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Direct Inhibitory and Indirect Stimulatory Effects of RAGE Ligand S100 on sRANKL-Induced Osteoclastogenesis

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ABSTRACT

Diabetes results in increased fracture risk, and advance glycation endproducts (AGEs) have been implicated in this pathophysiology. S100 proteins are ligands for the receptor of AGEs (RAGE). An intracellular role of the S100 family member S100A4 (Mts1) to suppress mineralization has been described in pre-osteoblastic MC3T3-E1 cells. However, S100 proteins could have additional effects on bone. The goal of the current study was to determine effects of increased extracellular S100 on osteoclastogenesis. We first determined the direct effects of S100 on pre-osteoclast proliferation and osteoclastic differentiation. RANKL-treated RAW 264.7 cell proliferation and TRAP activity were significantly inhibited by S100, and the number and size of TRAP-positive multinucleated cells were decreased. We then determined whether S100 could affect osteoclastogenesis by an indirect process by examining effects of conditioned media from S100-treated MC3T3-E1 cells on osteoclastogenesis. In contrast to the direct inhibitory effect of S100, the conditioned media promoted RAW 264.7 cell proliferation and TRAP activity, with a trend toward increased TRAP-positive multinucleated cells. S100 treatment of the MC3T3-E1 cells for 14 days did not significantly affect alkaline phosphatase, M-CSF, or OPG gene expression. RANKL was undetectable in both untreated and treated cells. The treatment slightly decreased MC3T3-E1 cell proliferation. Interestingly, S100 treatment increased expression of RAGE by the MC3T3-E1 cells. This suggested the possibility that S100 could increase soluble RAGE, which acts as a decoy receptor for S100. This decrease in availability of S100, an inhibitor of pre-osteoclast proliferation, could contribute to osteoclastogenesis, ultimately resulting in increased bone resorption. J. Cell. Biochem. 107: 917–925, 2009.

KEY WORDS: S100/CALGRANULIN FAMILY; S100A4 (MTS 1); RAGE (RECEPTOR OF AGES); OSTEOCLASTOGENESIS; DIABETES MELLITUS

Patients with type 1 diabetes suffer from decreased bone mineral density, and decreased IGF-1 may be involved in the pathogenesis of the bone loss [Chiarelli et al., 2004]. In a mouse model in which diabetes was induced by streptozotocin, both bone formation and resorption were suppressed [Motyl et al., 2009]. Type 2 diabetes patients have a significantly higher incidence of fractures than normal healthy individuals, even at the same bone mineral density [Nicodemus and Folsom, 2001; Yamamoto et al., 2006]. Previous findings suggested that advanced glycation endproducts (AGEs) might be involved in the bone fragility in diabetic patients through stimulating bone resorption and inhibiting bone formation [Takagi et al., 1997; McCarthy et al., 1999; Ding et al., 2006]. Particularly, receptor of AGEs (RAGE)-deficient mice have a significantly increased bone mass and a decreased number of osteoclasts compared to wild-type mice [Ding et al., 2006]. AGE modification of β2-microglobulin conferred activity to stimulate secretion of the cytokines interleukin-1β, interleukin 6, and tumor necrosis factor-α from human macrophages, and AGE modification of β2-microglobulin increased effects on net calcium flux from neonatal mouse calvaria and the number of resorption pits formed by unfractionated bone cells containing osteoclasts [Miyata and Sprague, 1996; Miyata et al., 1996].

RAGE is a multiligand cell surface receptor that belongs to the immunoglobulin superfamily. RAGE was identified in bovine lung, and characterized based on its ability to bind AGEs [Neeper et al., 1992]. Six isoforms of RAGE receptor have been thus far identified. The dominant isoforms of the RAGE receptor are full-length RAGE, soluble RAGE, and dominant negative RAGE (DNRAGE) [Ding and Keller, 2005a]. Full-length RAGE is the most widely studied receptor, and signaling thorough full-length RAGE was shown to

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mediate the activation of nuclear factor κ-B (NF-κB), mitogen-activated protein kinase (MAPK), and Jun-N-terminal kinase (JUNK) through the intracellular domain [Sousa et al., 2000; Yeh et al., 2001; Okamoto et al., 2002; Dukic-Stefanovic et al., 2003; Haslbeck et al., 2004; Li et al., 2004]. Signaling through full-length RAGE is thought to be critical for chemotaxis, angiogenesis, inflammation, neurite outgrowth, apoptosis, and proliferation [Thornalley, 1998; Schmidt et al., 2000, 2001; Bucciarelli et al., 2002b]. A second major form of RAGE is soluble (secretory) RAGE. Soluble RAGE lacks the transmembrane domain, and is therefore released into the extracellular space. Since soluble RAGE retains the V-type domain, which is believed to be the principal site for ligand binding, soluble RAGE acts as a decoy receptor for RAGE ligands, inhibiting the activation of full-length RAGE by sequestering the RAGE ligands prior to their interaction with the RAGE receptor [Park et al., 1998; Thornalley, 1998; Schmidt et al., 2000, 2001; Bucciarelli et al., 2002a,b; Wear-Maggitti et al., 2004]. The least characterized form of RAGE is DNRAGE. DNRAGE lacks the short intracellular domain of full-length RAGE, and presumably competes for the binding of RAGE ligands and prevents the activation of full-length RAGE due to its lack of an intracellular domain [Ding and Keller, 2005a].

