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Insulin-like Growth Factor I Stimulates Proliferation and Fas-Mediated Apoptosis of Human Osteoblasts

Atsushi Kawakami, Tomoki Nakashima,* Masahiko Tsuboi, Satoshi Urayama, Naoki Matsuoka, Hiroaki Ida, Yojiro Kawabe, Hideaki Sakai,† Kiyoshi Migita, Takahiko Aoyagi,‡ Munetoshi Nakashima,§ Kenji Maeda,‡ and Katsumi Eguchi

*The First Department of Internal Medicine and *Department of Hospital Pharmacy, Nagasaki University School of Medicine, Nagasaki 852-8501; †Department of Pharmacology, Nagasaki University School of Dentistry, Nagasaki 852-8588; ‡Department of Orthopedics and §Department of Internal Medicine, National Ureshino Hospital, Saga, Japan*

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***In vitro* studies have shown that insulin-like growth factor I (IGF-I) is a potent growth factor for osteoblasts, although both bone formation and resorption are upregulated by IGF-I *in vivo*. To understand the difference in the action of IGF-I observed *in vitro* and *in vivo* experiments, we examined the effect of IGF-I on the proliferation and Fas-mediated apoptosis of human osteoblasts *in vitro*. Human osteoblastic cell line MG63 and human primary osteoblast-like cells obtained from biopsy specimens were used as human osteoblasts. Cells were cultured with or without various concentrations of IGF-I followed by determination of the proliferative response and Fas-mediated apoptosis. IGF-I dose dependently stimulated the proliferation of cultured human osteoblasts. Both Fas expression and the degree of anti-Fas IgM-induced apoptosis of human osteoblasts was also augmented by IGF-I. Furthermore, the cytotoxicity of Fas ligand (FasL) cDNA transformants against human osteoblasts was increased when IGF-I-stimulated osteoblasts were used as target cells, indicating that stimulation of IGF-I increased functional Fas expression on human osteoblasts as well as their proliferation. The addition of DEVD-CHO, a specific inhibitor of CPP32, to the culture resulted in a significant inhibition of Fas-mediated apoptosis of both unstimulated and IGF-I-stimulated osteoblasts, although it did not affect the proliferative response or Fas expression. Our data suggest that activation of CPP32 is necessary for Fas-mediated apoptosis of human osteoblasts, and treatment of IGF-I increased this signaling pathway. In contrast, regulation of proliferation and Fas expression of the cells were probably not affected by CPP32 activation. Our results suggest that IGF-I acts on cultured human osteoblasts by increasing their proliferation and induction of Fas-mediated apoptosis by neighbouring FasL$^+$ cells such as osteoclasts, thus probably functioning as a local coupling factor in the bone *in vivo*, stimulating both bone formation and resorption.**

Key Words: **osteoblasts, IGF-I, proliferation, Fas-mediated apoptosis, CPP32.**

Abbreviations used: IGF-I, insulin-like growth factor I; FasL, Fas ligand; DEVD-CHO: Asp-Glu-Val-Asp-aldehide.

Bone can remodel itself in response to changing physical demands and repair itself after injury (1-3). It has been known that the volume of bone are determined by the balance between two opposing processes, osteoblastic bone formation and osteoclastic bone resorption (i.e., coupling) (1-3). Therefore, to understand how bone volume changes in physiologic and pathologic conditions, it is important to understand the local mechanisms that regulate osteoblastic bone formation and osteoclastic bone resorption. Among such local mechanisms is the action of growth factors that act as autocrine or paracrine effectors of osteoblastic and osteoclastic proliferation and differentiation (1-3). Among these, IGF-I is a potent growth factor for osteoblasts as shown *in vitro* studies where it increases the proliferation and collagen synthesis of bone cells (1-3). In contrast, the administration of IGF-I in humans increases both serum procollagen peptides and urinary excretion of collagen cross-links (4, 5), indicating IGF-I-induced stimulation of both bone formation and remodeling *in vivo*.

