Decreased c-Src Expression Enhances Osteoblast Differentiation and Bone Formation

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Abstract. c-src deletion in mice leads to osteopetrosis as a result of reduced bone resorption due to an alteration of the osteoclast. We report that deletion/reduction of Src expression enhances osteoblast differentiation and bone formation, contributing to the increase in bone mass. Bone histomorphometry showed that bone formation was increased in Src null compared with wild-type mice. In vitro, alkaline phosphatase (ALP) activity and nodule mineralization were increased in primary calvarial cells and in SV40-immortalized osteoblasts from Src−/− relative to Src+/+ mice. Src-antisense oligodeoxynucleotides (AS-src) reduced Src levels by ~60% and caused a similar increase in ALP activity and nodule mineralization in primary osteoblasts in vitro. Reduction in cell proliferation was observed in primary and immortalized Src−/− osteoblasts and in normal osteoblasts incubated with the AS-src. Semi-quantitative reverse transcriptase-PCR revealed upregulation of ALP, Osf2/Cbfal transcription factor, PTH/PTHrP receptor, osteocalcin, and pro-alpha 2(I) collagen in Src-deficient osteoblasts. The expression of the bone matrix protein osteopontin remained unchanged. Based on these results, we conclude that the reduction of Src expression not only inhibits bone resorption, but also stimulates osteoblast differentiation and bone formation, suggesting that the osteogenic cells may contribute to the development of the osteopetrotic phenotype in Src-deficient mice.

Key words: osteopetrosis • Src • osteoblast • differentiation • bone formation

Introduction

Targeted disruption of the c-src gene in mice has been demonstrated to cause osteopetrosis (Soriano et al., 1991a). Although the sarcoma (Src) gene product is ubiquitous and highly expressed in several cell types, including platelets (Golden et al., 1986) and neurons (Pyper and Bolen, 1990), these mice develop only a bone phenotype (Soriano et al., 1991a). Therefore, much interest has been focused on the role of this gene in bone cells.

The Src family of nonreceptor tyrosine kinases consists of 10 proteins that contribute to many regulated events in cells (Thomas and Brugge, 1997). These proteins share a high homology and consist of a unique NH2-terminal domain containing a short SH4 motif for myristoylation and palmitoylation, a SH3 and a SH2 domain involved in protein–protein interactions, and a COOH-terminal SH1 tyrosine kinase domain (Resh, 1994; Wengling et al., 1997). Several substrates have been demonstrated to be tyrosine phosphorylated by Src, including transmembrane tyrosine kinase receptors (platelet-derived growth factor [PDGF], epidermal growth factor [EGF], and macrophage colony stimulating factor [CSF]-1), focal adhesion associated kinases (focal adhesion kinase, proline-rich tyrosine kinase2, Cdk-activating kinase), adhesion molecules (vinculin), and soluble enzymes (endo-lase, phosphoglyceromutase, and lactate dehydrogenase) (Hunter and Cooper, 1985; Parson and Parson, 1997).
The recognized defect in Src−/− mice consists in the alteration of the bone resorbing cell function. Increased numbers of osteoclasts at the bone surface, which are inactive and lack a ruffled border, are remarkable features of this bone phenotype (Boyce et al., 1992; Horne et al., 1992; Lowe et al., 1993). Impairment of osteoclastic bone resorption leads to decreased bone remodeling, which results in small size, failure in incisor eruption, thickened growth plate, poorly developed cortex, persistence of endochondral primary spongiosa with widening and extension of trabecular bone in the distal metaphysis and diaphysis, and reduced bone marrow tissue that fills the very little remaining space of the bone cavity (Soriano et al., 1991a).