The S100/calgranulin family is a calcium-binding family of proteins having the EF-hand motif, and comprised at least 21 members [Donato, 2003]. RAGE is a central cell surface receptor for S100 family proteins, although it is unclear whether it is the sole receptor for S100 proteins. Interaction of S100 family proteins with full-length RAGE triggers cellular activation, through activation of transcription factor NF-κB or increased expression of the anti-apoptotic protein Bcl-2 [Hofmann et al., 1999; Huttunen et al., 2000]. The effects of S100 proteins are likely to be tissue-specific and dose dependent [Huttunen et al., 2000]. Nanomolar concentrations of S100B were shown to induce trophic effects in RAGE-expressing neurons [Kligman and Marshak, 1985; Van Eldik and Zimmer, 1987; Winningham-Major et al., 1989], whereas micromolar concentrations of S100B induced apoptosis in an oxidant-dependent manner [Hu et al., 1996]. The gene expression of the S100 family protein, S100A4 (Mts1), is found to be regulated in the course of osteoblast differentiation and to have a crucial role in mineralization [Duarte et al., 2003]. S100A4 is an 11-kDa protein, and a typical member of S100 family. It was originally isolated from a metastatic tumor cell line, and is thought to be a potential marker for metastasizing adenocarcinomas [Ebralidze et al., 1989]. There is evidence that S100A4 has both intracellular and extracellular functions. Intracellular S100A4 was shown to regulate the function of p53 tumor suppressor [Grigorian et al., 1993, 2001] and to interact with the heavy chain of nonmuscle myosin II in metastatic tumor cells [Kriajevska et al., 1994; Ford and Zain, 1995; Li et al., 2003]. The role of extracellular S100A4 protein was reported in astrocytes and endothelial cells. When applied extracellularly, S100A4 acts as a potent cytokine and stimulates neurite outgrowth in astrocytes [Novitskaya et al., 2000] and angiogenesis in endothelial cells [Ambartsumian et al., 2001]. However, the role of extracellular S100 protein in osteoclastogenesis is still unclear. We were therefore interested in determining how S100 protein affects sRANKL-induced osteoclastogenesis, either directly, or indirectly through cytokines secreted from osteoblasts. Determining the role of extracellular S100 protein in osteoclastogenesis is also important for understanding the ligand–RAGE interaction in bone and potentially for its contribution to bone fragility in diabetes mellitus.

MATERIALS AND METHODS

CELL CULTURE

RAW264.7 mouse monocyte/macrophage lineage cells (American Type Culture Collection) and MC3T3-E1 pre-osteoblastic cells (American Type Culture Collection) were maintained in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin–streptomycin (Invitrogen) at 37°C, 5% CO_2. Media were replaced every 3 days, and the cells were subcultured weekly for use in experiments within 20 passages. For experiments, RAW 264.7 cells were seeded at 125 cells/well on 96-well plates. MC3T3-E1 cells were seeded at 250 cells/well on 96-well plates for MTT or LIVE/DEAD assays and seeded at concentrations ranging from 1,000 to 500,000 cells/dish on 100 mm dishes for RT-PCR experiments, the initial cell plating density being based on the intended length of culture. Cells were pre-incubated at 37°C, 5% CO_2 for 24 h, and continuously incubated in the presence or absence of indicated concentration of S100 protein (Calbiochem, San Diego, CA), a widely used ligand for RAGE [Hofmann et al., 1999; Vincent et al., 2007]. The medium was changed twice a week in the presence or absence of S100 for the indicated period. For osteoclastogenesis analysis, RAW 264.7 cells were pre-incubated at 37°C, 5% CO_2 for 24 h, and incubated with or without 200 nM S100 protein in the presence of 30 ng/ml human soluble RANKL (Peprotech, Rocky Hill, NJ). The media were replaced at day 4 and the cells were incubated for 7 days total with or without treatments.

For conditioned medium (CM) studies, we utilized CM from pre-osteoblastic MC3T3-E1 cells that had either been untreated or treated with S100 protein. MC3T3-E1 cells were incubated with or without 200 nM S100 protein for up to 15 days. The culture medium was then changed to medium without S100, which was incubated with the MC3T3-E1 cells for additional 6 h. This medium was then used for treatment of osteoclastic RAW 264.7 cells with or without the addition of RANKL.

