H-Ras/Mitogen-activated Protein Kinase Pathway Inhibits Integrin-mediated Adhesion and Induces Apoptosis in Osteoblasts*

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We have studied the relevance of H-Ras and its downstream effectors to osteoblast functions. 1) Purified human osteoblasts highly expressed integrins β1, αv, α5, α6, and the activation epitope of β1. However, these molecules were markedly down-regulated on osteoblasts transfected with expression vector encoding fully activated H-RasV12, H-RasV12T35S, activating Raf-1/mitogen-activated protein kinase (MAPK), or an active Raf-1 but not on cells having H-RasV12Y40C, a phosphoinositide 3-kinase (PI3K)-binding mutant. 2) Although osteoblasts transfected with expression vector encoding fully activated H-RasV12, H-RasV12T35S, or Raf-1 became annexin-V high/PIlow after 24 and 72 h incubation, the expression of H-RasV12, H-RasV12T35S, or Raf-1 failed to proliferate, whereas those with H-RasV12Y40C proliferated well. 3) Osteoblasts bearing H-RasV12, H-RasV12T35S, or Raf-1 failed to proliferate, whereas those with H-RasV12Y40C proliferated well. (4) The up-regulation of Fas and down-regulation of Bcl-2 were observed in osteoblasts expressing H-RasV12, H-RasV12T35S, or Raf-1. (5) Most of the cells having H-RasV12, H-RasV12T35S, or Raf-1 became annexin-Vhigh/propidium iodide (PI)high or low and terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL)high/PIlow after 24 and 72 h incubation, respectively. Thus, we propose that H-Ras signals followed by Raf-1/MAPK pathway but not PI3K not only reduces β1-mediated adhesion of osteoblasts to matrix proteins but induces apoptosis presumably through the Fas up-regulation and Bcl-2 down-regulation.

Various biological processes such as proliferation, apoptosis, adhesion, cytokine production, and chemotaxis are tightly regulated by intracellular signaling. Recent evidence indicates that small G proteins (guanine nucleotide-binding regulatory proteins) control signaling pathways critical for such diverse cellular functions by activating multiple effector molecules. Bone metabolism in health and disease is based on a self-regulating cellular event. Osteoblasts play a central role in bone formation by synthesizing multiple bone matrix proteins and differentiating into bone cells and also regulate osteoclast maturation by producing bone-resorbing cytokines and by directing cell attachment, resulting in bone resorption (1–4). Such diverse functions of osteoblasts are induced or regulated by multiple soluble factors including growth factors, hormones, and prostaglandins. Recent findings imply that many critical factors such as estrogen, parathyroid hormone, glucocorticoid, prostaglandins, tumor necrosis factor-α, and chemokines control osteoblast functions by binding to their cognate G-protein-coupled receptors and/or subsequent small G-proteins-mediated signaling (5–9). However, little is known regarding the mechanisms of the integration of G-proteins in controlling osteoblast functions.

Among several small G-proteins, Ras has been characterized as a central molecule for the regulation of signal transduction pathways in various types of cells (10–14). It is noteworthy that Ras proteins both physically and functionally couple with multiple effectors including Raf-1/mitogen-activated protein kinase (MAPK),1 Raf guanine nucleotide dissociation stimulator, phosphoinositide 3-kinase (PI3K), protein tyrosine kinases, and small GTPases. Namely, Ras proteins are molecular switches similar to a “hub,” which radiates multiple signaling pathways critical for diverse cellular functions. This is a dynamic phenomenon involving an array of protein-protein interactions modulated by chemical modifications, structural rearrangements, and intracellular relocalizations. Thus, Ras proteins are activated by multiple extracellular stimuli and are involved in regulatory biological processes from the outside of the cell to its interior through a complex array of downstream effectors, thereby controlling a variety of cellular responses such as proliferation, apoptosis, adhesion, and cytokine/matrix production. However, the relevance of Ras to signaling and functions in osteoblasts remains unclear.

