Impairment of Bone Healing by Insulin Receptor Substrate-1 Deficiency*

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Takashi Shimoaka‡, Satoru Kamekura‡, Hirotaka Chikuda‡, Kazuto Hoshi‡, Ung-il Chung§, Toru Akune‡, Kenjiro Maruyama‡, Toshitaka Komori‡, Michihito Matsumoto‡, Wataru Ogawa‡, Yasuo Terauchi‡‡, Takashi Kadowaki‡‡, Kozo Nakamura‡, and Hiroshi Kawaguchi‡ ‡‡

From the Departments of Orthopaedic Surgery, Tissue Engineering, and **Metabolic Diseases, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo, Tokyo 113-8655, Department of Molecular Medicine, Osaka University Medical School, Suita, Osaka 565-0871, and Division of Diabetes, Digestive and Kidney Diseases, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

Insulin receptor substrate-1 (IRS-1) is an essential molecule for intracellular signaling of insulin-like growth factor (IGF)-1 and insulin, both of which are potent anabolic regulators of bone and cartilage metabolism. To investigate the role of IRS-1 in bone regeneration, fracture was introduced in the tibia, and its healing was compared between wild-type (WT) mice and mice lacking the IRS-1 gene (IRS-1−/− mice). Among 15 IRS-1−/− mice, 12 remained in a non-union state even at 10 weeks after the operation, whereas all 15 WT mice showed a rigid bone union at 3 weeks. This impairment was because of the suppression of callus formation with a decrease in chondrocyte proliferation and increases in hypertrophic differentiation and apoptosis. Reintroduction of IRS-1 to the IRS-1−/− fractured site using an adenovirus vector significantly restored the callus formation. In the culture of chondrocytes isolated from the mouse growth plate, IRS-1−/− chondrocytes showed less mitogenic ability and Akt phosphorylation than WT chondrocytes. An Akt inhibitor decreased the IGF-I-stimulated DNA synthesis of chondrocytes more potently in the WT culture than in the IRS-1−/− culture. We therefore conclude that IRS-1 deficiency impairs bone healing at least partly by inhibiting chondrocyte proliferation through the phosphatidylinositol 3-kinase/Akt pathway, and we propose that IRS-1 can be a target molecule for bone regenerative medicine.

In efforts to develop more advanced skeletal regenerative medicine through genetic manipulation, we have been attempting to identify genes implicated in bone and cartilage formation in vivo. Healing of bone fracture is composed of complex multistep processes involving a variety of cellular events for bone and cartilage regeneration (1, 2). Under the periosteum adjacent to the fracture gap, undifferentiated mesenchymal cells start differentiation directly to cells of osteoblastic lineage for the membranous ossification, whereas in granulation tissue inside the fracture gap, these mesenchymal cells undergo endochondral bone formation; they differentiate first into chondrocytes to form cartilage which is subsequently replaced by calcified tissues. The size and quality of fracture callus that determine the mechanical property of the fracture site are mostly dependent on the latter process. Because the endochondral bone formation also takes place in embryonic development and in skeletal growth after birth, understanding the molecular mechanism of fracture healing may not only help treat non-union and delayed union of fracture itself but also help advance bone regenerative medicine.

Insulin-like growth factor-1 (IGF-I)1 plays important roles in the anabolic regulation of bone and cartilage metabolism (3). Osteoblasts and chondrocytes produce this growth factor, express its receptor, and respond to it (3, 4). IGF-I appears essential for normal bone development because deletion of IGF-I or its receptor leads to a reduction in bone size at birth (5, 6). Clinically, patients with Laron syndrome caused by IGF-I deficiency exhibit growth retardation and osteoporosis (7). IGF-I is also reported to be expressed during fracture healing and to stimulate it, suggesting a role as an autocrine/paracrine factor potentiating bone regeneration (8, 9). Insulin also plays important roles in the anabolic regulation of bone and cartilage metabolism (10). Although the anabolic effect of insulin on bone may be primarily related to its ability to stimulate osteoblast proliferation, that on cartilage may involve the acceleration of chondrocyte differentiation (11, 12). Patients with insulin deficiency as exemplified by type 1 diabetes mellitus are associated with osteoporosis (13, 14). Diabetes has also been shown to impair fracture healing, which is restored by treatment with insulin in both humans and animals (2, 15, 16).

Both IGF-I and insulin initiate cellular responses by binding to distinct cell-surface receptor tyrosine kinases that regulate a variety of signaling pathways controlling metabolism, growth, and survival. Insulin receptor substrates (IRSs) are essential substrates of the receptor tyrosine kinases, which integrate the pleiotropic effects of IGF-I and insulin on cellular function (17, 18). The mammalian IRS family contains at least four members: ubiquitous IRS-1 and IRS-2, adipose tissue-predominant IRS-3, and IRS-4.

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†† To whom correspondence should be addressed: Dept. of Orthopaedic Surgery, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo 113-8655, Japan. Tel.: 81-3-3815-5411 (ext. 30473 or 33376); Fax: 81-3-3818-4082; E-mail: kawaguchi-ort@h.u-tokyo.ac.jp.

‡‡ To whom correspondence should be addressed: Dept. of Orthopaedic Surgery, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo 113-8655, Japan. Tel.: 81-3-3818-4082; E-mail: kawaguchi-ort@h.u-tokyo.ac.jp.