Although the precise mechanisms that control the number of bone cells are not clear at present, the involvement of apoptosis in the regulation of the amount of bone has recently been speculated (6-11). In this regard, we recently showed the importance of Fas-mediated apoptosis of human osteoblasts in regulating osteoblast cell number (10, 11). Cocultivation studies using time-lapse recordings suggested cellular interac-

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tions between osteoblasts and osteoclasts including the retraction of osteoblasts by osteoclasts, lead to bone resorption (12, 13). Since bone formation is coupled to bone resorption, and IGF-I increases bone turnover *in vivo* (4, 5), we speculated that IGF-I does not only increase the proliferation of osteoblasts but stimulates the induction of apoptosis by neighbouring cells such as osteoclasts, thus acting as a local regulator of the bone *in vivo*.

In the present study, we investigated the effects of IGF-I on cultured human osteoblasts, with regard to proliferation and Fas-mediated apoptosis (induced by anti-Fas IgM and FasL cDNA transformants).

MATERIALS AND METHODS

Cell culture. Human osteoblastic cell line MG63 and human primary osteoblast-like cells obtained from normal bone of subjects who underwent corrective surgery for accidental injury, were used in the present study, as described previously by our laboratory (10, 11). None of the patients had any known metabolic bone disease or endocrine disorder. A signed consent was obtained from each patient.

Effect of IGF-I on proliferation of MG63 and primary osteoblast-like cells. The proliferative response of MG63 and primary osteoblast-like cells was determined using a ^{3}H-thymidine incorporation assay. Briefly, the cells (5×10^3/well) were plated in 96-well flat-bottomed microtiter plates (Falcon 3072, Becton Dickinson, Oxnard, CA) in RPMI1640 supplemented with 2% BSA with or without various concentrations of rIGF-I (Fujisawa Pharmaceuticals Co., Osaka) for indicated time intervals. Twenty-four hours prior to the termination of cell culture, each well was pulsed with 0.5 μCi of ^{3}H-thymidine (New England Nuclear, Boston, MA) and harvested on glass filter, using a semiautomatic cell harvester (Labo Mash, Labo Science, Tokyo). The radioactivity of each sample was determined in a liquid scintillation counter (Aloka, LSC-5100, Tokyo). In some experiments, we added a CPP32 specific inhibitor, Ac-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO, 200 μM, Peptide Institute, Inc., Osaka) to the culture medium and the proliferative response of the cells was also examined.

Fas expression and anti-Fas IgM-mediated apoptosis of MG63 and primary osteoblast-like cells. We examined the expression of Fas on cultured MG63 and primary osteoblast-like cells as described previously (10, 11). Briefly, the cells were cultured for indicated time intervals in RPMI1640 supplemented with 2% BSA in the presence or absence of various concentrations of rIGF-I with or without DEVD-CHO (200 μM). After incubation, Fas expression on the cells was detected by an indirect immunofluorescence method using anti-human Fas monoclonal antibody (mAb, IgG1, MBL, Nagoya) and phycoerythrin (PE)-conjugated anti-mouse IgG (MBL) as the second reagent by in a flow cytometer (Epics-Profile-II, Coulter Immunology, Hialeah, FL).

Cultured MG63 and primary osteoblast-like cells were also examined for anti-Fas IgM-induced apoptosis using ^{51}Cr release assay and Hoechst 33258 dye (Wako Pure Chemical Industries, Osaka) staining. ^{51}Cr (Amersham International, Amersham)-labeled MG63 and primary osteoblast-like cells (5×10^3/well) cultured in the presence or absence of various concentrations of rIGF-I with or without DEVD-CHO (200 μM) for indicated time intervals, were incubated for additional eight hours with either control mouse IgM (1,000 ng/ml, Seikagaku Co., Tokyo) or anti-Fas IgM (1,000 ng/ml, MBL) in 96-well flat-bottom microtiter plates (Falcon 3072, Becton Dickinson) in a total volume of 200 μl of RPMI1640 supplemented with 2% BSA. After incubation, the plates were centrifuged, and 100 μl-aliquots of the supernatants were assayed for radioactivity using a gamma counter. The spontaneous release of ^{51}Cr was determined by incubating the target cells with the medium alone, whereas the maximum release was determined by adding Triton X-100 to a final concentration of 1%. The percentage of specific lysis was determined as follows:

$$\text{Lysis (\%)} = \frac{\text{Experimental } ^{51}\text{Cr release} - \text{Spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release} - \text{Spontaneous } ^{51}\text{Cr release}} \times 100$$

Apoptotic cells were detected using Hoechst 33258 dye staining. For this purpose, MG63 and primary osteoblast-like cells treated with either control mouse IgM or anti-Fas IgM were fixed with 2% glutaraldehyde solution for 10 minutes and stained with 0.2 mM Hoechst 33258 dye to visualize the localization of DNA. The cells were examined under a fluorescence microscope to determine fragmentation of nuclei and/or condensation of chromatin (AHB-LB, Olympus, Tokyo).

