Calcitonin-induced NF-κB Activation Up-regulates Fibronectin Expression in MG63 Osteosarcoma Cells

IOANNA TSAGARAKI[1], CONSTANTINOS PHENEKOS[2], EFFIE TSILIBARY[1] and ATHINA TZINIA[1]

[1]*Institute of Biology, National Center for Scientific Research Demokritos, Athens, Greece;*
[2]*Department of Endocrinology, Diabetes and Metabolism, Red Cross Hospital, Athens, Greece*

Abstract. *Salmon calcitonin has been used extensively as a therapeutic tool in the regulation of bone remodeling. However, there is a growing body of evidence indicating that the calcitonin peptides are involved in regulation of cell growth, differentiation, survival and tissue development. In the present study, we investigated the effect of calcitonin in cell matrix interactions in MG63 cell line. Our results demonstrated that calcitonin increases cell growth of MG63 osteosarcoma cells in parallel with serine/threonine protein kinase B (AKT/PKB) activation. Moreover, calcitonin induced up-regulation of fibronectin expression in a nuclear factor-kappa B (NF-κB)-dependent manner, accompanied by enhanced enzymatic activity of matrix metalloproteinase-9 (MMP-9) and increased expression of tissue inhibitors of MMP-1 and -2. MMP-9 stimulation with calcitonin was accompanied by an increase in protein expression of the α5β1 integrin receptor. To our knowledge, our results demonstrate, for the first time, that calcitonin is a potent inducer of fibronectin, an extacellular matrix component that is suggested to have a pro-oncogenic and healing effect, in a NF-κB-dependent manner.*

Calcitonin is a peptide 32 amino acids long, produced by C cells of mammalian thyroid. The main physiological role of calcitonin involves regulation of bone remodeling, through inhibition of bone resorption by osteoclasts and regulation of sodium, phosphate and calcium levels. Calcitonin has been used clinically to treat osteoporosis and humoral hypercalcemia of malignancy. However, recent studies indicate that calcitonin plays a major role in several cellular processes including cell survival, cell growth and proliferation of several cell types including osteosarcoma cell lines. Moreover, it regulates cell invasion and metastasis in several cell lines and is involved in wound healing (1-3).

Calcitonin mediates its effects through binding to its cell surface receptor, calcitonin receptor (CTR) and subsequent activation of several signaling mechanisms which include protein kinase B (PKB), extracellular signal-regulated kinases 1/2 (ERK1/2), Ca^{2+}-PKC, cyclic AMP-Protein Kinase A (cAMP-PKA) and phospholipases C, D and A_2 (4). In addition, calcitonin mediates its effects partly through regulation of extracellular matrix (ECM) components, such as the degradation of type II collagen (5).

One of the major components of the ECM highly expressed in the bone is fibronectin, the expression of which is controlled by a number of transcription factors including nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) (6). Fibronectin is a high molecular weight glycoprotein consisting of two nearly identical (250 kDa) subunits which are covalently linked near their C-termini by two disulfide bonds. Each subunit contains subdomains known as type I, II and III repeats which contain binding sites for other ECM proteins, including cell-surface receptors. Fibronectin is synthesized in a soluble form mainly in the liver and released into the circulation, or in an insoluble form disulfide-bonded to cross-linked fibrils and is subsequently deposited in the ECM. Fibronectin interaction with integrins regulates several cellular effects, including cell proliferation and apoptosis, as well as cell invasion and metastasis of several cancer cell lines (7, 8). Moreover, recent reports attribute pro-oncogenic effects of fibronectin that partly involve increased expression and enzymatic activity of matrix metalloproteinases (MMPs) (9). The MMPs are a family of zinc proteases which degrade components of the ECM, including collagen and fibronectin. Apart from ECM proteolysis, MMPs have been implicated in regulation of several physiological and pathological processes. The expression of MMPs is regulated at the transcriptional level, whereas secretion and activity are controlled in a very precise manner by interaction with tissue inhibitors of metallo-proteinases (TIMPs) (10).

Correspondence to: Athina Tzinia, Institute of Biology, National Center for Scientific Research Demokritos, Ag. Paraskevi, Athens, Greece. Tel: +30 2106503569, Fax: +30 2106511767, e-mail: atzin@bio.demokritos.gr

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In the present study, we investigated the effect of calcitonin on cell matrix interactions of cultured MG63 osteogenic sarcoma cells. In particular, we examined the effect of calcitonin in regulation of expression of ECM components, since they play a significant role in determining cellular morphology and behavior.