1 The abbreviations used are: IGF, insulin-like growth factor; IRS, insulin receptor substrate; WT, wild-type; BMC, bone mineral content; HE, hematoxylin-eosin; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; TUNEL, terminal transferase dUTP nick end labeling; AxLacZ, adenovirus vector carrying β-galactosidase gene; DMM, Dulbecco’s modified Eagle’s medium; P53K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; IGF-IR, insulin-like growth factor-1 receptor; SHC, Src homology collagen; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; FBS, fetal bovine serum; TdR, [3H]thymidine; P53K, phosphatidylinositol 3-kinase.
FIG. 1. X-ray features of bone healing in WT and IRS-1-/- mice. A, the fracture model used in this study. After exposing the right tibia of 8-week-old mice, a transverse osteotomy was performed at the midshaft with a bone saw. The bone marrow cavity was then stabilized with an intramedullary nail. B, time course of the fracture healing in representative WT and IRS-1-/- mice. C, X-ray features of the fracture sites of all WT mice (n = 15) and IRS-1-/- mice (n = 15) at 3 weeks after the fracture. Bone union was completed in all 15 WT mice, whereas in IRS-1-/- mice, 12 out of 15 showed non-union (the 12 panels at left).

IRS-3, and IRS-4 which is expressed in the thymus, brain, and kidney. We reported previously that IRS-1 and IRS-2 are expressed in bone (19, 20). Our further studies on bone metabolism of mice lacking the IRS-1 gene (IRS-1-/- mice) or the IRS-2 gene (IRS-2-/- mice) revealed that IRS-1 is important for maintaining bone turnover (19), whereas IRS-2 is important for maintaining predominance of anabolic function over catabolic function of osteoblasts (20). Regarding the role of these molecules on bone growth, IRS-1, but not IRS-2, seems to play an important role in the growth plate function, because IRS-1-/- mice were about 20–30% shorter in limbs and trunk, whereas IRS-2-/- mice were normal in size as compared with wild-type (WT) littermates (19–22). These data raise an interesting possibility that IRS-1 may be essential for endochondral ossification. To assess this possibility, the present study investigated the role of IRS-1 in bone healing and its mechanism by an in vitro fracture model and an in vivo culture system.

EXPERIMENTAL PROCEDURES

Animals—Mice in a C57BL/6CBA hybrid background were generated and maintained as reported previously (21). WT and IRS-1-/- male littermates generated from the intercross between heterozygous IRS-1-/- mice were compared. All experiments were performed according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

Fracture Model—Twenty five male mice at 8 weeks of age were used in each group. Under general anesthesia with pentobarbital (0.5 mg/10 g body weight, Sigma), the bilateral hind limbs were shaved and sterilized. A 15-mm incision was made longitudinally over the right leg, and a blunt dissection of the muscle was made to expose the tibia. The midpoint of the tibia was marked with a surgical marker, and a transverse osteotomy was performed using a bone saw (Volvere GX, Tochigi, Japan). The fracture was repositioned, and then the full-length of the bone marrow cavity was internally stabilized with an intramedullary nail using the inner pin of a spinal needle of 22- or 23-gauge diameter depending on the size of the cavity. After irrigation with saline, the skin was sutured. The left tibia (unfractured side) was sham-operated and an intramedullary nail of the same size as the control was inserted. No external fixation was used, and the animals were allowed unrestricted activity as well as diet and water ad libitum. For histological analyses, animals were killed at 1 week (n = 4/group), 3 (n = 3/group), and 6 weeks (n = 3/group) after the operation by diethyl ether, and bilateral tibiae were excised. After extracting the intramedullary nail gently so as not to injure the fracture site, the soft tissue surrounding the tibia, except for the soft callus around the fracture site, was removed.

Radiological Analysis—X-ray pictures of the right tibiae of WT and IRS-1-/- mice (n = 15 each) were taken at 0 (immediately after the operation), 1–3, 6, and 10 weeks after the operation under general anesthesia using a soft x-ray apparatus (CMB-2; Softex Co., Tokyo, Japan). To determine whether there was bone union, bony bridging on radiographs was evaluated by individuals who were blinded with regard to the genotype of mice.

Measurement of Callus Area and Bone Mineral Content (BMC)—Area and BMC of the entire bilateral tibia were measured by a single energy x-ray absorptiometry utilizing a bone mineral analyzer for small animals (PIXimus, Lunar Co., Ltd., WI) at 0 (immediately after the operation), 1–4, and 6 weeks after the operation. A preliminary experiment revealed that the intramedullary nail did not affect the BMC value. The gain of area and the % gain of BMC during observation periods as compared with those at time 0 were calculated for both fractured and unfractured sides, and the differences were compared between WT and IRS-1-/- mice.

Histological Analysis—Specimens of the harvested tibiae were fixed with 4% paraformaldehyde in 0.1 mol/liter phosphate buffer, pH 7.4, at 4 °C overnight. After decalcification with 4.13% EDTA at 4 °C for 14 days, the tibiae were dehydrated with an increasing concentration of ethanol, embedded in paraffin, and cut into 4-μm-thick sections. The sections were stained with hematoxylin-eosin (HE) or toluidine blue.