We have previously reported that H-Ras plays a pivotal role in integrin-mediated adhesion and proliferation of lymphocytes (15, 16). Here we have studied the relevance of H-Ras and its downstream effectors to functions of osteoblasts by shedding light upon the difference of Raf-1/MAPK and PI3K. The current report demonstrates that H-Ras/Raf-1/MAPK pathways might be involved in down-regulation of integrins and integrin-mediated adhesion of osteoblasts to matrix proteins, whereas PI3K pathways might be involved in modulating cell cycle progression and cell death.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; OC, osteocalcin; COLL-I, type I collagen; ALP, alkaline phosphatase; mAb, monoclonal antibody; IL, interleukin; ERK, extracellular signal-regulated kinase; Ab, antibody; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; ABC, antibody; anti-body; FN, fibronectin; LM, laminin; VN, vitronectin; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling; LFA, leukocyte function-associated antigen.
ated adhesion to matrix proteins as well as induction of apoptosis presumably via Fas/Bcl-2 systems in human-purified osteoblasts.

EXPERIMENTAL PROCEDURES

Purification of Human Osteoblastic Cells—Osteoblast-like cells were purified from metaphyseal trabecular bone in the proximal femur of five osteoarthritis patients during total hip arthroplasty by the established procedures of Russell and colleagues (17–19). All five patients were female (mean age: 57.3 ± 8.6 years). After removing pieces of cortical bone, articular cartilage, and soft connective tissue, the fragments were cut into small pieces and washed extensively. The bone explants were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS) (GIBCO) in 25-cm² culture flasks (Falcon, Lincoln Park, NJ) at a humidified 5% CO₂ atmosphere. When cell monolayers were confluent after the 6–8-week culture, the explants were removed and the cells were replated and incubated, which resulted in new cellular outgrowth and eventually a confluent monolayer of cells. At confluence, the cells were trypsinized, passed at a 1:3 split ratio, and recultured. The medium was changed twice each week, and the cells were used after 3–7 passages. The obtained cells showed a flattened polygonal shape with multiple spindles and possessed characteristics of osteoblast-like phenotype including osteocalcin (OC), bone sialoprotein, type I collagen (COL-1), and bone alkaline phosphatase (ALP) as described previously (18).

Antibodies and Other Reagents—The following monoclonal antibodies (mAbs) were used for detection of immunoglobulin Ig (in the preparation of staining and analysis of cell surface or cytoplasmic molecules and adhesion assays as follows: control mAb thy-1.2 (ATCC, Manassas, VA); human integrin β1 (CD29) mAb MAB13; human αv (CD49f) mAb MAR16 (provided by K. Yamada, National Institutes of Health, Bethesda, MD); human αv (CD49d) mAb NIH49d-1; human αv (CD49f) mAb NIH49f-1 (provided by S. Shaw, National Institutes of Health); anti-tightly binding sites of human integrin β1 mAb HUTS-21 (provided by F. Sanchez-Madrid, The Princess Hospital, Madrid, Spain) (20); human integrin β2 (CD29) mAb Lia1 (Immunotech, Marseille, France); human integrin β3 (CD49e) mAb P1B7 (Fuji, Japan); anti-human bone ALP mAb ALP-mAb (provided by M. Miura, Mitsubishi Chemical BCI, Tokyo, Japan); anti-human OC mAb 10B (provided by K. Hosoda, Teijin, Tokyo, Japan); anti-human IL-6 mAb M2Q-13A5 (Fujisawa); anti-human P50 (CD95) mAb DX2 (Fujisawa); and Bcl-2 mAb Ab-1 (Cosmo-Bio, Tokyo, Japan).