Cytotoxicity of FasL cDNA transformants against MG63 and primary osteoblast-like cells. Detection of the cytotoxic activity of FasL cDNA transformants (14, FasL K562 transformants, kindly provided by Dr. Paul Anderson, Department of Medicine, Division of Rheumatology, Immunology, and Allergy, Brigham & Women's Hospital, Boston, MA) against human osteoblasts was performed as previously described (10, 11). Briefly, either neo K562 transformants or FasL K562 transformants were co-cultured with ^{51}Cr-labeled MG63 or primary osteoblast-like cells at an effector to target ratio (E/T ratio) of 1 (5×10^3 of MG63 or primary osteoblast-like cells and 5×10^3 of transformants/well) in 96-well microtiter plates (Costar 3779, Cambridge, MA) in a total volume of 200 μl of RPMI1640 supplemented with 5% FBS. Following incubation for 4 hours, the plates were centrifuged, and the percentage of specific release was determined as mentioned above.

Statistical analysis. Data were expressed as mean ± SD. Differences between two values were tested for statistical significance using the Student's *t*-test. A p value less than 0.05 was selected as the level of significance.

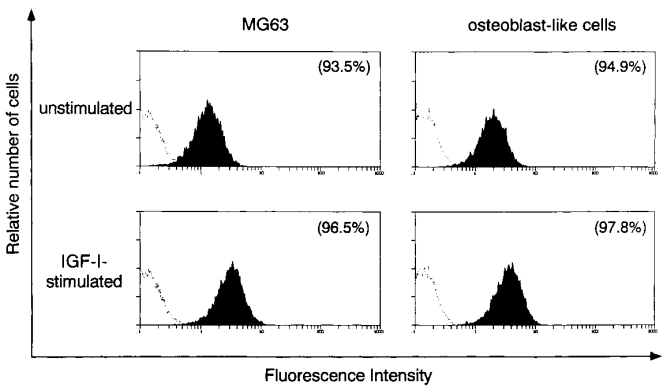

FIG. 1. Effect of IGF-I on Fas expression on human osteoblasts. Human osteoblasts (MG63 or primary osteoblast-like cells) were cultured with or without 50 ng/ml of IGF-I for 48 hours. After incubation, Fas expression of the cells was examined as described in the text. Results shown are representative data of five experiments. *Left panel,* MG63. *Right panel,* primary osteoblast-like cells. *Hatched line,* isotype-matched negative control. *Solid line,* anti-Fas mAb. Numbers in parentheses represent the percentage of positive cells.