Immunohistochemistry—Immunohistochemical localizations of IRS-1, IRS-2, type X collagen, and proliferating cell nuclear antigen (PCNA) were examined in 4-μm dewaxed paraffin sections. After dehydration, the sections were treated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min at room temperature. After blocking by PBS containing 1% bovine serum albumin (Sigma) for 1 h at room temperature, the sections were incubated in polyclonal rabbit antibody against IRS-1, IRS-2, or type X collagen (Santa Cruz Biotechnology) or monoclonal mouse antibody against PCNA (PC10, Sigma) (23), at a dilution of 1:100 for 24 h at 4 °C. As negative controls, we used non-immune rabbit IgG and mouse IgG of the same dilution instead of the primary antibodies. Then the sections were rinsed in PBS and incubated with the horseradish peroxidase-conjugated goat antibody against rabbit IgG (Dakopatts, Glostrup, Denmark) for immunohistochemistry of IRS-1, IRS-2, and type X collagen, and with the horseradish peroxidase-conjugated goat antibody against mouse IgG (EY Laboratories, Inc., San Mateo, CA) for that of PCNA, respectively, for 1 h at room temperature. After washing with PBS, the sections were immersed in a diamobenzidine solution for 10 min at room temperature to visualize immunoreactivity. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using an Apoptosis in Situ Detection kit (Wako Pure Chemical Co., Ltd., Osaka, Japan) according to the manufacturer's instructions.

Generation of Adenoviruses and Gene Transfer—The recombinant adenovirus vector carrying human IRS-1 gene engineered to express hemagglutinin tag at its N terminus was constructed using an Adeno- virus Expression Vector kit (Takara Shuzo Co., Ltd., Shiga, Japan) following the manufacturer's protocol. The adenovirus vector carrying β-galactosidase gene (AxLacZ) was kindly provided by Dr. I. Saito (University of Tokyo). Two days after the operation, a 1 × 1010 plaque-forming units suspension of AxIRS-1 or AxLacZ was injected into the fracture site of IRS-1-/- mice as described previously (24, 25). The same dose of AxLacZ was also injected to WT mice as a positive control. Animals were sacrificed at 1 week (n = 3/group) and 3 weeks (n = 3/group) after the injection. To confirm the infection efficiency, expression of lacZ was examined by histochemical staining by X-gal staining.
buffer (1 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide) (Wako).

Isolation and Culture of Mouse Growth Plate Chondrocytes—Chondrocytes were isolated from epiphyseal growth plates of WT and IRS-1−/− mice at 3.5 weeks of age. Mice were sacrificed, and tibiae were harvested and cleaned of periostium in an aseptic manner. Tibiae were pretreated with 0.3% collagenase in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Sigma) at 37 °C for 5 h, and matrix debris was removed by filtering through a 70-µm cell strainer (BD Biosciences). Chondrocytes were pelleted by centrifugation and washed twice with PBS. Cells were plated in 6-well dishes at a density of 5,000 cells/cm² and grown to confluency in DMEM containing 10% FBS and antibiotics in a humidified CO₂ incubator.

**X-gal Staining of Chondrocytes Isolated from Transgenic Mice with Type I Collagen Promoter or Type II Collagen Promoter Driving the lacZ Gene**—To confirm the purity of chondrocytes isolated by the method above, transgenic mice expressing osteoblast- or chondrocyte-specific marker gene construct (the 2.3-kb fragment of the a1(II) collagen gene promoter or the 1.0-kb fragment of a1(II) collagen promoter and 0.6-kb enhancer) linked to the Escherichia coli lacZ gene were used (26, 27). Expression of lacZ was examined by histochemical staining with X-gal. Cells were isolated from the growth plates of transgenic mice by the method above and were cultured for 2 days. They were rinsed in PBS twice and fixed with 0.25% glutaraldehyde in PBS on ice for 10 min. After fixed samples were washed in PBS, staining was carried out by the X-gal staining buffer described above at 37 °C overnight.

**Western Blot Analysis**—To examine the IRS-1 and IRS-2 protein levels, chondrocytes isolated from WT and IRS-1−/− growth plates described above were plated in 6-well plates at a density of 10⁵ cells/well and incubated in DMEM containing 10% FBS for 24 h. For comparison, we also examined the protein levels in primary osteoblasts that were isolated from neonatal mouse calvariae and cultured in α-minimum Eagle’s medium containing 10% FBS as described previously (28). To investigate the signaling pathways through phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs), primary chondrocytes were pre-incubated in DMEM containing 10% FBS for 24 h, and treated with IGF-I (10 nM) in the presence and absence of LY294002 (10 µM), PD98059 (10 µM), and SB203580 (10 µM) (all from Calbiochem-Novabiochem) for 30 min. Cells were lysed with TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 2 mM Na3VO4, 1 mM aminomethylbenzenesulfonate fluorido, and 10 µg/ml aprotinin), and the protein concentration in the cell lysate was measured using a Protein Assay Kit II (Bio-Rad). Equivalent amounts (20 µg) of cell lysates were electrophoresed by 8% SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% bovine serum albumin, the membrane was incubated with polyclonal rabbit antibodies against IRS-1 and IRS-2 as described above, Akt, phospho-Akt, extracellular signal-regulated kinase (ERK), phospho-ERK, p38, and phospho-p38 MAPK (all from Cell Signaling Technology, Inc. Beverly, MA) and against actin (Sigma). Immunoreactive bands were visualized using the ECL chemiluminescence reaction (Amersham Biosciences) following the manufacturer’s instructions. Signals were quantified by densitometry (Bio-Rad).

**DNA Synthesis and Proliferation Assays**—DNA synthesis and proliferation of isolated chondrocytes were determined by the [3H]thymidine (TdR) uptake and the growth curve, respectively. For the former assay, primary chondrocytes from WT or IRS-1−/− mice were inoculated at a density of 5 × 10⁴ cells/well in a 24-multiwell plate and cultured to confluency in DMEM, 10% FBS for 2 days. Serum was withheld for 12 h before adding the experimental medium with or without IGF-I (10 nM), LY294002 (1, 3, and 10 µM), PD98059 (1, 3, and 10 µM), and SB203580 (1, 3, and 10 µM). Uptake of [3H]TdR (1 Ci/ml in the medium) added for the final 2 h was measured at 18 h. For the growth curve assay, primary chondrocytes from WT or IRS-1−/− mice were inoculated at a density of 10⁶ cells/well in 6-multiwell plates in DMEM, 10% FBS and cultured with or without IGF-I (10 nM). The number of cells/well was counted 1, 3, 5, 7, and 9 days after the seeding.