The obtained oligonucleotides, a human active form of H-Ras expression plasmid, pEF-BOS-HA-RasV12 and a human Raf-1-binding/matrix cellular signal-regulated kinase (ERK) subfamily of MAPK-activating form of H-Ras expression plasmid, pEF-BOS-HA-RasV12T35S, a human Raf-1-non-binding form of H-Ras expression plasmid, pEF-BOS-HA-RasV12E37G, a human P54k binding/activating form of H-Ras expression plasmid, pEF-BOS-HA-RasV12T40C, and a human active form of Raf-1 expression plasmid, pEF-BOS-HA-Raf-1 were introduced into osteoblasts using a cationic lipid-mediated transfection method (21–23). Oligonucleotides and plasmids were mixed with 5 μl of LipofectAMINE 2000 (Invitrogen) and incubated for 15 min at room temperature. The oligonucleotide and liposome complex was added to osteoblasts plated in a 6-well culture dish (3 × 10⁵ cells/well, Falcon) and incubated in a 10% FCS containing DMEM for 24–72 h. The concentration of oligonucleotides in the conditioned medium was 2.2 μM, and the expression of each H-Ras was confirmed by staining with anti-HA Ab using flow cytometer. The transfection efficiency of pEF-BOS-HA-RasV12 into osteoblasts was 50–80% detected by anti-HA Ab and differed among donors. Marked difference of the transfection efficiency among all of the used mutants of H-Ras was not observed in COS cells (data not shown).

Flow Microfluorometry—Staining and flow cytometric analyses of osteoblasts with or without transfaction of several plasmid as mentioned above were performed using a FACScan (BD Pharmingen) and standard procedures as described previously (26). 2 × 10⁵ cells were incubated with negative control mAb thy-1.2, integrin β1 mAb, anti-ligand binding sites of β1 mAb, αv mAb, αv mAb, αv mAb, αv mAb, αv mAb, αv mAb, anti-Fas mAb in FACS medium containing of Hank’s balanced salt solution (SB, Tokyo, Japan), 0.5% human serum albumin (HAS) (Yoshitomi, Osaka, Japan), and 0.2% NaCl (Sigma) for 30 min at 4°C. The cytoplasmic antigens of osteoblasts, which were pretreated with cell permeabilization kit (Caltag, Burlingame, CA), were stained with anti-Bcl-2 mAb, anti-OC mAb, anti-ALP mAb, or anti-IL-6 mAb in FACS medium for 30 min at 4°C. After washing the cells three times with FACS medium, they were further incubated with fluorescein iso-

RESULTS

Expression of H-RasV12, H-RasV12T35S, or Active Raf-1 Reduced Expression of Integrins β1, αυ, αυ, αυ, and Activated Epitope of β1 on Osteoblasts—Initially, we assessed the ability of H-Ras and its mutants to regulate integrins on osteoblasts that were transfected ectopically in the cells. Purified human osteoblastic cells (osteoblasts) were then highly expressed an integrin β1, as recognized by anti-convexity mAb CD29 (27) and MAB13 (Fig. 1). However, the expression of β1 was decreased on osteoblasts expressing H-RasV12 but not on those expressing mock plasmid. β1 requires an active configuration to bind to its ligand, a process that can be induced by a variety of stimuli, and can be assessed by HUTS-21 mAb, which reacts with a ligand-induced binding site located on the β1 (20). Osteoblasts spontaneously expressed the ligand binding sites of
\[ \text{H-Ras MAPK on Osteoblasts} \]

**FIG. 1.** Integrin \( \beta_1 \), \( \alpha_\tau \), \( \alpha_\delta \), and \( \alpha_6 \) were inhibited by expressing H-Ras\(^{V12} \) on osteoblasts. Osteoblasts transfected with or without the expression vectors encoding H-Ras\(^{V12} \) or a mock plasmid were analyzed for the expression of \( \beta_1 \) (CD29) as recognized by MAB13 mAb (A), an activated form of \( \beta_1 \) as recognized by HUTS-21 mAb (B), \( \alpha_\tau \) (CD49d) by NIH49d-1 mAb (C), \( \alpha_6 \) (CD49e) by MAB16 mAb (D), \( \alpha_\delta \) (CD49c) by P1B5 mAb (E), and \( \alpha_6 \) (CD49f) by NIH49f-1 mAb (F) using flow cytometry. The data are expressed as the mean percentage and mean \( \pm \) S.E. of the number of molecules expressed per one cell calculated by standard QIFKIT beads from five different experiments using five different donors.