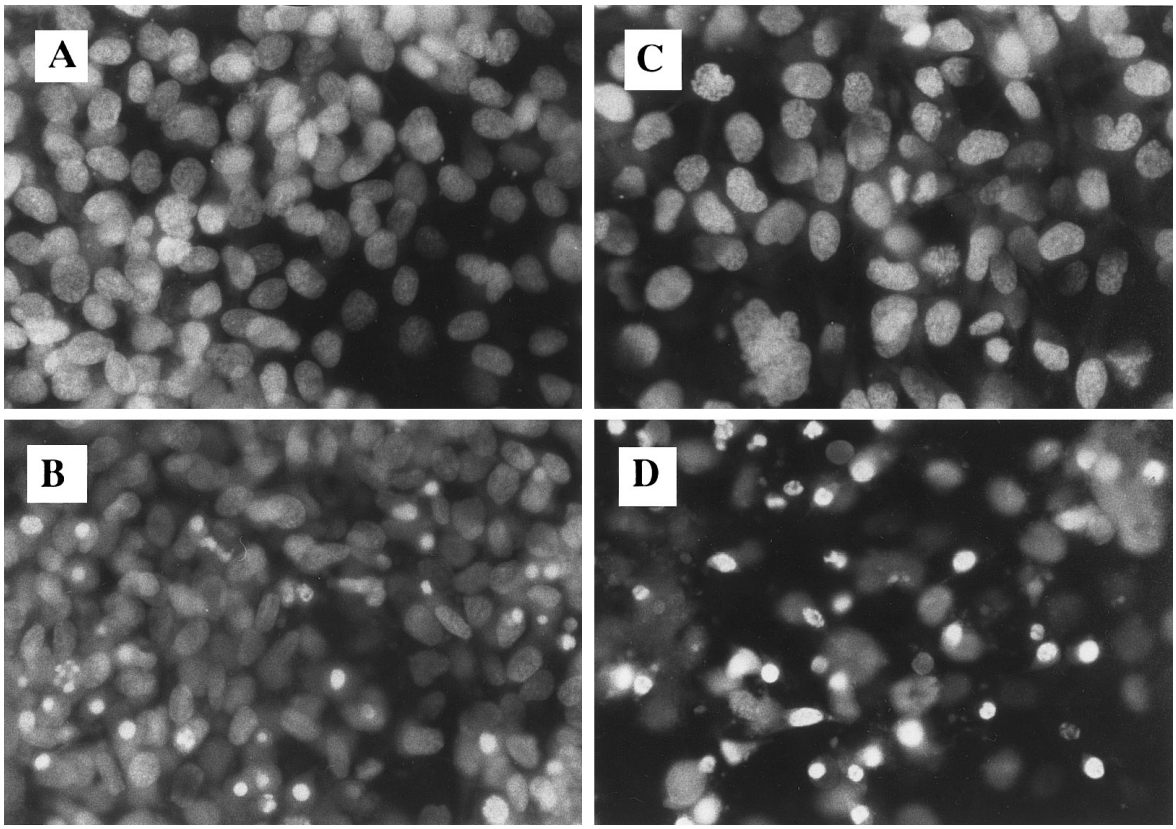

FIG. 2. Fas-mediated apoptosis of MG63 determined by Hoechst 33258 dye staining. MG63 were cultured with or without 50 ng/ml of IGF-I for 48 hours. After incubation, Fas-mediated apoptosis of the cells by anti-Fas IgM was examined as described in the text. (A) Unstimulated MG63 treated with control IgM. (B) Unstimulated MG63 treated with anti-Fas IgM. (C) IGF-I-stimulated MG63 treated with control IgM. (D) IGF-I-stimulated MG63 treated with anti-Fas IgM. Note that apoptotic cells were found in B and D, and the percentage of these cells was greater in D than in B. Magnification: 400×. Results shown are representative data of six experiments.

RESULTS

Effect of IGF-I on Proliferation and Fas-Mediated Apoptosis of Human Osteoblasts

We initially examined the proliferative effect of IGF-I on human osteoblasts, demonstrating that the addition of IGF-I to MG63 significantly increased the proliferation of cells, and the maximum effect was observed when the cells were cultured for 48 hours in the presence of 50 ng/ml of IGF-I (unstimulated: 5495 ± 225 cpm vs IGF-I-stimulated: 14010 ± 505 cpm, respectively). The same results were obtained using primary osteoblast-like cells (data not shown). In addition, culture of MG63 or primary osteoblast-like cells with 50 ng/ml of IGF-I for 48 hours resulted in clear augmentation of Fas expression on these cells (Fig. 1). We next examined Fas-mediated apoptosis of human osteoblasts using anti-Fas IgM. As shown in Fig. 2, the addition of anti-Fas IgM resulted in apoptosis of a small percentage of unstimulated MG63, while treatment with IGF-I significantly increased this process although spontaneous apoptosis of the cells in culture was not found with or without the addition of IGF-I. Similar results were noted following the use of primary osteoblast-like cells (data not shown). These data suggested that treatment of human osteoblasts with IGF-I increased functional Fas expression.

Effect of DEVD-CHO on Proliferation and Fas-Mediated Apoptosis of Human Osteoblasts

We next examined the effect of DEVD-CHO on the proliferative response of human osteoblasts. The addition of DEVD-CHO did not influence the baseline proliferation or IGF-I-induced increased proliferation of MG63 (unstimulated MG63 without DEVD-CHO: 5650 ± 260 cpm vs unstimulated MG63 with DEVD-CHO: 5310 ± 264 cpm, IGF-I-stimulated MG63 without DEVD-CHO: 13950 ± 590 cpm vs IGF-I-stimulated MG63 with DEVD-CHO: 13528 ± 605 cpm, respectively). Furthermore, DEVD-CHO did not change Fas expression on human osteoblasts cultured with or without IGF-I (Table 1). In contrast, DEVD-CHO clearly inhibited Fas-mediated apoptosis of unstimulated human osteoblasts (Table 2). Furthermore, the addition