TARTRATE-RESISTANT ACID PHOSPHATASE (TRAP) ASSAY, TRAP, AND HOECHST STAIN

RAW 264.7 cells grown on 96-well plate were fixed with 10% (v/v) formalin and washed with 95% (v/v) ethanol. They were then incubated in 100 μl phosphatase substrate (3.7 mM *p*-nitrophenyl phosphate [Sigma, St. Louis, MO] in 50 mM citrate buffer, pH 4.6) in the presence of 10 mM sodium tartrate (General Chemical Division of Allied Chemical, Morristown, NJ) at room temperature for 30 min. After the incubation, the solution was removed from each well and reacted with 0.1 ml of 0.1 N NaOH. Absorbance at 410 nm was read with a Dynatech MR 5000 microplate reader (Dynatech Laboratories, Alexandria, VA). After the assay, the cells were stained for TRAP with 0.1 mg/ml naphthol AS-MX phosphate (Sigma) and 0.6 mg/ml fast red violet LB salt (Sigma) in 0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate. Following this step, the cells

were stained (Hoechst) with 5 μg/ml bisbenzimide (Hoechst 33342; Sigma) in phosphate-buffered saline (PBS) for 15 min in darkness.

MTT ASSAY

At the end of the experiment, 30 μl of 4 mg/ml MTT (Sigma) solution was added to each 96-well of RAW 264.7 cells or MC3T3-E1 cells, and the mixture was incubated for 30 min at 37°C, 5% CO_2. Absorbance at 570 nm was read with a Dynatech MR 5000 microplate reader. This assay, which measures reduction of the tetrazolium compound MTT into a colored formazan product by the mitochondria of living cells, was used as a surrogate measure of cell proliferation.

LIVE/DEAD ASSAY

RAW 264.7 cells grown on 96-well plates were rinsed with PBS, and stained with a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) according to the manufacturer's instructions. Briefly, 0.4 μM ethidium homodimer and 0.4 μM Calcein-AM dissolved in PBS were added to the wells, and the mixture was incubated for 45 min at room temperature. Viable cells fluoresced green and dead/dying cells fluoresced red in this assay. Microscope images were recorded and quantification of cell numbers was achieved with a "cell counter" plug-in of Image J software.

SEMI-QUANTITATIVE ANALYSIS OF MRNA EXPRESSION BY RT-PCR

Total RNA was isolated from cells using the RNeasy mini-prep method (Qiagen, Germantown, MD), and first strand cDNA was synthesized in 20-μl reactions using the Promega reverse transcription system (Promega, Madison, WI) according to the manufacturer's instructions. The 20 μl of first strand cDNA reaction was diluted to 100 μl with TE buffer and 2.5 μl of this reaction mixture was used for PCR amplification using Platinum *Taq* polymerase (Invitrogen) or Ex *Taq* polymerase (TaKaRa Bio, Inc., Osaka, Japan) and the following mouse gene-specific primers: RAGE sense, 5′-TTGTCGATGAGGGGACTTTC-3′, and antisense, 5′-AGCTCTGACCGCAGTGTAAAG-3′ generated a 304-bp fragment; S100A4 sense, 5′-CAGGCAAAGAGGGTGACAAG-3′ and antisense, 5′-GGCAATGCAGGACAGGAAGA-3′ generated a 194-bp fragment [Rocken et al., 2003]; alkaline phosphatase (ALP) sense, 5′-GCCCTCTCCAAGACATATA-3′ and antisense, 5′-CCATGATCACGTCGATATCC-3′ generated a 373-bp fragment [Qu et al., 1998]; macrophage-colony stimulating factor (M-CSF) sense, 5′-CGAGTCAACAGAGCAACCAAAC-3′ and antisense, 5′-GAGTGGCTTTTGGGAAGCAG-3′ generated a 498-bp fragment; osteoprotegerin (OPG) sense, 5′-AAAGCACCCTGTAGAAAACA-3′ and antisense, 5′-CCGTTTTATCCTCTCTACACTC-3′ generated a 257-bp fragment [Huang et al., 2000]; GAPDH sense, 5′-GGGGTGAGGCCGGTGCTGAGTAT-3′ and antisense, 5′-CATTGGGGGTAGGAACACGGAAGG-3′ generated a 459-bp fragment [Yoshida et al., 2006]. Thermal cycling conditions were: 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (M-CSF and RAGE); 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s (OPG and ALP); and 28 cycles of 94°C for 30 s and 55°C for 30 s, and 72°C for 60 s (S100A4 and GAPDH). PCR products were resolved by 2.0% agarose gel electrophoresis and visualized by ethidium bromide on a UV transilluminator. Integral density of the PCR products was determined by Image J software.

STATISTICS

Significance was determined by analysis of variance and Tukey's post hoc test.