Osteoclast differentiation and bone resorption are also dependent on cells of the osteoblast lineage (Rodan and Martin, 1981; Suda et al., 1997). However, Lowe et al. (1993) demonstrated that osteoblasts derived from c-src knockout mice successfully contributed to normal osteoclast differentiation and showed unremarkable morphological features relative to wild-type mice. This lead to the conclusion that the inherited defect is exclusively with mature osteoclasts and is autonomous from the bone marrow microenvironment. However, a detailed molecular analysis of osteoblast function has not been performed. A recent examination of the skeletal phenotype in older Src−/− mice has indicated that bone mass continues to increase with age, suggesting a continued imbalance between bone resorption and formation (Amling et al., 2000). Therefore, this present study was aimed at determining whether inhibition of Src expression in vivo or in vitro lead to alterations in osteoblast and bone formation, a feature which could contribute to the osteopetrotic phenotype, together with the decreased bone resorption.

Materials and Methods

Materials

DME, FCS, penicillin, streptomycin, and trypsin were from GIBCO BRL. Sterile plasticware was from Becton Dickinson. Anti-Src and anti-actin pAbs and HER-2/cationic secondary antibodies were from Santa Cruz Biotechnology, Inc. Antibody L-123 to osteopontin (Fisher et al., 1995) was donated by Dr. Larry Fisher (Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Synthetic totally phosphorothioated oligodeoxynucleotide (ODN) sequences (Tanaka et al., 1996) were as follows: antisense (AS-src), 5′-GGGCTTGGCTTGTTGCTGCCCAT-3′ and sense (S-src), 5′-ATGGCCAGCAACAAGAAGCAAGCCC-3′. ODNs were purchased from M-Medical. Enhanced chemiluminescence kit was from Amersham Pharmacia Biotech. Primers and reagents for reverse transcriptase (RT)-PCR were from Promega. Benzyl peroxide was from Polyscience Inc. PCR were from Promega. Benzyl peroxide was from Polyscience Inc. All other reagents were of the purest grade from Sigma-Aldrich.

Histology and Histomorphometry

3- and 10-wk-old Src+/+ and Src−/− mice were injected with calcine (20 mg/ kg body weight) followed by the same dose of demeclocycline at a 5-d interval. Animals were killed 2 after the second injection and tibiae were dissected out, fixed in 3.7% formaldehyde in PBS, and embedded in methyamethacrylate by a procedure modified from Baron et al. (1983). Specifically, bones were dehydrated in graded acetone for 1 h in each of 70%, 90%, and twice in 100% acetone then infiltrated and embedded in 80% activated methyamethacrylate (Jowsey et al., 1965), 20% dibutylphthalate, and 4% benzyl peroxide. 5-μm sections were stained with toluidine blue or unstained, with a coverslip on top, for analysis of fluorochrome labels. Histomorphometric analysis of the secondary spongiosa was carried out according to standard procedures (Parfitt et al., 1987) using the Osteomeasure system (OsteoMetrics, Inc.) in a region 200 μm below the chondro-osseous junction to the diaphysis.

Osteoblast Cultures

Primary cultures of calvarial osteoblasts were prepared by a modification of the sequential collagenase/trypsin digestion method (Robey and Termine, 1985). In brief, calvaria were removed from 7–9-d-old CD1 mice or B6, 129/Sv and Src−/− and Src+/+ mice, cleaned free from soft tissue, and digested with 1 mg/ml Clostridium histolyticum type IV collagenase and 0.025% trypsin for 20 min at 37°C in HBSS with gentle agitation. The procedure was repeated three times and cells from the second and third digestions were plated in petri dishes and grown to confluence in DME supplemented with antibiotics and 10% FCS. At confluence, cells were trypsinized by the standard procedure and plated in wells for experiments. The cells obtained with this method were positive for alkaline phosphatase (ALP) activity and expression of the osteoblast markers, parathyroid hormone/parathyroid hormone–related peptide (PTH/PTHrP) receptor and Osf2/Cbfa1 transcription factor, and of bone matrix proteins, osteopontin and bone sialoprotein II.

Immortalized Osteoblasts

Permanent osteoblast cell lines were prepared from Src+/+ and Src−/− mice (B6, 129/Sv) and immortalized by infection with the SV40 virus, according to Soriano et al. (1991b). Cells were grown in DME supplemented with 10% FCS and antibiotics in standard culture conditions. Cells were fed twice a week, trypsinized at confluence, and split 1:5.