**FIG. 2.** \( \beta_1 \) and an activated form of \( \beta_1 \) were inhibited by expressing H-Ras\(^{V12} \) on osteoblasts. Osteoblasts transfected with or without the expression vectors encoding H-Ras\(^{V12} \), H-Ras\(^{V12}\)Y40C, H-Ras\(^{V12}\)T35S, H-Ras\(^{V12}\)E37G, or active Raf-1, respectively, were analyzed for the expression of \( \beta_1 \) (A) and an activated form of \( \beta_1 \) using flow cytometry (B). The data are expressed as the mean percentage and the mean \( \pm \) S.E. of the number of molecules expressed per one cell calculated by standard QIFKIT beads from three different experiments using three different donors.

\[ \text{H-Ras MAPK on Osteoblasts} \]

\[ \beta_1 \] as recognized by HUTS-21 mAb, whereas osteoblasts bearing H-Ras\(^{V12} \) but not mock plasmid expressed significantly lesser amounts of the sites on the cell surface. The expression of \( \alpha \) subunits was also screened on osteoblasts. Osteoblasts expressed \( \alpha_\tau \) (CD49c), a receptor for LM; \( \alpha_\delta \) (CD49d), a receptor for FN and vascular cell adhesion molecule-1; \( \alpha_6 \) (CD49e), a receptor for FN; and \( \alpha_6 \) (CD49f), a receptor for LM. The expression of \( \alpha_\tau \), \( \alpha_\delta \), and \( \alpha_6 \) but not \( \alpha_\tau \) was reduced on osteoblasts expressing H-Ras\(^{V12} \), whereas these \( \alpha \) chains on osteoblasts expressing mock plasmid was comparable with \( \alpha \) chains on control osteoblasts.

Because H-Ras exhibits its actions through binding to a set of effector proteins involved in Ras/Raf-1/MAPK and Ras/Pi3K pathways (10–14), it important to determine which Ras effector is required to induce \( \beta_1 \). It is noteworthy that osteoblasts expressing H-Ras\(^{V12}\)T35S mutant, which selectively binds to Raf-1 and activates Raf-1, reduce the expression of not only \( \beta_1 \) but also an activated form of \( \beta_1 \) (Fig. 2). In contrast, the expression of both \( \beta_1 \) and an activated form of \( \beta_1 \) on the cells expressing a H-Ras\(^{V12}\)Y40C mutant that selectively binds to P13K and a H-Ras\(^{V12}\)E37G mutant that does not bind to Raf-1 was comparable with their spontaneous expression on osteoblasts expressing a mock plasmid. Furthermore, osteoblasts expressing an active Raf-1 reduced the expression of \( \beta_1 \) and an activated form of \( \beta_1 \). These results imply that H-Ras signals, especially those mediated by Raf-1/ERK pathway, reduces \( \beta_1 \)-mediated adhesion of osteoblasts to matrix proteins such as FN, LM, and VN.

**Intracellular Expression of ALP, OC, and IL-6 Was Not Changed in Osteoblasts and Osteoblasts Expressing H-Ras Mutants**—The expression of intracellular ALP, OC, and IL-6 in osteoblasts was assessed. To this end, osteoblasts expressing H-Ras\(^{V12} \), H-Ras\(^{V12}\)Y40C, or H-Ras\(^{V12}\)T35S were fixed by formaldehyde and permeabilized by saponin and subsequent staining, and flow cytometric analyses of the cells were performed with the indicated mAbs using FACScan. Control osteoblasts and osteoblasts transfected with the expression vectors encoding H-Ras\(^{V12} \), H-Ras\(^{V12}\)Y40C, or H-Ras\(^{V12}\)T35S,
respectively, exhibited similar levels of intracellular ALP, OC, and IL-6 (Fig. 4). The secretion of IL-6 from osteoblasts expressing H-RasV12, H-Ras V12Y40C, or H-Ras V12T35S did not change (data not shown). These results suggest that not only the differentiation of osteoblasts but also the production of one bone-resorbing cytokine IL-6 is not affected by the expression of H-Ras or its mutants.

Expression of H-RasV12 or H-RasV12T35S Suppressed Proliferation of Osteoblasts—The proliferation assay of control osteoblasts or osteoblasts transfected with the expression vectors encoding H-RasV12, H-RasV12Y40C, or H-RasV12T35S, respectively, with cell permeabilization kit, staining and flow cytometric analyses of the cells were performed with anti-human bone-type ALP mAb ALP-mAb, anti-OC mAb 10B and anti-IL-6 mAb, and fluorescein isothiocyanate-conjugated second Ig using FACScm. The data are expressed as the mean percentage and mean ± S.E. of the number of molecules expressed per one cell calculated by standard QIFKIT beads from three different experiments using three different donors.