RESULTS

DIRECT EFFECT OF S100 PROTEIN ON RAW 264.7 CELLS

Studies were carried out to test the hypothesis that S100 protein promotes osteoclastogenesis. We first determined whether there was a direct effect of S100 protein on osteoclastogenesis. Studies were carried out with RAW 264.7 mouse monocyte/macrophage lineage cells, which differentiate into multinucleated mature osteoclasts in the presence of receptor activator of nuclear factor-κB ligand (RANKL). RAW 264.7 cells were incubated with or without 200 nM S100 protein in the presence of sRANKL for 7 days and TRAP activity was measured as a marker of osteoclastic differentiation. Unexpectedly, S100 significantly decreased sRANKL-induced TRAP activation (Fig. 1A). There was also a decrease in TRAP-positive multinucleated cell formation (Fig. 1B) and an observable decrease in the size of the multinucleated cells (Fig. 1C). The proliferation of RAW 264.7 cells determined by the surrogate MTT assay was directly inhibited by S100 as well (Fig. 1D).

INDIRECT EFFECT OF S100 PROTEIN ON RAW 264.7 CELLS

Since it has been reported that RAGE-deficient mice have a significantly increased bone mass and a decreased number of osteoclasts compared to wild-type mice [Ding et al., 2006], findings inconsistent with the direct inhibitory effect we observed, we hypothesized that S100 protein might stimulate osteoclastogenesis in an indirect manner, that is, through increasing osteoblastic secretion of a stimulator of osteoclast differentiation such as RANKL or M-CSF, or by decreasing the secretion of the decoy receptor OPG. To test this hypothesis, we utilized CM from pre-osteoblastic MC3T3-E1 cells treated with S100 protein. MC3T3-E1 cells were incubated with or without 200 nM S100 protein for up to 15 days. Culture medium was then changed to medium without S100, which was incubated with the MC3T3-E1 cells for additional 6 h. This medium was then used for the treatment of osteoclastic RAW 264.7 cells.

In contrast to the direct inhibitory effect of S100 on the RAW 264.7 cells, medium from S100-treated MC3T3-E1 cells significantly promoted the proliferation of RAW 264.7 cells determined by both the Live/Dead assay (Fig. 2A) and the MTT assay (data not shown). CM from S100-treated MC3T3-E1 cells also stimulated osteoclastogenesis as determined by TRAP assay (Fig. 2C). There was a trend toward an increase in the number of TRAP + multinucleated cells (Fig. 2D) although this was not statistically significant at the $P < 0.05$ level.

DIRECT EFFECT OF S100 PROTEIN ON PROLIFERATION OF MC3T3-E1 CELLS

Since S100 protein directly inhibited RAW 264.7 cell proliferation, we investigated whether S100 protein inhibited proliferation of MC3T3-E1 cells. MC3T3-E1 cells were incubated with or without 100 or 200 nM of S100 protein for up to 14 days, and cell proliferation rate was determined by MTT assay. There was no

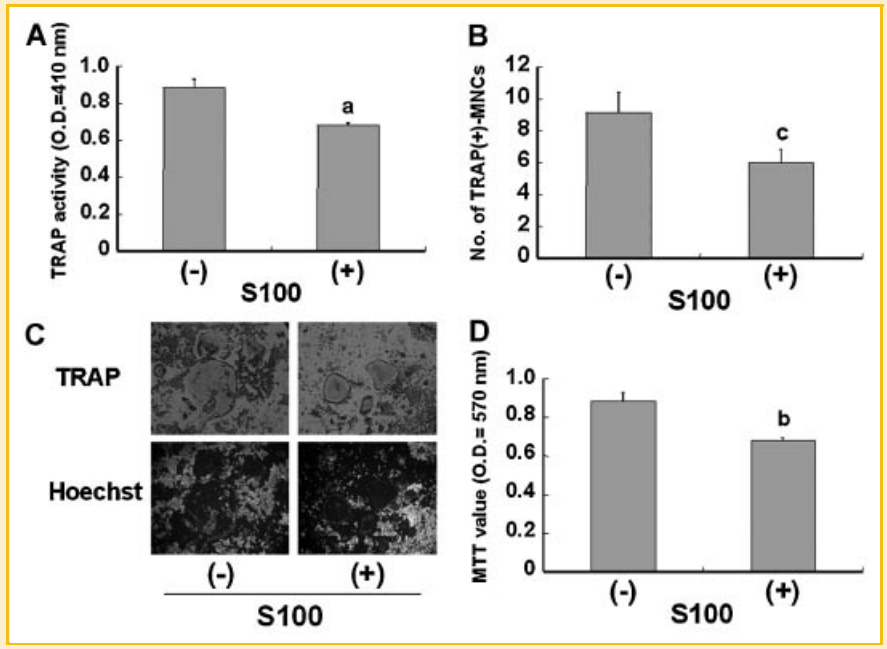

Fig. 1. Direct/inhibitory effects of S100 protein on RAW 264.7 cells. RAW 264.7 cells were incubated with or without 200 nM S100 in the presence of sRANKL for 7 days. Quantitative results are per well and represent mean ± SEM of six replicates. [a]$P < 0.001$ versus control (no S100), [b]$P < 0.01$ versus control (no S100). A: TRAP activity of RAW 264.7 cells was inhibited by S100. B: TRAP-positive multinucleated cell formation was inhibited by S100. TRAP-positive cells with three or more nuclei were counted. C: TRAP-positive multinucleated cells tended to be smaller with S100 treatment. Representative pictures for TRAP stain and Hoechst stain (nuclear stain) are shown. D: Proliferation of RAW 264.7 cells was inhibited by S100.