**Statistical Analysis**—Means of groups were compared by analysis of variance, and significance of differences was determined by post-hoc testing using Bonferroni’s method.

**RESULTS**

**Radiological Findings**—Fig. 1A shows an x-ray feature of the fracture model that we used in this study. This model was confirmed to show the bone healing process similar to that in
humans in a definite temporal sequence by the time course of x-ray examination in WT mice (Fig. 1 B, upper panel). Callus formation could be detected at 1 week, and bony bridging at the fracture site was completed 2 or 3 weeks after the fracture. After the callus size and density reached maximum around 3 weeks, they decreased gradually due to bone remodeling up to 10 weeks. In IRS-1−/− mice, however, neither callus formation nor bridging between the fracture stumps was seen at the early stage, and the fracture site became atrophic without bone union at 10 weeks. Fig. 1C shows x-ray features of the fracture site of all 15 mice in each of WT and IRS-1−/− groups at 3 weeks after fracture. In WT mice, bone union with substantial hard callus formation was observed in all 15 animals. In IRS-1−/− mice, however, fracture healing was extremely impaired, and 12 out of 15 mice showed no bone union (the 12 panels at left). Although bone union was seen in 3 IRS-1−/− mice (the 3 panels at right), the callus looked much smaller and fainter than that of WT mice.

The time course of the number of animals with fracture union determined by bony bridging on x-ray revealed that 4 WT mice achieved bone union at 2 weeks after the operation, and all 15 mice did so at 3 weeks. However, only 3 IRS-1−/− mice showed bone union at 3 weeks, but the other 12 animals remained in a non-union state even at 10 weeks (Fig. 2A).

**Callus Area and BMC**—To quantify the callus formation, differences in the gain of area and the % gain of BMC between the fractured and unfractured tibiae were measured by a bone densitometer (Fig. 2, B and C). Significant differences between WT and IRS-1−/− mice were seen from 1 to 4 weeks in the callus area, and from 2 weeks to the end in the BMC. In WT mice, both parameters were increased at the early stage of healing due to the acceleration of the modeling
process and decreased thereafter due to remodeling. In IRS-1−/− mice, these parameters remained at low levels throughout the observation period. At the later stage in the IRS-1−/− mice, the fracture site became displaced due to non-union, and at 10 weeks more than half the IRS-1−/− mice showed severe displacement which was beyond evaluation. To exclude the possibility of other parts of tibiae outside the fracture site affecting BMC, BMC at the bilateral femurs and distal third tibiae was measured at 3 and 6 weeks. Because there were no differences between the fractured and unfractured sides (data not shown), the BMC decrease in IRS-1−/− mice was caused by the decrease in that of the callus itself.

Histological Findings—To assess the involvement of IRS-1 and IRS-2 in bone healing, we examined the localizations of these proteins at the fracture site in WT and IRS-1−/− mice at 3 weeks after fracture (Fig. 3A). Immunohistochemical analysis of WT callus revealed that IRS-1 was localized at various cells including chondrocytes and fibroblasts, although IRS-2 immunoreactivity was very faint. In IRS-1−/− mice, both IRS-1 and IRS-2 proteins were barely detectable at the fracture site, suggesting that there was no compensatory up-regulation of IRS-2 by IRS-1 deficiency.

Fig. 3B shows the temporal comparison of histology between WT and IRS-1−/− fracture sites. At 1 week after fracture, tissue reaction was seen in the large areas around the fracture gap in WT mice, whereas in IRS-1−/− mice it was markedly decreased, resulting in the reduced size of soft callus. At 3 weeks, mineralized and hard callus due to endochondral ossification was abundantly observed in WT mice, whereas fibrous tissue remained at the fracture gap in IRS-1−/− mice. At 6 weeks, bony union after the remodeling was completed in WT mice. However, in IRS-1−/− mice, fibrous tissue still remained in the fracture gap, indicating a state of non-union. Thus, the impairment of fracture healing in IRS-1−/− mice was seen from the early stage of fracture healing, at which time the required amount of soft callus must be formed. To learn the cellular and molecular mechanisms of impaired bone healing at the early stage due to IRS-1 deficiency, we performed more detailed histological analyses of the fracture callus at 1 week (Fig. 4). Although the size of soft callus was much smaller in IRS-1−/− mice than in WT mice, the chondrocyte differentiation in this small cartilage of IRS-1−/− mice was more advanced than that of WT mice as shown by the toluidine blue staining (Fig. 4, A and B). Type X collagen, a marker for hypertrophic chondrocytes, was much more prevalent in IRS-1−/− cartilage than in WT cartilage (Fig. 4, C and D). We further investigated the proliferation and apoptosis of chondrocytes by PCNA and TUNEL stainings, respectively. PCNA-positive proliferative cells were found massively at the WT callus, although they were faint and scant in the IRS-1−/− callus (Fig. 4, E and F). In contrast, TUNEL-positive apoptotic cells were hardly visible in WT, whereas they were abundant in hypertrophic chondrocytes of IRS-1−/− (Fig. 4, G and H). These findings indicate that the suppression of callus formation in the IRS-1−/− fracture site was associated with a decrease in chondrocyte proliferation and increases in hypertrophic differentiation and apoptosis.