ODNs

AS-src and S-src ODNs were diluted in culture medium in 100-μM stock solutions and stored in aliquots at −20°C until used. They were then diluted to the final concentration in medium and administered to the cells for 24 h–3 wk, according to the experimental design. The most effective concentration was 1 μM. Lower concentrations of AS-src had little effect, whereas concentrations >1 μM often resulted in nonspecific changes, which were also observed in S-src-treated cultures. Experiments in which osteoblasts were incubated with a mixture of ODNs and 5 μg/ml of the uptake enhancer lipofectamine resulted in effects similar to cultures incubated with the ODNs alone. Therefore, all the experiments illustrated below were performed at 1 μM of both AS-src and S-src ODNs without uptake enhancer.

Immunoblotting

Cells were lysed in ice-cold 0.1% SDS containing protease inhibitors and 1 mM sodium orthovanadate, and stored at −80°C until used. Protein content was measured using the Bradford method, and then 25–60 μg of cell protein in reducing sample buffer was subjected to 10% SDS-PAGE. Proteins were then transferred to nitrocellulose filter papers and probed with the primary antibody overnight at 4°C, followed by the secondary antibody for 1 h at room temperature. Bands were revealed by enhanced chemiluminescence detection.

RT-PCR

RNA was prepared from osteoblasts using the acid phenol technique. For RT-PCR, 1 μg/ml of total RNA was reverse-transcribed using Moloney-murine leukemia virus RT and the equivalent of 0.1 μg was added to PCR reactions. These were carried out in a final volume of 20 μl buffer containing 200 μM deoxynucleoside triphosphates, 1.5 mM MgCl2, 10 pM of each primer, and 1 U of Thermus aquaticus DNA polymerase. PCR conditions and primer pairs used are listed in Table I. For quantitative analysis, primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used along with the primers for the gene being analyzed. 10–30 μl of the PCR-amplified products were analyzed on 1–1.5% agarose gels containing ethidium bromide.

DNA Staining

To stain the DNA and morphologically evaluate apoptosis, cells were fixed in Carnoy’s fixative (methanol/glacial acetic acid, 3:1), incubated for 30 min in 0.5 μg/ml bis-benzimide, which specifically binds the adenine-thymine regions of the nucleic acid, rinsed twice in distilled water, mounted in glycerol/PBS, 1:1, and observed by conventional epifluores-
At the end of incubation, mineralization was detected with von Kossa staining. Media were then replaced with mineralizing media (DME supplemented with 10% FCS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, with or without 10^{-7} M dexamethasone) containing, where indicated, 1 μM AS-Src or S-Src, and cells were cultured for an additional 3 wk. Media were then replaced with fresh media containing the test compounds every 3 d. As-Src or S-Src, and cells were cultured for an additional 3 wk. Media were then replaced with mineralizing media (DME supplemented with 10% FCS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, with or without 10^{-7} M dexamethasone) containing, where indicated, 1 μM AS-Src or S-Src, and cells were cultured for an additional 3 wk. Media were then replaced with fresh media containing the test compounds every 3 d.

**Cell Proliferation**

Osteoblasts were plated in 24-well multiplates and grown until confluent. Cells were then treated with the test compounds for 48 h and incubated for the final 4 h with 1 μCi/ml [3H]thymidine (specific activity 14.3 Ci/mmole). At the end of incubation cells were solubilized in 1 ml of 1% SDS to which 10 μl of 10 mg/ml BSA was added as a carrier protein, and precipitated by addition of 100 μl of 100% TCA. After incubation for 30 min at 4°C, the TCA-precipitable material was pelleted by centrifugation at 955 x g for 10 min. The TCA precipitate was then dissolved in 5 ml of 10 mg/ml BSA and the radioactivity was measured using a scintillation spectrophotometer. A Scintiverse TCA precipitate was then dissolved in 5 ml of 10 mg/ml BSA and the radioactivity was measured using a scintillation spectrophotometer. A Scintiverse TCA precipitate was then dissolved in 5 ml of 10 mg/ml BSA and the radioactivity was measured using a scintillation spectrophotometer.

