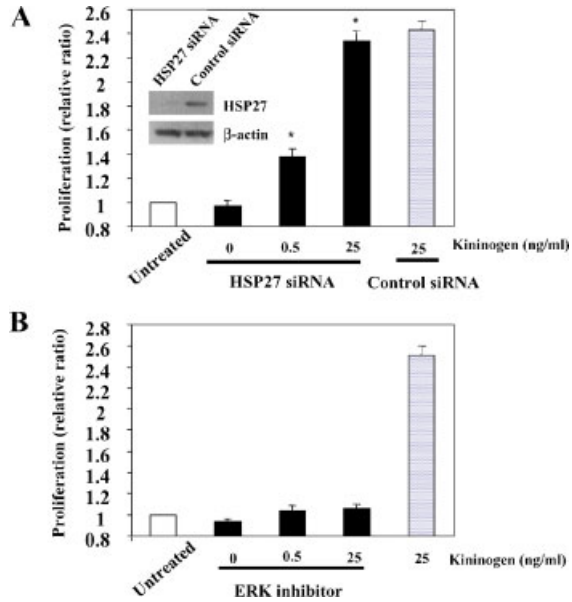

Fig. 5. KNG stimulate pagetic stromal cell proliferation through ERK signaling pathway. **A**: siRNA suppression of HSP27 expression did not affect KNG stimulated proliferation of pagetic marrow stromal cells. HSP27 or control siRNA (10 nM) was transiently transfected into pagetic stromal cells, and examined cell proliferation. Western blot analysis confirms the suppression of HSP27 expression (Inset). **B**: ERK activation inhibitor peptide suppressed the KNG stimulated proliferation of pagetic stromal cells. Pagetic marrow stromal cells were treated with ERK activation inhibitor peptide (25 μM) for 4 h at 37°C, and then stimulated with KNG (0.5, 25 ng/ml) for an additional 48 h and assayed for cell proliferation as described ($P < 0.05$).

to block the expression of HSP27 and ERK activation in these cells, respectively. As shown in Figure 5A, siRNA suppression of HSP27 expression did not affect the KNG stimulated proliferation of these cells. However, inhibition of ERK phosphorylation completely abolished the KNG stimulated cell proliferation (Fig. 5B). These results suggest that KNG enhances proliferation of Paget's bone marrow stromal/preosteoblast cells through ERK signaling pathway.

KNG Inhibits Etoposide-Induced Apoptosis in Paget's Bone Marrow Stromal Cells

It has been reported that HSP27 inhibits cellular apoptosis by preventing cytochrome c-triggered caspase-3 activation [Garrido et al., 1999]. Therefore, we further examined the effect of KNG on etoposisde-induced apoptosis and the role of HSP27 in Paget's bone marrow derived stromal/preosteoblast cells. Paget's stromal cells were transiently transfected with HSP27 siRNA and treated with KNG (25 ng/ml)

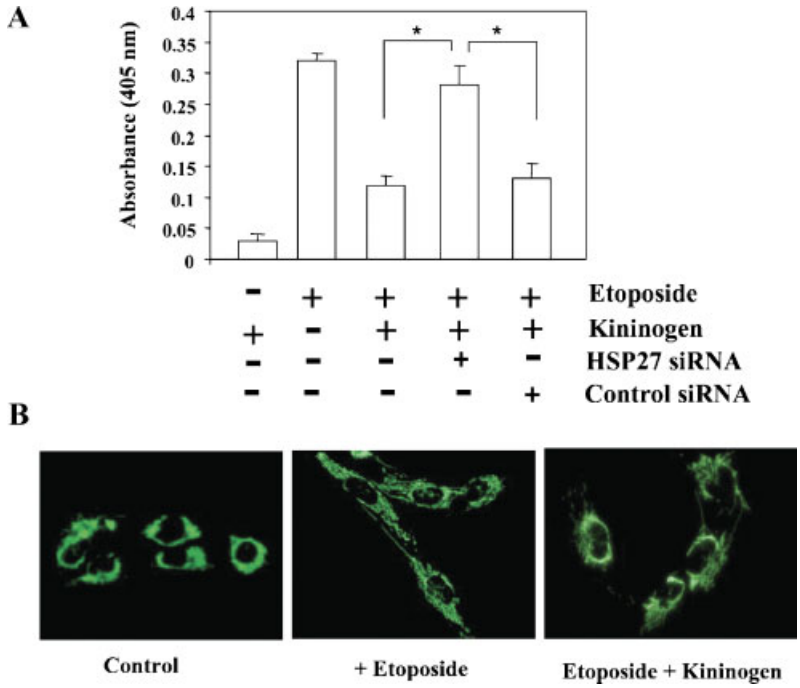

Fig. 6. Effect of KNG on etoposide-induced apoptosis in pagetic bone marrow stromal cells. **A**: Paget's bone marrow stromal cells were transiently transfected with HSP27 siRNA or control siRNA, and stimulated with or without KNG in the presence or absence of etoposide (25 μM) for 24 h. Total cell lysates obtained were assayed for caspase-3 activity was measured as described in methods. **B**: Immunofluorescence staining of cytochrome c distribution in KNG treated control cells, etoposide alone and KNG + etoposide combination treatment to pagetic marrow stromal cells.