Restoration by Reintroduction of IRS-1 in the IRS-1−/− Fracture—To confirm that the impairment in fracture healing was due simply to the IRS-1 deficiency in these mice, we injected an adenovirus vector carrying IRS-1 gene (AxIRS1) or lacZ (AxLacZ) to the fracture site 2 days after the fracture. The transgene expression was confirmed by the X-gal staining in and around the AxLacZ-injected fracture sites, which was positively observed intracellularly in various differentiation stages of mesenchymal cells (data not shown). AxIRS1 reintroduction to the IRS-1−/− fractured site restored the callus area at 1 and 3 weeks, and the callus BMC at 3 weeks after the injection (Fig. 5A). Although the restorations did not fully reach the levels of the WT callus injected with AxLacZ, AxIRS1 produced a substantial callus at 1 week (Fig. 5B), and all 3 IRS-1−/− mice injected with AxIRS-1 showed bony union at 3 weeks, whereas all those injected with AxLacZ remained in a non-union state. It was therefore confirmed that the IRS-1 deficiency in situ caused the impaired fracture healing.

Analyses of Cultured Primary Chondrocytes—To clarify fur-
ther the cellular mechanisms underlying these abnormalities, we used our original method to isolate chondrocytes from the growth plate. We first confirmed the purity of the growth plate chondrocytes by the X-gal staining of cultured chondrocytes isolated by this method from the transgenic mice expressing the lacZ reporter gene driven by a promoter fragment of type I collagen (COL1-LacZ) or type II collagen gene (COL2-LacZ) (26, 27). More than 99% of the cells isolated from transgenic mice with the type II collagen promoter were stained X-gal-positive, indicating positive lacZ transgene expression, whereas none of the cells from type I collagen promoter-driving transgenic mice exhibited the expression (Fig. 6A). These results demonstrate that almost all cells isolated from the growth plate by this method are cells of chondrocyte lineage and are not contaminated by other cells such as osteoblasts or fibroblasts, which express type I collagen.

To confirm the expression patterns of IRS-1 and IRS-2 as shown in Fig. 3A, we examined the protein levels in the growth plate chondrocytes by Western blotting using the same antibodies as Fig. 3A (Fig. 6B). WT chondrocytes, but not IRS-1-/- chondrocytes, were shown to express the IRS-1 protein; however, neither WT nor IRS-1-/- chondrocytes expressed IRS-2. This was not due to the lack of immunoreactivity of the IRS-2 antibody since IRS-2 protein could be detected in primary osteoblasts, the positive control cells that we studied previously (20).

We then compared DNA synthesis and proliferation of the primary chondrocytes between WT and IRS-1-/- by the [3H]TdR uptake (Fig. 6C) and the growth curve (Fig. 6D), respectively. IRS-1-/- chondrocytes showed significantly reduced [3H]TdR uptake as compared with WT chondrocytes in the control culture (Fig. 6C). Both IGF-I and insulin significantly stimulated the DNA synthesis of WT chondrocytes but not that of IRS-1-/- chondrocytes. On the other hand, the stimulation by FGF-2 was similarly seen in both WT and IRS-1-/- chondrocytes, indicating that impaired DNA synthesis in cultured IRS-1-/- chondrocytes was specific to IGF-I and insulin. The growth curve for 9 days after the seeding confirmed the impaired mitogenic ability of IRS-1-/- chondrocytes (Fig. 6D). The number of these chondrocytes was significantly lower than that of WT chondrocytes at 7 days and thereafter in the control cultures, and the difference was enhanced in the presence of IGF-I.

Signal Transduction in Cultured Primary Chondrocytes—To provide some insight into signaling pathways that are affected by the IRS-1 deficiency in chondrocytes, we compared the activation of PI3K/Akt and MAPKs, the main signals lying downstream of IRS-1 in the WT and IRS-1-/- growth plate chondrocytes (Fig. 7A). Western blot analyses of the WT chondrocytes revealed that IGF-I induced phosphorylations of Akt and ERK but not that of p38 MAPK. Specific inhibitors, LY294002, PD98059, and SB203580, were confirmed to inhibit the phosphorylations of Akt, ERK, and p38 MAPK, respectively. In the IRS-1-/- chondrocytes, the Akt phosphorylation by IGF-I was reduced as compared with that of WT chondrocytes; however, phosphorylations of ERK and p38 MAPK were similar between WT and IRS-1-/- chondrocytes. The decrease in the Akt phosphorylation by the IRS-1 deficiency was confirmed by quantitative analyses using densitometry in independent Western blotting of three separate experiments (Fig. 7B). These results indicate that the IRS-1 deficiency led to impairment of the PI3K/Akt pathway but not the MAPK pathways.

To examine further the involvement of these signaling pathways in the mitogenic action of IGF-I on the WT and IRS-1-/- chondrocytes, the inhibitors above were added to the chon-
DISCUSSION

Although IRS-1 and IRS-2 are known to be essential for intracellular signaling of IGF-I/insulin, these two adaptor molecules have distinct biological roles and are differentially expressed in a variety of cells. Regarding glucose homeostasis, IRS-1 plays an important role in the metabolic actions of insulin mainly in skeletal muscle and adipose tissue, whereas IRS-2 does so in the liver (29, 30). For bone metabolism, although both are expressed in osteoblasts, they play distinct roles in the anabolic function of IGF-I and insulin (19, 20). The present study, however, failed to detect the IRS-2 expression in isolated chondrocytes or fracture callus. Our previous studies also revealed that IRS-2 was not expressed in the epiphyseal cartilage (31) and that IRS-2−/− mice showed normal epiphyseal cartilage and skeletal growth as opposed to IRS-1−/− mice (20). We therefore speculate that IRS-2 is much less important than IRS-1 in bone regeneration through endochondral ossification such as bone healing and skeletal growth. In fact, our preliminary experiment showed no abnormality of bone healing in IRS-2−/− mice (data not shown).