**Nodule Mineralization**

Osteoblasts were plated in 24-well multiplates and grown until confluent. Media were then treated with the test compounds for 48 h and incubated for the final 4 h with 1 μCi/ml [3H]thymidine (specific activity 14.3 Ci/mmole). At the end of incubation cells were solubilized in 1 ml of 1% SDS to which 10 μl of 10 mg/ml BSA was added as a carrier protein, and precipitated by addition of 100 μl of 100% TCA. After incubation for 30 min at 4°C, the TCA-precipitable material was pelleted by centrifugation at 955 g. The TCA-precipitate was then dissolved in 5 ml of 10 mg/ml BSA and counted in a scintillation spectrophotometer.

**ALP Activity**

ALP activity was evaluated histochemically and biochemically, using the Sigma kits n.85 and 104-LS, respectively, according to the manufacturer’s instruction (Sigma-Aldrich).

**Densitometric Analysis**

Scanning densitometry of the density of interest was performed using the Molecular Analyst software for the model 670 scanning densitometer (Bio-Rad Laboratories) to obtain the arbitrary density units. Normalization for immunoblotting and RT-PCR was performed using as internal controls the reference genes β-actin and GAPDH, respectively. The “gene of interest/reference gene” density ratio was then computed and represented in graphs.

**Statistics**

Data are expressed as mean percentage ± SEM of at least three independent experiments. Statistical significance was computed by the unpaired Student’s t test. A P value < 0.05 was conventionally considered statistically significant.

**Results**

**In Vivo Histomorphometry**

Histomorphometric analysis of 3- and 10-wk-old Src−/− and Src+/+ mice was carried out to determine whether bone formation was increased in the absence of Src in vivo (Fig. 1). As observed previously, both trabecular bone volume (BV/TV) and osteosurface area (OcS/BS) were significantly increased in Src−/− mice. Bone formation was assessed by examining fluorochrome labels incorporated during mineralization in the same mice and an ~50% increase in bone formation rate (BFR/BS) was observed at both 3 and 10 wk, which, for the most part, was due to an increase in mineralizing surface (MS/BS) rather than a change in mineral appositional rate (MAR). In contrast, the increase in osteoblast surface, expressed as a ratio of bone surface, in Src−/− mice was only significantly elevated at 3 wk.

Since BV/TV and trabecular number (TbN) are both dramatically increased in osteopetrotic Src−/− mice, and since OcS/BS is expressed as a ratio of osteoblast surface to total bone surface, any effect of Src deficiency on the number of osteoblasts may be masked by the large extent of bone surface in Src−/− mice (trabecular bone surface was ~3.4 times that of wild-type mice). To take this change into account, the total number of osteoblasts was also assessed by measuring osteoblast number relative to the area of secondary spongiosa (ObN/TA), as well as the total bone surface lined with osteoblasts (ObPm). Indeed, when the increased trabecular bone surface is taken into account, we found that the total number of osteoblasts, indicated by both ObN/TA and ObPm was significantly increased in Src−/− mice, about twofold at both 3 and 10 wk. As expected, osteoblast number and total osteosurface were both dramatically increased in Src−/− mice. Thus, Src−/− mice exhibit not only a cell autonomous defect in bone resorption (Soriano et al., 1991a; Lowe et al., 1993), but also a dramatic increase in osteoblast numbers and bone formation, changes that would contribute significantly to the osteopetrotic phenotype and explain the continued increase in bone mass in the knockout mice (Am- ling et al., 2000).
ule formation was significantly increased (approximately twofold, Fig 2 b) in Src null primary osteoblasts relative to wild-type cells. No morphological changes were observed in Src\(^{-/-}\) osteoblasts, and DNA staining by bis-benzimide revealed the same numbers of apoptotic cells as the wild-type cells (Src\(^{+/+}\); 8.4 ± 0.7%; Src\(^{-/-}\); 7.7 ± 0.8% of the total number of nuclei; \(n = 3; P = 0.6\)). Therefore, these results suggest that the increased osteoblastic function observed in vivo in Src\(^{-/-}\) mice is cell autonomous.