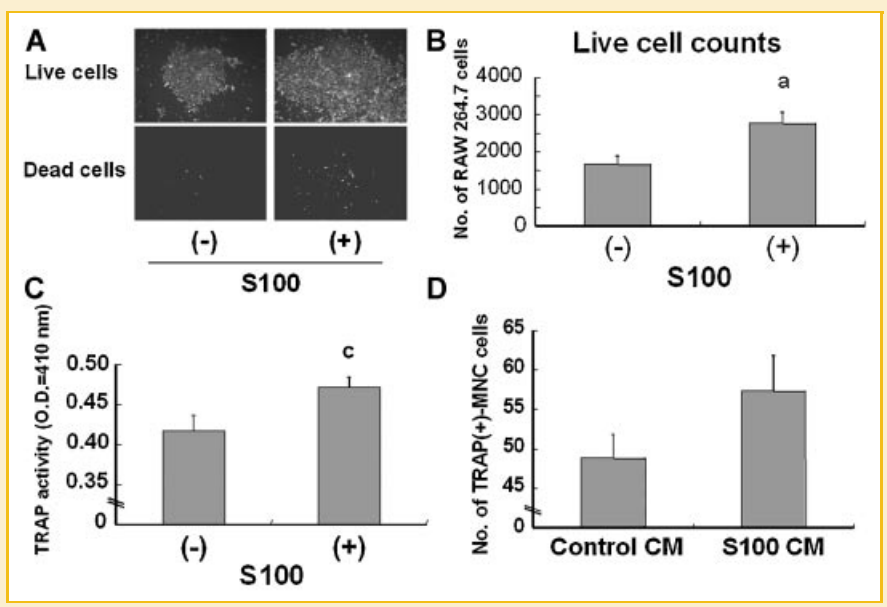

Fig. 2. Indirect/stimulatory effects of S100 protein on RAW 264.7 cells. MC3T3-E1 cells were incubated with or without 200 nM S100 protein for 14 days, and then medium was changed to S100-free medium. After 6 h incubation of the MC3T3-E1 cells in S100-free medium, the conditioned medium was harvested. RAW 264.7 cells were incubated with the conditioned medium in the presence or absence of 30 ng/ml sRANKL and then proliferation and osteoclastogenesis were determined. Quantitative results are per well and represent mean ± SEM of six replicates. A,B: Conditioned medium from MC3T3-E1 cells treated with 200 nM S100 protein for 7 days led to a statistically significant increase in RAW 264.7 cell proliferation as determined by LIVE/DEAD staining. A: Representative pictures of LIVE/DEAD stained cells. B: The number of Live RAW 264.7 cells determined by counting after LIVE/DEAD staining. [a]$P < 0.05$ versus control (no S100). C: TRAP activity of RAW 264.7 cells was increased by conditioned medium from MC3T3-E1 cells treated with 200 nM S100 protein for 5 days. D: The number of TRAP-positive multinucleated cells tended to be increased by treatment with conditioned medium from MC3T3-E1 cells treated with 200 nM S100 protein for 5 days.

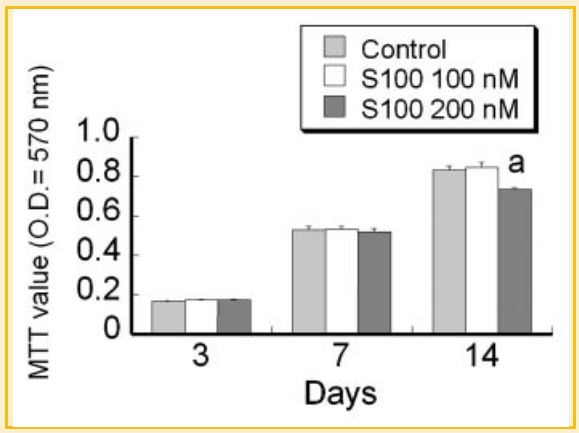

Fig. 3. Pre-osteoblastic MC3T3-E1 cell proliferation was inhibited by S100 protein. MC3T3-E1 cells were incubated with or without 100 or 200 nM S100 protein for up to 14 days as indicated, and cell proliferation rate was determined by MTT assay. Results represent mean ± SEM of six replicates. $^{a}P < 0.01$ versus control (no S100).

significant effect of 100 nM S100 protein on cell proliferation during the observation period, but 200 nM S100 protein slightly but significantly inhibited MC3T3-E1 cell proliferation at day 14 (Fig. 3).