Intracellular expression of ALP, OC, and IL-6 in osteoblasts. After treating the osteoblasts or osteoblasts transfected with the expression vectors encoding H-RasV12, H-RasV12Y40C, or H-RasV12T35S, respectively, with cell permeabilization kit, staining and flow cytometric analyses of the cells were performed with anti-human bone-type ALP mAb ALP-mAb, anti-OC mAb 10B and anti-IL-6 mAb, and fluorescein isothiocyanate-conjugated second Ig using FACScm. The data are expressed as the mean percentage and mean ± S.E. of the number of molecules expressed per one cell calculated by standard QIFKIT beads from three different experiments using three different donors.

Expression of H-RasV12 or H-RasV12T35S Reciprocally Regulated of Expression of Fas and Bcl-2—Fas is known to be involved in apoptosis, whereas Bcl-2 is essential to proliferative responses (28). These molecules on osteoblasts expressing H-RasV12, H-RasV12Y40C, H-RasV12T35S, or active Raf-1 were observed with anti-Fas mAb DX2 or anti-Bcl-2 mAb Bcl-2/100 using FACScan. Control osteoblasts expressed both cell surface Fas and intracellular Bcl-2 (Fig. 6). Of note, the expression of H-RasV12, H-RasV12T35S, or an active Raf-1 further augmented Fas expression on osteoblasts, whereas it completely inhibited intracellular Bcl-2. In contrast, the expression of H-RasV12Y40C did not change Fas and Bcl-2 levels in osteoblasts. Taken together, our observation of increased expression of Fas and reduced expression of Bcl-2 in osteoblasts expressing H-RasV12, H-RasV12T35S, or an active Raf-1 suggests that the cells might be apoptotic at least partially mediated by Fas.

Expression of H-RasV12T35S Induced Apoptosis of Osteoblasts—Accordingly, we assessed apoptotic features of control osteoblasts and osteoblasts expressing H-RasV12T35S. Annexin-V/PI-staining indicates that the majority of control osteoblasts expressing H-RasV12T35S were annexin-Vhigh/PIlow, namely early apoptotic after 24 h incubation (Fig. 7). The percentage of annexin-Vhigh/PIlow osteoblasts expressing an active Raf-1 was also significantly increased within 24 h (Table I). Furthermore, PI/TUNEL staining of the osteoblasts indicates that half of the osteoblasts expressing H-RasV12T35S were
The main findings obtained in this study are as follows. 1) Osteoblasts adhere to matrix protein such as FN, LM, and VN in a \( \beta_1 \) integrin-dependent manner. However, the osteoblasts transfected with the expression vector encoding fully activated H-Ras\(^{V12} \) mutant or H-Ras\(^{V12T}35S \), which selectively binds to Raf-1 and activates Raf-1/MAPK, failed to adhere to them, whereas the expression of H-Ras\(^{V12Y}40C \) mutant, which selectively binds to PI3K, or H-Ras\(^{V12E}37G \) mutant, which does not bind to Raf-1, did not affect the adhesion. 2) The expression levels of cell surface \( \beta_1, \alpha_4, \alpha_5, \) and \( \alpha_6 \) and of ligand-binding activation epitope of \( \beta_1 \) were decreased on osteoblasts expressing H-Ras\(^{V12} \), H-Ras\(^{V12T}35S \), or an active Raf-1. 3) The expression of any of the H-Ras mutants did not affect on the intracellular expression of ALP, OC, and IL-6 in osteoblasts. 4) The osteoblasts expressing H-Ras\(^{V12} \), H-Ras\(^{V12T}35S \), or active Raf-1 failed to proliferate, whereas control osteoblasts and osteoblasts expressing H-Ras\(^{V12Y}40C \) or H-Ras\(^{V12E}37G \) proliferated well. 5) The up-regulation of Fas and down-regulation of Bcl-2 were observed in osteoblasts expressing H-Ras\(^{V12} \) or H-Ras\(^{V12T}35S \).