We then used SV40-immortalized osteoblasts from Src\(^{-/-}\) or Src\(^{+/+}\) animals to further investigate this possibility. As expected, expression of the SV40 large T antigen did not modify the pattern of Src expression, with no protein in Src\(^{-/-}\) cells, whereas normal protein levels were observed in Src\(^{+/+}\) osteoblasts (Fig. 3 a). These immortalized osteoblasts showed low ALP activity in standard culture conditions. However, ALP activity spontaneously increased in long-term cultures, with greater activity observed in Src\(^{-/-}\) versus Src\(^{+/+}\) cells at any time point (Fig. 3 b). In addition, ALP activity was stimulated by treatment with the differentiating agent dexamethasone, with, once again, a higher level of ALP in Src\(^{-/-}\) relative to Src\(^{+/+}\) cells (Fig. 3 b).

In contrast to primary cultures, immortalized Src\(^{+/+}\) cells failed to form mineralized nodules in vitro, independent of time in culture and treatment with dexamethasone (Fig. 3 c). In contrast, Src\(^{-/-}\) cells formed a few small mineralized nodules even in the absence of dexamethasone, whereas in the presence of dexamethasone these cultures underwent macroscopic nodule mineralization clearly visible by week 3 of culture (Fig. 3 c).

Taken together these findings suggest that Src deletion favors osteoblast differentiation and function in vitro and in vivo and in a cell autonomous manner. Therefore, Src may play a negative role in osteoblast differentiation and/or function. To test this hypothesis and elucidate whether the increased osteoblastic function was directly related to Src deletion, we reduced Src expression in normal calvarial osteoblasts harvested from B6, 129\(^{sv}\)/or mice, as described in Materials and Methods, and cultured for detection of ALP activity and nodule mineralization. (a) 90% confluent osteoblasts were cultured for 1 wk, fixed, and stained histochemically to detect ALP activity using the Sigma kit n. 85. ALP-positive cells were counted and converted into a percentage versus the total number of cells. Data are the mean ± SEM of three independent experiments. ***P < 0.001 versus Src\(^{+/+}\). (b) 90% confluent osteoblasts were cultured for 3 wk in the presence of ascorbic acid and \(\beta\)-glycerophosphate, as described in Materials and Methods. Cultures were then fixed and mineralized (dark) nodules were detected by von Kossa staining. Quantitative analysis was performed by scanning densitometry, as described in Materials and Methods. Cultures were then fixed and mineralized (dark) nodules were detected by von Kossa staining. Quantitative analysis was performed by scanning densitometry, as described in Materials and Methods. Cultures were then fixed and mineralized (dark) nodules were detected by von Kossa staining. Quantitative analysis was performed by scanning densitometry, as described in Materials and Methods. Cultures were then fixed and mineralized (dark) nodules were detected by von Kossa staining. Quantitative analysis was performed by scanning densitometry, as described in Materials and Methods. Cultures were then fixed and mineralized (dark) nodules were detected by von Kossa staining. Quantitative analysis was performed by scanning densitometry, as described in Materials and Methods.
rial osteoblasts by the use of AS-src shown previously to inhibit Src synthesis in osteoclasts and affecting Src-dependent functions (Tanaka et al., 1996). In osteoblasts treated for 24 h with 1 μM AS-src or with the control sense (S-src) sequence, we found that AS-src reduced Src protein level by ~60%, whereas the control oligonucleotide was inactive (Fig. 4).

Using this assay, we determined whether treatment with the AS-src would result in alterations of the osteoblast proliferation/differentiation pattern. The incorporation of [3H]thymidine into the TCA-precipitable material derived from osteoblasts exposed to the ODNs for 48 h was significantly decreased by AS-src, but not by S-src, producing a significant 40% inhibition of cell proliferation (Fig. 5 a). A similar reduction of [3H]thymidine incorporation was observed in primary (Fig. 5 b) as well as in SV40-immortalized (Fig. 5 c) Src−/− versus Src+/+ osteoblasts.