CHANGES IN mRNA EXPRESSION IN THE COURSE OF DIFFERENTIATION OF PRE-OSTEOBLASTIC MC3T3-E1 CELLS

Since it appeared that extracellular S100 protein stimulated osteoclastic RAW 264.7 cell proliferation through growth factors secreted from pre-osteoblastic MC3T3-E1 cells, we were interested in determining whether extracellular S100 protein stimulated expression of genes that could affect osteoclastogenesis, including RANKL, OPG, and M-CSF. To further investigate the response, we determined whether S100 treatment affected the expression of S100A4 and RAGE in the MC3T3-E1 cells.

Since previous studies [Duarte et al., 2003] had shown that S100A4 changes during MC3T3-E1 cell differentiation, we first characterized the changes occurring during differentiation. MC3T3-E1 cells were grown in regular medium for 2 or 31 days, and mRNA expression was determined by conventional RT-PCR. As expected, mRNA expression of the differentiation marker, ALP, increased over time (Fig. 4A). OPG mRNA expression increased over time as well, whereas M-CSF gene was unaffected. Unfortunately, we could not detect RANKL mRNA expression (data not shown) although we used the same primer sets as previously reported [Huang et al., 2000; Ikeda et al., 2001]. Consistent with the previous report, in which S100A4 mRNA expression was shown to decrease as pre-osteoblastic MC3T3-E1 cells differentiated [Duarte et al., 2003], mRNA expression of S100A4 decreased over time. RAGE gene expression was higher at 31 days than at 2 days.

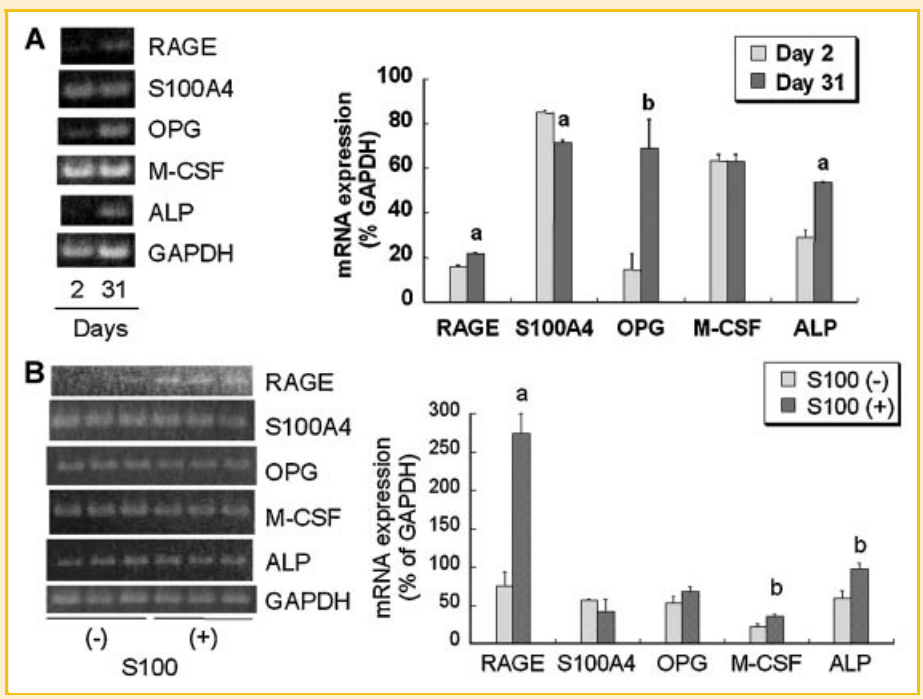

Fig. 4. A: Changes in mRNA expression in the course of differentiation of pre-osteoblastic MC3T3-E1 cells. MC3T3-E1 cells were grown in regular medium for 2 or 31 days, and mRNA expression was determined by RT-PCR. Images were analyzed by Image J software, and each integral density was determined. Background value was subtracted from each sample value, and this value was normalized by the value of GAPDH. Results represent mean ± SEM of three replicates (right panel). Representative images were shown in the left panel. $^{a}P < 0.01$, $^{b}P < 0.05$ versus 2-day treatment. B: RAGE mRNA expression was significantly increased by S100 in pre-osteoblastic MC3T3-E1 cells, whereas S100A4, M-CSF, ALP, and OPG mRNA expression were unaffected: MC3T3-E1 cells were incubated with or without 200 nM S100 protein for 14 days, and mRNA expression was determined by RT-PCR. Images (left) were analyzed by Image J software, and each integral density was determined. Background value was subtracted from each sample value, and this value was normalized by the value of GAPDH. Results represent mean ± SEM of three replicates (right panel). $^{a}P < 0.001$, $^{b}P < 0.05$ versus control (no S100).

RAGE mRNA expression was increased by S100 protein in pre-osteoblastic MC3T3-E1 cells, whereas S100A4, M-CSF, ALP, and OPG mRNA expression were unaffected.