in the presence or absence of etoposide (25 μM) and assayed for caspase-3 activation. As shown in Figure 6A, KNG treatment significantly inhibits etoposide-induced caspase-3 activity in pagetic stromal cells. Furthermore, siRNA suppression of HSP-27 expression significantly decreased KNG inhibition of etoposide-induced caspase-3 activity in these cells. Evident from the immunoflurenscence staining, etoposide treatment to pagetic marrow stromal cells demonstrated a diffused pattern of cytochrome c staining further confirms apoptosis in these cells. However, etoposide treatment in the presence of KNG (25 μM) demonstrates low levels of cytochrome c diffusion compared to etoposide alone treated cells (Fig. 6B). These results indicate functional role for HSP27 in KNG inhibition of apoptosis in pagetic marrow stromal/preosteoblastic cells.

DISCUSSION

Enhanced levels of IL-6, M-CSF, and endothelin-1 have been associated with PD, implicated in its pathogenesis and indicator of disease activity. Although PD is localized, bones not clinically involved with PD appear to show increased bone remodeling. For example, the increased levels of IL-6 in the peripheral blood of patients with PD may in part explain the increased bone remodeling seen in bones not clinically involved with PD. Therefore, it is necessary to define a pathologic role of systemic factors that are upregulated in patients with PD. In the present study, we have identified elevated levels of KNG expression in serum of patients with PD. Since KNG appears to be the major glycosylated peptide that is detected at the level of total protein analysis of patient's serum, we further determined KNG influence on pagetic bone marrow derived stromal/pre-osteoblast cells and associated signaling mechanism. The KNG is a multifunctional inflammatory cytokine which is composed of a 362-amino acid heavy chain, the 9-residue bradykinin sequence, and one 255-amino acid light chain [Takagaki et al., 1985]. KNG being a glycosylated secretory molecule, posttranslational regulatory mechanisms may be responsible for enhanced levels of serum KNG in patients with PD. The KNG localizes on the surface of endothelial cells, platelets, and

neutrophils. It has also been demonstrated that KNG purified from bovine milk stimulates proliferation of osteoblastic cells; however, the molecular signaling mechanism is unclear [Yamamura et al., 2000]. Although our results indicate KNG treatment results in high levels of HSP27 phosphorylation in pagetic stromal cells, we observe no significant change in RANKL expression in these cells. We have recently demonstrated that HSF-2 is a downstream target of fibroblast growth factor-2 (FGF-2) to induce RANKL expression in stromal/preosteoblast cells [Roccisana et al., 2004]. HSP are molecular chaperones activated upon cellular stress/stimuli [Snoeckx et al., 2001]. HSPs have been shown to prevent inflammatory damage through production of antiinflammatory cytokines [van Eden et al., 2005]. Several members of the *HSP* gene family have been reported to exhibit differential expression during stromal/preosteoblast differentiation. The differences in HSP expression are consistent with involvement in mediating a series of regulatory events functionally related to the physiologic control of cell growth and differentiation [Shakoori et al., 1992]. Our results using siRNA suppression of HSP27 did not significantly affect KNG stimulation of pagetic marrow stromal cell proliferation. However, inhibition of ERK phosphorylation completely abolished the KNG stimulated cell proliferation suggesting that KNG enhances proliferation of pagetic stromal cells through ERK signaling pathway. Therefore, KNG may play an important role in modulating marrow stromal cell proliferation/differentiation. Although KNG exerts similar effect on normal and pagetic marrow derived stromal cells with respect to HSP27 and ERK activation, our results indicate that KNG stimulates pagetic marrow derived stromal cell proliferation efficiently compared to normal bone marrow derived cells. It is possible that the pagetic marrow stromal cells are either indirectly or directly affected by the elevated systemic factors and chronic exposure to cytokines produced in the focal lesions. Alternatively, pagetic cells may be more sensitive to KNG stimulation due to an intrinsic genetic defect in patients with PD.

HSPs have been implicated with anti-apoptotic role in mammalian cells. Recent evidence further indicates that rapid phosphorylataion of HSP27 is required for cell adhesion and suppression of apoptosis in renal epithelial cells [de Graauw et al., 2005]. In the present study, KNG-induced HSP27 activation and suppression of etoposide-induced caspase-3 activity suggest antiapoptotic role for KNG in pagetic marrow stromal/preosteoblastic cells. In support of our results, recently Kaschina et al. (2004), demonstrated that KNG deficiency results in enhanced caspase-3 mediated cellular apoptosis. In summary, KNG modulate pagetic bone marrow stromal/preosteoblast cell proliferation through ERK signaling pathway and suppress etoposide-induced apoptosis through enhanced HSP27 phosphorylation. Therefore, enhanced levels of KNG in patients with PD further implicate a pathophysiologic role for KNG in PD.

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