The effect of a reduction in Src expression could be to promote differentiation, which would only secondarily decrease cell proliferation. To determine whether inhibition of Src expression could favor osteoblast differentiation, we analyzed specific markers of the osteoblast phenotype. Treatment of osteoblasts with AS-src increased the number of ALP-positive osteogenic cells, whereas S-src was again inactive relative to control (Fig. 6 a). Further confirmation of enhancement of ALP activity was demonstrated by the quantitative analyses, using histochemical (Fig. 6 b) and biochemical (Fig. 6 c) assays, which showed a greater than twofold increase of the enzyme activity in AS-src versus S-src−treated and control cultures.

We then measured nodule mineralization in the presence of the ODNs, which was added repetitively to the medium during the entire length of the culture period (see Materials and Methods). Fig. 7 shows that AS-src, but not S-src, was able to stimulate nodule mineralization over a
period of 3 wk, whether or not cells were cultured in the presence of dexamethasone (Fig. 7).

We then examined whether Src deficiency affected the expression of marker genes of the osteoblast phenotype. For this purpose we performed semiquantitative RT-PCR using primers for the housekeeping gene GAPDH along with primers for the gene being analyzed and measured the relative ratios by densitometry. Fig. 8 shows that the transcription of ALP was increased by AS-src, but not by S-src. In addition, two other osteoblast markers, the Osf2/Cbfa1 transcription factor and the PTH/PTHrP receptor, were similarly transcriptionally upregulated by the AS-src versus controls and S-src–treated cultures (Fig. 8). Finally, the matrix proteins osteocalcin (OCN) and pro-alpha 2(I) collagen (COL1A2) were significantly increased by the AS-src. Interestingly, similar increases were observed in primary (Fig. 9) as well as in immortalized (Fig. 10) Src

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increase in the number of osteoblasts and in the rate of formation, both in vivo and in vitro. Our in vivo observations show that Src deletion or decreased expression leads to a marked increase in osteoblast differentiation and bone formation. Primary osteoblasts derived from the calvaria of Src−/− mice show an increased expression of various osteoblast markers, decreased proliferation, and an accelerated rate of mineralized nodule formation. Similar results were obtained with Src−/− immortalized osteoblast cell lines and with primary osteoblasts treated with Src-antisense oligonucleotides (AS-Src). Thus, Src deletion leads to a cell autonomous increase in osteoblast differentiation and bone formation. These results suggest that under wild-type conditions Src plays a negative role in these processes.

Despite its heterogeneity, osteopetrosis is defined as an osteoclast disease. Defects in bone resorption, due to either defective osteoclast differentiation or to defective function of the cell, are the cause of all forms of osteopetrosis (Whyte, 1999). This is in contrast with osteosclerosis, another pathological situation in which bone mass is abnormally high, where it is assumed that the defect is increased bone formation rather than decreased bone resorption (Whyte, 1999). However, these two abnormalities in bone cell differentiation and/or function are not mutually exclusive. So far, little has been done to determine whether, notwithstanding the osteoclast defects, osteoblasts could also contribute to the phenotype in some forms of osteopetrosis (Marks et al., 1989; Shalhoub et al., 1994).