**FIG. 5.** Proliferation of osteoblasts was inhibited by expression of H-Ras\(^{V12T}35S \). Proliferation assay of control osteoblasts or osteoblasts transfected with the expression vectors encoding H-Ras\(^{V12} \), H-Ras\(^{V12Y}40C \), H-Ras\(^{V12T}35S \), H-Ras\(^{V12E}37G \), or active Raf-1 was performed. After these osteoblasts were incubated in DMEM containing 10% FCS for 24 h (open bar) and 72 h (hatched bar), cells were stained with TetraColor One including tetrazolium and electron carrier mixture for detecting cell proliferation. The optical density value was measured by enzyme-linked immunosorbent assay plate reader at 450 nm. The data are expressed as the mean \pm S.E. in osteoblasts from triplicate wells of a representative result among five different donors. OD, optical density.

**TABLE I**

| Osteoblasts                  | Annexin-V\(^{\text{high}}/\text{PI}^{\text{low}} \) cells (%) | %     |
|-----------------------------|-------------------------------------------------------------|-------|
| Without transfection        | 15.7 ± 5.4                                                  |       |
| With H-Ras\(^{V12} \)       | 33.4 ± 8.2**                                                |       |
| With H-Ras\(^{V12T}35S \)   | 41.2 ± 4.6**                                                |       |
| With active Raf-1            | 45.8 ± 16.2**                                               |       |
H-Ras\textsuperscript{V12}T35S compared with control cells. 6) The osteoblasts expressing H-Ras\textsuperscript{V12}, H-Ras\textsuperscript{V12}T35S, or active Raf-1 were apoptotic, because most of them were annexin-V\textsuperscript{high}/PI\textsuperscript{high} to low after 24-h incubation and TUNEL\textsuperscript{high}/PI\textsuperscript{low} after 72-h incubation. Thus, we propose that H-Ras signals, especially those followed by Raf-1/MAPK pathway but not by PI-3K, not only reduces \(\beta_3\)-mediated adhesion of osteoblasts to matrix proteins but induces apoptosis via the Fas up-regulation and Bcl-2 down-regulation.

Regeneration is a process common in keeping homeostasis of several tissues and is also essential to bone metabolism designated bone remodeling. During bone remodeling cycle, osteoblasts play a central role not only in bone formation by synthesizing multiple bone matrix proteins but also in bone resorption by regulating osteoclast maturation and activation (1–4). Integrins are a superfamily of cell surface receptors involved in cell-cell and cell-matrix adhesion. Functional osteoblasts, which adhere to matrix via integrins in the “formation” phase, are achieved by combining the ability to create mechanically functional adhesion to matrices or opposing cells and signal-transducing capabilities. Signals from matrices transduced by integrins play critical roles in regulating gene expression, tissue-specific differentiation, and survival of primary osteoblasts and fibroblasts (2, 29, 30). Such functions of osteoblasts during the remodeling cycle are determined by hormones, cytokines, prostaglandins, and growth factors, most of which transduce signals by binding to their cognate G-protein-coupled receptors and/or subsequent small G-proteins-mediated signaling (5–9). Among several small G-proteins, Ras has been characterized as a central molecule for the regulation of signal transduction pathways in various types of cells (10–14). We here observed that H-Ras signals, especially those followed by Raf-1/MAPK pathway but not by PI-3K, reduces integrins \(\beta_1, \alpha_4, \alpha_6, \text{ and } \alpha_6\) and also inhibits ligand-binding activation epitope of \(\beta_1\) on the surface of osteoblasts and subsequent \(\beta_3\)-mediated adhesion of osteoblasts to matrix proteins without changing the synthesis of matrix proteins and IL-6. H-Ras/Raf-1 pathway appeared to be involved in osteoblast adhesion to FN, VN, and LM, although it did not mediate the adhesion to COLL-I, a major bone matrix component. Although further evidence is required, we suppose that the pathway could function well when osteoblasts encounter with the circumstance in which the ratio of FN, VN, or LM is increased in bone matrix rather than usual COLL-I-enriched matrix.