TABLE 1

Effect of DEVD-CHO on Fas Expression on Cultured Human Osteoblasts

Treatment of cells		Mean fluorescence intensity of Fas expression	
IGF-I	DEVD-CHO	MG63	Primary osteoblast-like cells
−	−	1.85 ± 0.09	2.25 ± 0.10
−	+	1.83 ± 0.1 (NS)	2.22 ± 0.11 (NS)
+	−	3.2 ± 0.17	3.89 ± 0.20
+	+	3.25 ± 0.16 (NS)	3.94 ± 0.18 (NS)

Data are mean ± SD of five experiments. NS: not significant, compared with control [DEVD-CHO (−)].

of DEVD-CHO significantly suppressed Fas-mediated apoptosis of IGF-I-stimulated human osteoblasts (Table 2, Fig. 3).

Cytotoxicity of FasL Transformants against Human Osteoblasts

Finally, we examined the cytotoxic effects of FasL transformants against human osteoblasts. As shown in Table 3, although neo K562 transformants did not kill MG63, FasL K562 transformants showed a clear cytotoxic activity against unstimulated MG63, and the use of IGF-I-stimulated MG63 increased the cytotoxicity. The cytotoxic activity of FasL K562 transformants was significantly suppressed following the addition of hFas-Fc (data not shown) or the use of DEVD-CHO-treated MG63 as target cells (Table 3). Similar results were obtained when primary osteoblast-like cells were used as target cells (data not shown).

DISCUSSION

Although the precise role of apoptosis in bone cells *in vivo* is not clear at present, apoptosis of human osteoblasts was recently demonstrated *in vivo* in humans near joints affected by rheumatoid arthritis (15). Since bone formation rate at local sites primarily depends on the number of osteoblasts (1-3), it is suggested that IGF-I, which is stored in large quantities in the bone (1-3), acts on human osteoblasts, stimulates both the proliferation and apoptosis of these cells, and stimulates bone remodeling *in vivo.*

IGF-I dose-dependently activated the proliferation of human osteoblasts, at concentrations that can be achieved during *n vivo* IGF-I administration (4, 5). We recently showed that Fas-mediated apoptosis was in-

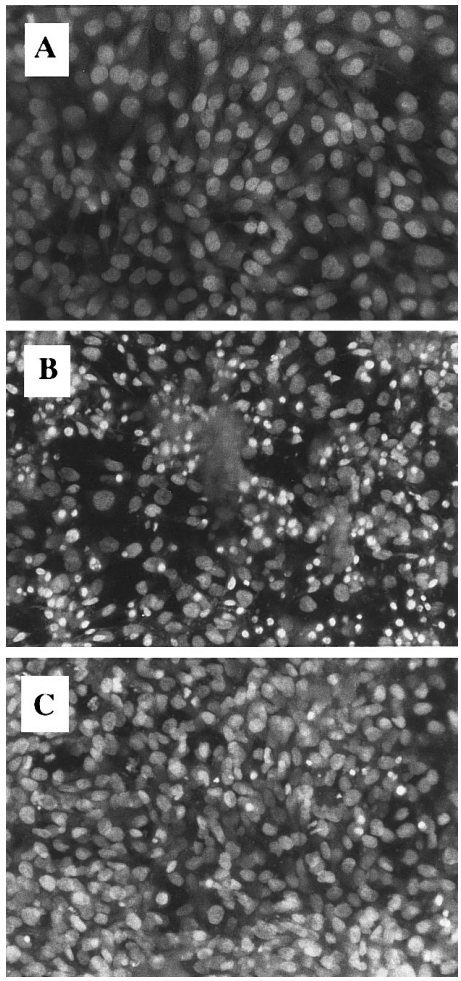

FIG. 3. Inhibition of Fas-mediated apoptosis of MG63 by DEVD-CHO determined by Hoechst 33258 dye staining. MG63 were cultured with 50 ng/ml of IGF-I for 48 hours in the presence or absence of 200 μM of Ac-DEVD-CHO. After incubation, the cells were further cultured with 1 μg/ml of anti-Fas IgM for 8 hours, and apoptotic cell death was examined as described in the text. (A) IGF-I-stimulated MG63 treated with control IgM. (B) IGF-I-stimulated MG63 treated with anti-Fas IgM. (C) MG63 stimulated with IGF-I in the presence of Ac-DEVD-CHO, then treated with anti-Fas IgM. Note that the percentage of apoptotic cells was significantly suppressed by the addition of Ac-DEVD-CHO. Results shown are representative examples of five experiments. Magnification: 400×.