We have recently performed histomorphometric analyses in aging osteopetrotic Src−/− mice and found that bone mass continues to increase with time in the absence of Src. In adult animals, this continued bone formation in the face of impaired resorption eventually leads to the development of extreme osteopetrosis and extramedullary hematopoiesis (Amling et al., 2000). However, this study did not involve Src deletion.
not allow us to determine whether bone formation was at a normal level, albeit higher than the defective bone resorption, or whether it was increased above the normal level of bone formation. Although both situations would lead to a progressive increase in bone mass, the mechanisms involved are quite distinct. Only in the case where bone formation is higher than normal would the osteoblast potentially be defective. Our dynamic histomorphometric data clearly show that this is the case in the Src knockout mice. We observed a marked increase in most parameters of bone formation in these mutant mice; the bone formation rate increased to 150% of control values and the osteoblast number and surface more than doubled. The increased bone formation in vivo appeared to relate mainly to increased osteoblast number rather than increased matrix production, which may indicate increased proliferation, accelerated differentiation, or extended lifespan of the osteoblast. Thus, not only is bone formation maintained, despite the decrease in bone resorption known to be present in these animals (Soriano et al., 1991a; Boyce et al., 1992; Lowe et al., 1993), but it is actually increased above the normal level, explaining the progressive increase in bone mass with time observed in this study and a previous one (Amling et al., 2000). Most interestingly, our in vitro data indicate that these changes are cell autonomous, since Src−/− cultured primary osteoblasts or immortalized cells as well as normal osteoblasts treated with AS-Src show a decrease in proliferation, an increase in the expression of osteoblast markers, and an increase in the ability to form mineralized nodules in culture. Since osteoblast apoptosis is not decreased in Src−/− primary osteoblasts, it is unlikely that the osteoblast lifespan is markedly extended in the absence of Src. Therefore, the increase in the number of osteoblasts in vivo is mostly due to increased differentiation.

Whether or not the precursor pool is also induced to proliferate cannot be determined in vivo. However, in vitro Src deletions lead to decreased cell proliferation. One possibility is that the early precursor pool is not present in the cultures such that increased differentiation depletes the pool of preosteoblasts, whereas in vivo the precursor pool could be continuously replenished at the same rate as cells move along their differentiation pathway. Therefore, we propose that the increase in bone mass and bone formation observed in vivo is likely to be the result of a cell-autonomous enhancement of osteoblast differentiation when Src is deleted or when its expression is markedly depressed, as in our antisense experiments. This suggests that under normal circumstances, c-Src is a negative regulator of osteoblast differentiation and bone formation.

On the basis of the in vivo observations, we attempted to investigate whether Src could affect the osteoblasts in vitro. Primary calvarial cells harvested from Src-deficient mice indeed showed a greater differentiated phenotype relative to their wild-type counterpart. Our observation that ALP activity and nodule mineralization in culture was enhanced in Src−/− versus Src+/+ strongly supports our hypothesis. Moreover, we further addressed this issue and analyzed several functional and molecular aspects of osteoblast differentiation in two additional osteoblast models, which were more readily available in culture. These were (a) calvarial cells harvested previously from Src deficient or wild-type mice and subsequently immortalized in culture with the SV40 large T antigen, and (b) normal calvarial osteoblasts where Src deficiency was achieved by introduction into the cell of a Src-antisense oligonucleotide (AS-src). As described below, the fact that the same findings were confirmed in all the osteoblast models used in this study reasonably supports our hypothesis.

Immortalized calvarial cells showed only a weak osteoblast phenotype under basal conditions. However, spontaneous increase in ALP activity could be seen in long-term cultures. This is the time when the cells achieve postconfluence, a condition which in our hands represents a stimulus towards osteoblast differentiation (Migliaccio et al., 1998). In addition, these cells showed normal responsiveness to glucocorticoids, which significantly stimulated ALP activity. A remarkable feature of immortalized Src-deficient cells was a greater ALP activity, at any time of culture and treatment with dexamethasone, relative to normal Src expressing osteoblasts. Higher levels of ALP activity confirmed the presence of a more differentiated phenotype, as indicated by the increased matrix mineralizing ability that could be observed in Src-deficient cells but not in wild-type immortalized osteoblasts.

Experiments performed with calvarial cells harvested from normal mice where Src expression was downregulated by the phosphorothioated Src antisense ODN further confirmed direct involvement of Src in inhibiting osteoblast differentiation. AS-src is a complementary nucleic acid sequence that hybridizes to target the AUG translational start site of the Src mRNA and form an RNA–DNA duplex, resulting in the block of translation of the mRNA into the protein, and activation of RNase H, which destroys the duplex (Alama et al., 1997). This ODN has been successfully used previously to inhibit Src expression in mouse osteoclasts (Tanaka et al., 1996). In the same study, the sense ODN was found to be an appropriate control, which was also confirmed here. The antisense-specific reduction of Src levels demonstrated that the changes observed were indeed due to the antisense sequence-specific properties of AS-src and not to sequence-independent effects (Wagner, 1995; Stein, 1997; Bennett, 1998). In this model, Src expression was not completely inhibited (~60%). However, enhanced ALP activity and nodule mineralization were observed relative to the control cultures. Therefore, we believe that increased ALP activity and nodule mineralization ability reflect conditions directly related to the deficiency in Src expression in osteoblasts.