To test the hypothesis that S100 protein affects secretion of factors from osteoblasts to stimulate pre-osteoclast proliferation, we investigated whether mRNA expression of genes involved in pre-osteoclast proliferation was changed by treatment of MC3T3-E1 cells with S100 protein.

MC3T3-E1 cells were incubated with or without 200 nM S100 protein for 14 days, and mRNA expression was determined by semi-quantitative RT-PCR. Interestingly, S100 treatment resulted in a significant, nearly fourfold increase in RAGE. S100A4 and OPG were unaffected by S100 treatment. There were small increases in M-CSF and ALP (Fig. 4B), and RANKL mRNA expression was again undetectable by RT-PCR (data not shown).

DISCUSSION

To further understand the basis for bone fragility in diabetes patients, the direct effects of extracellular S100 protein on osteoclastogenesis, as well as the indirect effects of extracellular S100 protein, released from osteoblasts, on the osteoclast precursor cells were determined. S100A4 was previously found to be produced by pre-osteoblastic MC3T3-E1 cells and to inhibit matrix mineralization in cultures of MC3T3-E1 cells [Duarte et al., 2003]. Since S100A4 was shown to be secreted from monocytes [Rammes et al., 1997] and glial cells [Van Eldik and Zimmer, 1987; Davey et al., 2001], and when present extracellularly, to affect neurite outgrowth [Novitskaya et al., 2000] and angiogenesis [Ambartsumian et al., 2001], we hypothesized that S100A4 could be secreted from pre-osteoblastic MC3T3-E1 cells and affect osteoclastogenesis. We also hoped to understand the mechanism of ligand–RAGE signaling in bone through determining the role of extracellular S100 protein on osteoclastogenesis.

We initially hypothesized that S100 protein might stimulate osteoclastogenesis because S100 proteins have been demonstrated to be natural ligands for RAGE [Hofmann et al., 1999; Huttunen et al., 2000; Schmidt et al., 2000, 2001; Bucciarelli et al., 2002b] and a RAGE-knockout mouse model was shown to have increased bone mass and a decreased number of osteoclasts [Ding et al., 2006; Zhou et al., 2006]. However, in this, the first study to investigate the role of extracellular S100 protein on osteoclastogenesis, the results (Figs. 1 and 5A) revealed that the direct effect of S100 protein (200 nM) on RAW 264.7 pre-osteoclastic cells was to inhibit S100 sRANKL-induced TRAP activity and TRAP-positive multinucleated cell formation. Lower concentrations (0.2–20 nM) did not have significant effects (data not shown). The proliferation of both RAW 264.7 (Fig. 1) and MC3T3-E1 cells (Fig. 3) was also directly inhibited by S100 protein. These results led us to hypothesize that S100 protein might stimulate osteoclastogenesis indirectly, that is, through cytokines secreted from osteoblasts treated with S100 protein. CM from MC3T3-E1 cells treated with S100 protein increased TRAP activity compared to CM from MC3T3-E1 cells treated with regular medium; the CM from the S100-treated cells also stimulated proliferation of pre-osteoclasts, suggesting that S100 protein stimulates osteoblasts to produce and secrete one or more osteoclast growth factors (Figs. 3 and 5B).

Since the cytokines RANKL and M-CSF are essential and sufficient for basal osteoclastogenesis [Suda et al., 1999; Boyle et al., 2003; Pixley and Stanley, 2004], and OPG is also critical because it acts as a decoy receptor for RANKL [Kostenuik and Shalhoub, 2001], we determined whether S100 protein could affect expression of those genes (Fig. 4). Before doing these experiments, we examined gene expression during differentiation in MC3T3-E1 cells to establish the responsiveness of these genes and to determine whether the previously reported change in S100A4 during differentiation [Duarte et al., 2003] could be replicated in our cultures. For this experiment, we compared gene expression in undifferentiated MC3T3-E1 cells cultured for just 2 days and in