Because histological examinations suggested the central role of abnormal chondrocyte functions in the impairment of bone healing by the IRS-1 deficiency, we focused on the chondrocyte culture to study cellular and molecular mechanisms. For this study, we avoided using the conventional chondrocyte culture system derived from the rib cartilage of neonatal mice (32), because most of the cartilage belongs to the permanent cartilage that does not undergo endochondral ossification but maintains cartilage phenotypes. Instead, we succeeded in isolating chondrocytes from the growth plate with more than 99% purity, as confirmed by the X-gal staining of cultured cells from transgenic mice expressing osteoblast- or chondrocyte-specific reporter gene driven by the type I collagen promoter gene fragment (COL1-LacZ) or the type II collagen promoter gene fragment (COL2-LacZ). LacZ activity was visualized as blue with X-gal staining. B, Western blottings for the IRS-1 and IRS-2 expressions in the growth plate chondrocytes and calvarial osteoblasts derived from WT and IRS-1−/− mice. Antibodies for IRS-1 and IRS-2 (α-IRS-1 and α-IRS-2) were the same as those used in the immunohistochemical analyses (Fig. 3A). C, DNA synthesis determined by [3H]Tdr uptake in chondrocytes isolated from WT and IRS-1−/− growth plates cultured with and without insulin (100 nm), IGF-I (10 nm), or FGF-2 (10 nm) for 18 h. Data are expressed as the mean (bars) ± S.E. (error bars) for 6 wells/group. #, p < 0.05; *, p < 0.01 versus WT culture. D, cell proliferation determined by growth curves of chondrocytes that were isolated from WT and IRS-1−/− growth plates and cultured in the presence and absence of IGF-I. Chondrocytes were inoculated at a density of 105 cells/well in 6-multwell plates, and the number of the cells/well was counted 1, 3, 5, 7, and 9 days after the seeding. Data are expressed as the mean (symbols) ± S.E. (error bars) for 6 wells/group. *, p < 0.01; significant decrease by the IRS-1 deficiency.
Intracellular signaling in the WT and IRS-1<sup>−/−</sup> growth plate chondrocytes. A, effects of IGF-I and specific inhibitors on phosphorylations of Akt, ERK, and p38 MAPK in cultured chondrocytes. The chondrocytes were isolated as above and cultured with and without IGF-I (10 nM) in the presence and absence of an Akt inhibitor LY294002 (LY, 1 μM), an ERK inhibitor PD98059 (PD, 10 μM), and a p38 MAPK inhibitor SB203580 (SB, 10 μM) for 30 min. Western blotting was performed as described under “Experimental Procedures.” B, quantitative analysis of phospho-Akt (p-Akt) levels determined by three independent Western blotting experiments including that above (A). The ordinate axis shows the intensity of the p-Akt band normalized to that of β-actin measured by densitometry. The graph indicates means (bars ± S.E. (error bars)) of the ratio values as compared with that of WT control group in three independent experiments. #, p < 0.05; significant decrease by the IRS-1 deficiency. C, effects of specific inhibitors on the mitogenic action of IGF-I in cultured primary chondrocytes. The primary chondrocytes were cultured with and without IGF-I (10 nM), LY294002 (1, 3, and 10 μM), PD98059 (1, 3, and 10 μM), and SB203580 (1, 3, and 10 μM). After 18 h of culture, DNA synthesis was determined by [3H]TdT uptake. Data are expressed as means (symbols) ± S.E. (error bars) for 8 wells/group, *, p < 0.01, significant inhibition by LY294002, PD98059, or SB203580.

The IRS-1<sup>−/−</sup> callus exhibited not only a decrease in proliferation of chondrocytes but also increases in hypertrophic differentiation and apoptosis (Fig. 4). This is compatible with our histological findings of the IRS-1<sup>−/−</sup> growth plate, which showed a decrease in the height of the proliferating zone and an early closure of the growth plate, resulting in a reduced longitudinal bone growth (31). Although the mechanism of the decrease in chondrocyte proliferation by the IRS-1 deficiency was shown by the growth plate chondrocyte cultures, that of the increase in differentiation or apoptosis remains unclarified. Because chondrocytes are known to start hypertrophic differentiation in synchrony with the cessation of proliferation, the acceleration of chondrocyte differentiation and the subsequent apoptosis seen in the IRS-1<sup>−/−</sup> fracture callus and growth plate might be secondary to the impairment of proliferation. However, there are several reports (33–38) showing that IRS-1 directly inhibits differentiation and apoptosis in hematopoietic and neuronal cells and that these inhibitions are partly associated with an increase in the cell size. Although the present in vivo studies did not find the change of cell size of chondrocytes by the IRS-1 deficiency, the possible involvement of direct action of IRS-1 signaling in differentiation and apoptosis cannot be denied. As mentioned above, studies on these aspects using a culture system of other primary chondrocytes in which both hypertrophy and apoptosis can be properly assessed will be the next task for investigation.