Increased ALP activity and mineral deposition were not the only features indicating a negative role of Src in osteoblast differentiation. Osteogenic cells are known to express various specific markers that distinguish them from other matrix-forming cells of mesenchyme origin. Among these, we have selected a few genes that characterize the osteoblast phenotype and have investigated their Src-dependent transcriptional regulation. We observed in Src-deficient osteoblasts, as well as in cells treated with AS-src, a significant upregulation of ALP, Osf2/Cbfa1 transcription factor, PTH/PTHrP receptor, and matrix proteins OCN and COL1A2 gene transcripts relative to normal Src-expressing cells. These markers are considered highly predictive of the differentiated osteoblast phenotype. The transcriptional regulation of these genes is not well characterized. The ALP gene promoter is known to
possess a consensus sequence (GGGCGG) for SP1 binding (Weiss et al., 1988), whereas at least three promoters have been described in the PTH/PTHrP receptor gene, one of which, the P2, is also found in mouse (Manen et al., 1998). In contrast, the promoter of the recently identified Os2/Cbfal transcription factor (Komori et al., 1997; Ducy et al., 1997) is still uncharacterized, and only regulation by bone morphogenetic proteins 4 and 7, vitamin D3, and Os2/Cbfal itself has been identified so far (Ducy et al., 1997, 1999; Tsuji et al., 1998). The COL1A2 promoter is known to possess SP1 and SP3 binding sites (Inh and Transjonska, 1997), whereas the OCN promoter is regulated by AP-1-related proteins and, interestingly, by the Os2/Cbfal transcription factor (Frendo et al., 1998; Stein, 1997). Therefore, it is possible that the comitant upregulation of expression of these genes in a condition of Src deficiency does not necessarily imply a direct relationship with Src, but rather an indirect regulation by Src-dependent intracellular signaling events.

Our results indicate that the modifications induced by Src deficiency in osteoblasts lead to the deposition of a matrix capable of greater and/or faster mineralization. Bone matrix is a mixture of extracellular proteins arranged to promote mineral deposition. Particular interest has been recently focused on osteopontin, whose expression has been found to be increased in ia (incisor absent), op (osteopetrotic), and tl (toothless) osteopetrotic rats (Shalhub et al., 1994; Marks et al., 1989), but reduced in mouse Src-/- fibroblasts (Chackalaparampil et al., 1996). Osteopontin is a sialoprotein of bone necessary for osteoclast attachment and bone resorption (Duong and Rodan, 1998), which signals the cell through the vitronectin receptors inducing changes in cytosolic free-calcium concentration and phosphorylation patterns (Duong and Rodan, 1998). The osteopontin gene promoter has a CCAAT box, located at -53 to -122 from the transcription start site, functioning as a v-Src response element; v-Src stimulates osteopontin expression in NIH 3T3 fibroblasts (Tezuka et al., 1996). However, Chackalaparampil et al. (1996) showed that in vivo assays normal levels of osteopontin accumulated in the bone matrix of Src-/- mice. Despite the increased deposition and mineralization of matrix in Src-/- cultures, we could not detect changes in osteopontin expression in osteoblasts treated with the AS-oc or in primary and immortalized osteoblasts derived from the Src-deficient mice.

In conclusion, our study provides evidence that Src-deficient osteoblasts exhibit a cell autonomous alteration that lead to increased osteoblast numbers and bone formation in vivo, as well as accelerated differentiation, and increased matrix production, and/or mineralization in vitro. These alterations contribute to the development of the osteopetrotic phenotype observed in Src-deleted mice. Thus, Src exerts a negative regulatory influence on osteoblasts and bone formation in normal cells.

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