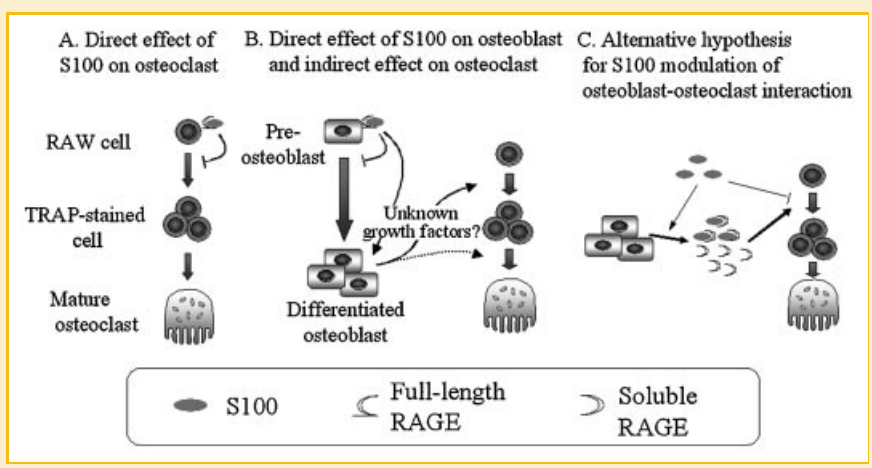

Fig. 5. Observed and hypothesized actions of S100 protein on bone cells. A: S100–RAGE signaling directly inhibits osteoclastogenesis. B: S100–RAGE signaling inhibits osteoblast proliferation, but promotes pre-osteoclast proliferation and osteoclastogenesis through factors secreted from osteoblasts. C: S100–RAGE signaling could promote osteoclastogenesis through osteoblast production of a decoy receptor, soluble RAGE, that prevents the inhibitory effects of S-100 on osteoclastogenesis.

MC3T3-E1 cell cultured for 31 days in α-MEM, containing phosphate and L-ascorbic acid, treatments that are indispensable for osteoblast differentiation [Ecarot-Charrier et al., 1983, 1988; Gerstenfeld et al., 1987; Bellows et al., 1991; Franceschi and Iyer, 1992; Franceschi et al., 1995]. mRNA expression of the osteoblast-differentiation marker ALP was higher in day-31 MC3T3-E1 cells than in day-2 cells, suggesting that day-31 cells were more differentiated than day-2 cells. S100A4 mRNA expression was higher in day-2 undifferentiated cells than in day-31 differentiated cells, confirming the previous report [Duarte et al., 2003]. Gene expression of OPG and RAGE was increased during differentiation, but M-CSF gene was unchanged.

Having established that changes in expression of the genes of interest could be detected with our PCR conditions, we determined the effect of extracellular S100 protein on gene expression in MC3T3-E1 cells. There was no effect of S100 protein on OPG mRNA expression (Fig. 4B). S100 protein also failed to affect S100A4 mRNA expression, suggesting that there was no autocrine regulation of S100A4. S100 treatment led to a slight increase in the expression of M-CSF and ALP. Interestingly, RAGE mRNA expression was significantly increased by S100 protein (Fig. 4B), indicating that the ligand can stimulate the expression of one or more forms of its receptor, RAGE, in the MC3T3-E1 cells. This effect of S100 protein is consistent with the finding that cells grown on AGE-modified collagen matrix have higher expression of RAGE [Cortizo et al., 2003]. The finding that S100 increases RAGE expression allows us to propose a novel alternative model (Fig. 5C) through which the S100 protein could have an indirect effect on proliferation of pre-osteoclasts. This model proposes that S100 might increase the production of soluble RAGE from the osteoblastic cells. Among the six isoforms for RAGE, soluble RAGE has been shown to be able to suppress full-length RAGE activation by sequestering RAGE ligands before they encounter the membrane form of RAGE [Park et al., 1998; Bucciarelli et al., 2002a; Wear-Maggitti et al., 2004]. Hanford et al. [2004] reported that soluble RAGE results from an alternative splicing of RAGE mRNA in humans, but soluble RAGE in the mouse results from a carboxyl-terminal truncation, suggesting that soluble RAGE isoform production would not be distinguishable from full-length RAGE at the level of mRNA expression. In the human brain, soluble RAGE mRNA was found to be at least threefold higher than other forms of RAGE [Ding and Keller, 2005b]. Based on these previous reports, we hypothesize that the observed increment of osteoblastic RAGE mRNA expression by treatment with S100 protein results in increased soluble RAGE, which competes with full-length RAGE on osteoclast membranes for S100 binding. Although S100 protein directly inhibits osteoclastogenesis, the increase in soluble RAGE, acting as a decoy receptor for S100 protein, could decrease the inhibitory effect of S100 protein on osteoclasts, and result in increased pre-osteoclast proliferation (Fig. 5C). Future studies involving the development of an assay for soluble RAGE in conditioned media are planned to investigate this possibility. The model does not exclude other possible factors that could stimulate the proliferation of the pre-osteoclastic cells. However, these were less compelling, since the effects on M-CSF and ALP gene expression were quite small, and RANKL was undetectable in the studies.

In summary, our findings, carried out in well-established osteoclast and osteoblast cell models, indicate that RAGE ligation by S100 protein directly inhibits osteoclastogenesis, but stimulates proliferation of osteoclast precursors and osteoclastogenesis by an indirect mechanism. Our results indicate that ligand–RAGE signaling in bone tissue is complex. We have proposed a model by which the increment of RAGE mRNA expression by S100 protein could be involved in the indirect effect of S100 protein. Our results are consistent with the possibility that the increased level of AGEs in diabetes patients directly reduces bone turnover by inhibiting proliferation of both osteoblasts and osteoclasts, but can indirectly stimulate proliferation and differentiation of osteoclast precursor cells, through mechanisms that could include an increase in soluble RAGE. The current findings thus contribute to our understanding the ligand–RAGE interaction in osteoclastogenesis, and to the pathophysiology of the fragility in diabetes patients.

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