The decreased [3H]TdT uptake in IRS-1<sup>−/−</sup> chondrocytes was seen not only in the stimulated culture by IGF-I or insulin but also in the control culture (Fig. 6C). This may be due to the blockage of signalings of endogenous IGF-I as an autocrine/paracrine factor in the culture. The concentrations of IGF-I in the culture medium were 0.79 ± 0.20 and 0.66 ± 0.15 nM (mean ± S.E.) in the control WT and IRS-1<sup>−/−</sup> cultures, respectively. In addition, our previous study has shown that serum IGF-I levels were similar between WT and IRS-1<sup>−/−</sup> mice, suggesting the absence of systemic compensation for impaired IRS-1 activity (19). Hence, the impaired fracture healing in IRS-1<sup>−/−</sup> mice might be due to the deficit of anabolic signaling of endogenous IGF-I produced by chondrocytes acting as an autocrine/paracrine factor. Impairment of IRS-1<sup>−/−</sup> fracture healing might partly be caused by systemic hormones, of which actions are mediated by IGF-I. Growth hormone is a well known stimulus of IGF-I production in a variety of tissues, including bone, and exerts its effects on bone mainly through IGF-I mediation (39). Parathyroid hormone also increases IGF-I production, and decreases of IGF-I signaling can block selective anabolic actions of parathyroid hormone on bone (40, 41). Similarly, other hormones with effects on bone such as cortisol (42), thyroid hormone (43), estrogen (44), and androgens (45) alter IGF-I levels in bone in a manner consistent with IGF-I playing a role in the actions of these hormones on bone.

It is noteworthy that skeletal phenotypes of IGF-I receptor (IGF-IR) and IGF-I-deficient mice are rather different from that of IRS-1<sup>−/−</sup> mice. First, they exhibit more severe growth retardation than do IRS-1<sup>−/−</sup> mice (5, 6, 46). More important, the closure of the growth plate is delayed in IGF-I<sup>−/−</sup> mice (5, 6), whereas it is accelerated in IRS-1<sup>−/−</sup> mice (31). These discrepancies might be due to the 66-kDa Src homology collagen (SHC), another adaptor protein phosphorylated by the IGF-IR activation, which is reported to be expressed in chondrocytes and osteoblasts (47, 48). SHC is known to associate with growth factor receptor-bound protein 2, p21 Ras, and a serine/threonine kinase cascade leading to the activation of ERK in chondrocytes (47, 49, 50). Our Western blotting data that IGF-I induced phosphorylations of both Akt and ERK, whereas IRS-1 deficiency decreased only the Akt phosphorylation in Fig. 7A, support the existence of SHC/ERK pathway in chondrocytes. Inhibition of this pathway by PD98059 may cause the suppression of the IGF-I mitogenic effect in both WT and IRS-1<sup>−/−</sup>
cultures (Fig. 7C). Most interesting, IGF-I induced the Akt phosphorylation not only in WT but also in IRS-1−/− chondrocytes (Fig. 7, A and B), suggesting the involvement of SHC/Ark pathway in a downstream signaling of IGF-IR activation. This pathway, however, seems functionally less important than IRS-1/Akt and SHC/ERK pathways, because the inhibition by LY294002 was not statistically significant in the IRS-1−/− culture (Fig. 7C). It is also important that the SHC signaling positively regulates apoptosis (51). A decrease in the apoptotic pathway through SHC may cause the delay of the growth plate closure in the IGF-I−/− mice, whereas the compensatory up-regulation of the SHC signaling might lead to accelerated apoptosis of chondrocytes at the fracture callus and the growth plate in IRS-1−/− mice. Studies on the skelental phenotype of the SHC-deficient mice will lead to elucidation of the differential regulation of bone regeneration by IRS-1 and SHC signalings.

We hereby conclude that the IRS-1 deficiency impairs bone healing at least partly by inhibiting the chondrocyte proliferation through the PI3K/Akt pathway, and we propose that IRS-1 can be a target molecule for bone regenerative medicine. There are several recent reports (52, 53) that used bone fracture models to identify in vivo signalings, such as tumor necrosis factor-α and cycloxygenase-2, which play essential roles in the osteogenic process. Because IRS-1 is an intracellular protein, we are planning to use the gene transfer into precursor cells by using conventional natural viruses or nonviral vectors that we are now developing (54). In the present study, the in vivo fracture system and the in vitro chondrocyte culture system were applied for the first time in combination to investigate the role of a certain molecule in knockout mice. This approach seems useful for elucidating a network of molecules implicated in bone regeneration.