TABLE 2

Inhibition of Fas-Mediated Apoptosis of Cultured Human Osteoblasts by DEVD-CHO

Treatment of cells		^{51}Cr release (%)	
IGF-I	DEVD-CHO	MG63	Primary osteoblast-like cells
−	−	13.5 ± 0.9	15.9 ± 1.2
−	+	3.8 ± 0.3*	4.6 ± 0.4*
+	−	42.8 ± 3.0	45.8 ± 3.2
+	+	12.8 ± 1.1*	13.5 ± 0.9*

Data are mean ± SD of five experiments. *$p<0.01$: compared with Ac-DEVD-CHO (−).

TABLE 3

Cytotoxicity of FasL K562 Transformants against MG63

Effector cells	Treatment of MG63		^{51}Cr release (%)
	IGF-I	DEVD-CHO	
neo K562	−	−	1.82 ± 0.09
	−	+	2.05 ± 0.13
	+	−	2.02 ± 0.11
	+	+	1.95 ± 0.1
FasL K562	−	−	18.5 ± 1.2
	−	+	5.0 ± 0.3*
	+	−	40.6 ± 2.5
	+	+	13.5 ± 0.7*

Data are mean ± SD of five experiments. *$p<0.01$: compared with data of Ac-DEVD-CHO (−).

duced in human osteoblasts *in vitro* although the spontaneous apoptosis of the cells was not determined in culture (10, 11). Spontaneous apoptosis of osteoblasts *in vitro* was not influenced by IGF-I. Therefore, in the next step, we examined the effect of IGF-I on Fas-mediated apoptosis of cultured osteoblasts. Constitutive Fas expression of osteoblasts was determined, and treatment with IGF-I stimulated both Fas expression and anti-Fas IgM-induced apoptosis of human osteoblasts.

Fas-mediated apoptosis of target cells *in vivo* is mediated by $FasL^+$ effector cell populations (16). In addition to anti-Fas IgM, FasL K562 transformants killed Fas^+ human osteoblasts, and the use of IGF-I-stimulated osteoblasts as target cells increased the cytotoxicity. These data suggest that the action of local IGF-I on osteoblasts *in vivo* includes increasing the sensitivity of Fas-mediated apoptosis of the cells by neighbouring $FasL^+$ cells. FasL is preferentially expressed on hematopoietic cells (17-19), and previous studies suggested that the origin of osteoclasts is hematopoietic stem cells (20), suggesting FasL expression on human osteoclasts. Previous studies have indicated the presence of cellular interactions between osteoblasts and osteoclasts, and that the position of osteoblasts could be occupied by osteoclasts following membrane ruffling or blebbing of osteoblasts, representing the morphological characteristics of apoptosis, induced by osteoclasts (12). These data suggest the induction of Fas-mediated apoptosis of human osteoblasts by $FasL^+$ osteoclasts, and that IGF-I-stimulation increased the sensitivity of Fas-mediated apoptosis of osteoblasts by neighbouring osteoclasts. Thus, these results probably represent the underlying mechanisms of the stimulatory effects of IGF-I on bone remodeling following IGF-I administration in humans.

Intracellular signalling pathways through IGF-I receptor are mediated by the activation of intrinsic tyrosine kinase of that receptor, promoting gene expression and cell proliferation (21). In contrast, activation of CPP32 is indispensable for the induction of Fas-mediated apoptosis (22). In the present study, neither the proliferative response nor Fas expression of osteoblasts induced by IGF-I was affected by CPP32 inhibitor, DEVD-CHO. These findings suggest the importance of kinase cascades in the IGF-I receptor in the proliferation and induction of Fas expression on osteoblasts without the activation of CPP32. In contrast to the lack of effect on proliferation and Fas expression, the addition of DEVD-CHO significantly suppressed Fas-mediated apoptosis of both unstimulated and IGF-I-stimulated osteoblasts, indicating that CPP32 is a major target molecule signalling Fas-mediated apoptosis of these cells, and that treatment with IGF-I increased this signalling pathway. These results support the notion that the use of certain agents, such as CPP32 inhibitor, could separate the proliferative effect of IGF-I in osteoblasts from the stimulatory effect on Fas-mediated apoptosis of these cells. Such treatment may provide a potentially new therapeutic use of IGF-I in patients with osteoporosis since it may allow a net proliferation of osteoblasts.