It was also reported that the expression of an active form of H-Ras and its effector kinase, Raf-1, in CHO cells stably expressing an active chimeric integrin suppressed the function of the chimeric \(\alpha_4\text{-}\alpha_6\), \(\beta_3\), and \(\beta_4\). The suppression of integrin function correlated with the activation of the Ras/Raf/MAPK kinase pathway (31). In contrast, we reported that H-Ras\textsuperscript{V12}Y40C mutant, which binds to PI3K in T cells, induce the activated form of leukocyte function-associated antigen (LFA)-1\(\alpha(\alpha_1\beta_2)\) and LFA-1-dependent adhesion to ICAM-1 (intercellular adhesion molecule 1) and that activation of LFA-1 is inhibited by PI3K inhibitors (15). We also found that the expression of active form of H-Ras induces the activation of the \(\beta_1\) in B cells (16). Accumulating evidence demonstrates that PI3K appears to play a central role in integrin triggering (27, 32, 33). One plausible explanation for such discrepant and complex nature of H-Ras functions can be considered to be that second signals induced by H-Ras may be differently involved in “on and off switch” for integrin triggering. Ras is known to be a hub that radiates multiple signaling pathway including Raf-1/MAPK and PI3K (10). From our findings and others, we propose that H-Ras-sensitive PI3K activation is involved in “on switch” for integrin functions, whereas the H-Ras/Raf-1/MAPK may function as an “off switch” for integrin functions.

We also observed that H-Ras signals, especially those followed by Raf-1/MAPK pathway but not PI3K, inhibits proliferation and induces apoptosis of osteoblasts presumably via the reciprocal regulation of Fas/Bcl-2 expression. Such a regulation of cell survival/apoptosis is an important determinant of the life span of cells in regenerating tissues including bone in which continuous bone remodeling keeps its homeostasis. More than half of the osteoblasts, which initially present at the remodeling sites and complete their bone-forming function, undergo apoptosis, and that the process can be modulated by growth factors and/or cytokines produced in the bone microenvironment and by exogenous administration of glucocorticoids (34, 35). Thus, apoptosis of osteoblasts is a fundamental regulatory event during bone tissue differentiation (36). Fas gene is known to be a target gene of p53 during apoptosis, and the p53 mediates down-regulation of Bcl-2 protein presumably by binding to a cis-acting p53-negative response element located in the 5'-untranslated region of the bcl-2 gene (37). In addition, anti-Fas antibody stimulates apoptosis of human osteoblastic MG-63 cells, and Bcl-2 prevents this change (34). Our observation of increased expression of Fas and reduced expression of Bcl-2 in osteoblasts expressing H-Ras\textsuperscript{V12} or H-Ras\textsuperscript{V12}T35S implies that the cells might be apoptotic at least partially mediated by Fas. Furthermore, the adhesion of osteoblasts to FN is required for the survival of osteoblasts and subsequent bone formation (38). The reduced \(\beta_3\)-mediated adhesion of osteoblasts to matrix proteins such as FN, which is induced by H-Ras/Raf-1/MAPK signals, might further augment apoptotic features of osteoblasts.

The potential importance of the balance between survival and apoptosis of osteoblasts during bone remodeling is well accepted. Taken together, we propose that H-Ras signals, especially those followed by Raf-1/MAPK pathway but not PI3K, not only reduces the expression of functionally active \(\beta_1\) and \(\beta_3\)-mediated adhesion of osteoblasts to matrix proteins but induces apoptosis presumably via the Fas up-regulation and Bcl-2 down-regulation, and that such a regulation of cell cycle

![Fig. 8](https://example.com/figure8.png)
arrest is an important determinant of the life span of cells in regenerating bone in which continuous remodeling keeps its homeostasis. As described, the functions of osteoblasts during the remodeling cycle are tightly regulated by hormones, cytokines, prostaglandins, and growth factors, most of which transduce small G-proteins-mediated signaling. Thus, the regulation of Ras-mediated signaling might also lead to novel pharmacotherapeutic strategies for osteoporosis and other pathologic conditions in which tissue mass diminution has compromised functional integrity.

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