REFERENCES

1. Bolander, M. E. (1992) Proc. Soc. Exp. Biol. Med. 200, 165–170
2. Kawaguchi, H., Kurokawa, T., Hanada, K., Hiyama, Y., Tamura, M., Ogata, E., and Matsumoto, T. (1994) Endocrinology 135, 774–781
3. Canalis, E. (1990) Bone (NY) 14, 273–276
4. Haroon, Z. (2001) Mol. Pathol. 54, 311–316
5. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Esfahatian, A. (1993) Cell 75, 59–72
6. Powell-Braxton, L., Hollingshead, L. P., Warburton, C., Dowd, M., Pitts-Meek, S., Dalton, D., Gillette, N., and Stewart, T. A. (1995) Genes Dev. 9, 2009–2117
7. Haroon, Z., Klinger, B., and Silbergeld, A. (1999) J. Bone Miner. Res. 14, 156–157
8. Trippe, S. B. (1998) Clin. Orthop. Relat. Res. 355, S201–S213
9. Schmidmaier, G., Wildemann, B., Heeger, J., Gabelstein, T., Flyvbjerg, A., Bail, H. J., and Raschke, M. (2002) Bone (NY) 31, 165–172
10. Thomas, D. M., Hardie, D. R., Rogers, S. D., Ng, K. W., and Best, J. D. (1997) Endocrinol. Metab. Clin North Am. 4, S–17
11. Shukunami, C., Shigeno, C., Atsumi, T., Ishizeki, K., Suzuki, F., and Hiraki, Y. (1996) J. Cell Physiol. 172, 216–223
12. Piepkorn, B., Kann, P., Forst, T., Andreas, J., Pfutzner, A., and Beyer, J. (1997) J. Biol. Chem. 272, 4349–4356
13. McCarthy, T. L., Centerella, M., and Canalis, E. (1999) Endocrinology 140, 5579–5586
14. Miyakoshi, N., Kasukawa, Y., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., Sekihara, H., Yoshioka, Y., and Komori, T. (2001) J. Cell Biol. 153, 87–100
15. Shimakawa, T., Ogasaewara, T., Yonamine, A., Chikazu, D., Kawano, H., Nakamura, K., Itoh, N., and Kawaguchi, H. (2002) J. Biol. Chem. 277, 7493–7500
16. Brunnin, J. C., Winnay, J., Cheatam, B., and Kahn, C. R. (1997) Mol. Cell. Biol. 17, 1513–1521
17. Yamazaki, T., Uto, K., Tamemoto, H., Ueki, K., Kaburagi, Y., Yamamoto-Honda, R., Takahashi, Y., Yoshiwasa, F., Aizawa, S., Akanuma, Y., Senenbergs, N., Yazaki, Y., and Kadokawa, T. (1996) Mol. Cell. Biol. 16, 3074–3084
18. Hoshi, K., Ogata, N., Shimakawa, T., Terauchi, Y., Kadokawa, T., Kenukoto, S., Chung, U., Ozawa, H., Nakamura, K., and Kawaguchi, H. (2004) J. Bone Miner. Res. 19, 211–222
19. Lefebvre, V., Garfullo, S., Zhou, G., Mesaranta, M., Vuorio, E., and de Crombrugghe, B. (1994) Matrix Biol. 14, 329–335
20. Valentini, B., and Baserga, R. (2001) Mol. Pathol. 54, 133–137
21. Moronne, A., Navarro, M., Rausch, A., Reiss, K., and Baserga, R. (2000) Oncogene 20, 4842–4852
22. Valentini, B., Navarro, M., Zanocco-Marani, T., Edmonds, P., McCormick, J., Moronne, A., Sacchi, A., Romano, G., Reiss, K., and Baserga, R. (2000) J. Biol. Chem. 275, 25451–25459
23. Peruzzi, F., Prisco, M., Desu, M., Saloni, G., Barisali, E., Roman, G., Calabretta, B., and Baserga, R. (1999) Mol. Cell. Biol. 19, 7203–7215
24. Hermangto, U., Zong, C. S., Li, W., and Wang, L. H. (2002) Mol. Cell. Biol. 22, 2345–2365
25. Pende, M., Kezna, S. C., Jaquet, M., Oorschot, V., Burcellin, R., Le Marchand-Brustel, Y., Klumperman, J., Thersens, B., and Thomas, G. (2000) Nature 408, 994–997
26. Okahs, C., Bengtsson, B. A., Isaksson, O. G., Andreasen, T. T., and Slootweg, M. C. (1998) Endocrinol. 190, 59–79
27. Canalis, E., Centerella, M., Borch, W., and McCarthy, T. L. (1989) J. Clin. Invest. 83, 60–65
28. Miyakoshi, N., Kasukawa, Y., Linhart, T. A., Baylink, D. J., and Mohan, S. (2001) Endocrinology 142, 3439–3456
29. McCarthy, T. L., Centerella, M., and Canalis, E. (1990) Endocrinology 126, 1569–1575
30. Herzog, B. K., Golden, L. A., Tarjan, G., Madison, L. D., and Stern, P. H. (2000) Nature 408, 188–197
31. Ernst, M., and Rodan, G. A. (1991) Mol. Endocrinol. 5, 1081–1089
32. Giuri, F., Hoofbauer, L. C., Conover, C. A., and Khosla, S. (1999) Endocrinology 140, 5579–5586
33. Arami, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, B., Johnson, R. S., and Kahn, C. R. (1994) Nature 372, 186–190
34. Shakhbozi, M., John, T., De Souza, P., Rahmanzadeh, R., and Merker, H. J. (1999) Biochem. J. 342, 615–623
35. Cavaziano, P., Palmier, G., Suzuki, A., and Benju, J. P. (1997) J. Bone Miner. Res. 12, 1975–1983
36. Lapczynski, W. (1999) Acta Biochim. Pol. 46, 501–560
37. Benito, M., Valverde, A. M., and Lorenzo, M. (1996) Int. J. Biochem. Cell Biol. 28, 499–510
38. Mignonne, E., Giorgio, M., Mele, S., Pelicci, G., Rebaldi, P., Pandolfi, P., Lanfrancone, L., and Pelicci, G. (1999) Nature 402, 309–313
39. Zhang, X., Schwartz, E. M., Young, A. D., Puzas, J. E., Rosier, R. N., and O’Keefe, R. J. (2002) J. Clin. Invest. 109, 105–115
40. Gerstenfeld, L. C., Cho, T. J., Kon, T., Asakawa, T., Tsay, A., Fitch, J., Barnes, G. L., Graves, D. T., and Einhorn, T. A. (2003) J. Bone Miner. Res. 18, 1584–1592
41. Itaka, K., Harada, A., Nakamura, K., Kawaguchi, H., and Katakai, K. (2002) Biomacromolecules 3, 841–845
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