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REFERENCES

1. Baylink, D. J., Finkelman, R. D., and Mohan, S. (1993) *J. Bone Miner. Res.* **8,** S565–572.
2. Hayden, J. M., Mohan, S., and Baylink, D. J. (1995) *Bone* **17,** s93–98.
3. Conover, C. A. (1996) *in* Principles of Bone Biology (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Eds.), pp. 607–618, Academic Press, San Diego, CA.
4. Johansson, A. G., Linch, E., and Ljunghall, S. (1992) *Lancet* **339,** 1619.
5. Ebeling, P. R., Jone, J. D., O'fallon, W. M., Janes, C. H., and Riggs, B. L. (1993) *J. Clin. Endocrinol. Metab.* **77,** 1384–1387.
6. Kameda, T., Ishikawa, H., and Tsutsui, T. (1995) *Biochem. Biophys. Res. Commun.* **207,** 753–760.
7. Kameda, T., Miyazawa, K., Mori, Y., Yuasa, T., Shiokawa, M., Nakamura, Y., Mano, H., Hakeda, Y., Kameda, A., and Kumegawa, M. (1996) *Biochem. Biophys. Res. Commun.* **220,** 515–519.
8. Kitajima, I., Nakajima, T., Imamura, T., Takasaki, I., Kawahara, K., Okano, T., Tokioka, T., Soejima, Y., Abeyama, K., and Maruyama, I. (1996) *J. Bone Miner. Res.* **11,** 200–210.
9. Kitajima, I., Soejima, Y., Takasaki, I., Beppu, H., Tokioka, T., and Maruyama, I. (1996) *Bone* **19,** 263–270.
10. Kawakami, A., Eguchi, K., Matsuoka, N., Tsuboi, M., Koji, T., Urayama, S., Fujiyama, K., Kiriyama, T., Nakashima, T., Nakane, P. K., and Nagataki, S. (1997) *J. Bone Miner. Res.* **12,** 1637–1646.
11. Nakashima, T., Sasaki, H., Tsuboi, M., Kawakami, A., Fujiyama, K., Kiriyama, T., Eguchi, K., Ichikawa, M., and Nagataki, S. (1998) *Endocrinology,* in press.

12. Ferrier, J., Xia, S-L., Lagan, E., Aubin, J. E., and Heersche, J. N. M. (1994) *J. Bone Miner. Res.* **9,** 1397–1405.
13. Vesely, P., Boyde, A., and Jones, S. J. (1992) *J. Anat.* **181,** 277–291.
14. Ida, H., Robertson, M. J., Voss, S., Ritz, J., and Anderson, P. (1997) *J. Immunol.* **159,** 2154–2160.
15. Kitajima, I., Shao, Q., Soejima, Y., and Maruyama, I. (1997) *in* Program of the 61st Annual Meeting of The American College of Rheumatology, p. S78. (Abstract)
16. Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993) *Cell* **75,** 1169–1178.
17. Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K., and Nagata, S. (1995) *J. Immunol.* **154,** 3806–3813.
18. Arase, H., Arase, N., and Saito, T. (1995) *J. Exp. Med.* **181,** 1235–1238.
19. Hahne, M., Renno, T., Schroter, M., Irmler, M., French, L., Bornand, T., MacDonald, H. R., and Tschopp, J. (1996) *Eur. J. Immunol.* **26,** 721–724.
20. Takahashi, N., Yamada, H., Yoshiki, S., Roodman, G. D., Mundy, G. R., Jones, S. J., Boyde, A., and Suda, T. (1988) *Endocrinology* **122,** 1373–1382.
21. LeRoith, D., Werner, H., Beitner-Johnson, D., and Roberts, C. T., Jr. (1995) *Endocr. Rev.* **16,** 143–163.
22. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380,** 723–726.