Materials and Methods

Antibodies and reagents. Polyclonal antibodies against TIMP-1 and TIMP-2, as well as P5D2, P1D6, P1B5 and LM609 monoclonal antibodies against β1, α5, α3 and αvβ3 integrin respectively, were purchased from Chemicon International (Temecula, CA, USA). Antibodies against PKB, phospho-PKB (Ser473), ERK1/2 Mitogen-activated protein kinases (MAPK), phospho-ERK1/2 MAPK (Thr202/Tyr204) were obtained from Cell Signaling Technology (Beverly, MA USA). NF-κB p65 subunit polyclonal antibody and monoclonal antibodies against fibronectin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibodies against β-tubulin and the reagents pyrrolidine dithiocarbamate (PDTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Luis, MO, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham Biosciences (Uppsala, Sweden). Salmon calcitonin (sCT) was a generous gift from Novartis, Athens, Hellas.

Cell culture and treatment. MG63 human osteogenic sarcoma cells (ATCC, Rockville, MD, USA) were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (all from Biochrom Seromed, Berlin, Germany). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were starved overnight in culture medium lacking FBS and salmon calcitonin was added at a concentration of 10 nM or 50 nM for 24 h. Where indicated, 100 μM PDTC was added 1 h prior to sCT treatment.

Isolation of nuclear extracts. Confluent cells were lysed in hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1× cocktail of protease inhibitors]. The cells were incubated on ice for 15 min and 0.4% NP-40 was added for cell lysis. Cell extracts were centrifuged at 10000 ×*g* for 2 min at 4˚C and the supernatants were harvested as the cytosolic fraction. Nuclear pellets were solubilized in nuclear lysis buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.02% NP-40, 0.5 mM PMSF and 1× cocktail of protease inhibitors) under agitation for 30 min at 4˚C. Samples were centrifuged at 10000 ×*g* for 5 min at 4˚C. Supernatants were harvested containing nuclear extracts and were dialysed in dialysis buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT). The nuclear extracts were stored at –70˚C until further use.

Immunoblotting and zymography. Confluent cells were lysed in lysis buffer (20 mMTris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 10 mM NaF, 1 mM Na_2VO_4, 1 mM PMSF, 1% Triton X-100 and 1× cocktail of protease inhibitors) for 60 min at 4˚C. Protein concentration of lysates was determined by the Bradford method (Pierce, Rockford, USA). Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by loading equal amounts of total cell lysates or serum-free conditioned media on 10% polyacrylamide gels under reducing conditions. The volume of conditioned medium loaded per lane was adjusted according to the cell number obtained at harvest. The resolved proteins were subsequently transferred to Hybond-ECL nitrocellulose membrane (Amersham) and immunoblotted against primary and secondary antibodies following the manufacturer's instructions. For gelatin zymography, aliquots of conditioned media harvested as above were analyzed on 10% SDS gels containing 0.1% gelatin under non-reducing conditions as previously described (11).

MTT assay for cell growth. To measure cell growth, the MTT colorimetric assay was used (12). Briefly, 5000 cells/well were seeded in a flat-bottomed 96-well plate and and were left for 24 h in serum-containing medium. The cells were starved overnight in culture medium lacking FBS and sCT (at a concentration of 10 nM or 50 nM) was then added and cells incubated for a further 24 h. The cells were then incubated with MTT solution (5 mg/ml) for 4 h at 37˚C and 5% CO_2. Subsequently, crystalline formazan precipitates were re-solubilized in DMSO and the absorption was measured at a wavelength of 570 nm in a microtiter plate reader. Cell growth was expressed as the percentage of viable cells relative to that of the untreated control.

Densitometric analysis. Densitometric analysis of images of western blots and zymograms was performed using image-processing software (Bioprofil Vilber Loumart, France).

Statistical analysis. Mean values were derived from experiments performed in triplicate. These values were compared using Student's *t*-test. Additionally, post-hoc testing, using the Newman-Keuls (SNK) test was used to compare for differences between the selected pairs of means. In all instances, $p<0.05$ was considered statistically significant.

Results

sCT increases the growth of MG63 cells with concomitant activation of PKB. Calcitonin has been shown to regulate cell growth in various cell lines both positively or negatively. Specifically, calcitonin was suggested to have wound-healing effects in bone, resulting in increased bone formation and increased alkaline phosphatase activity (13). The MTT assay was used to evaluate the effects of sCT on cell growth of MG63 cells. Our results showed that treatment of MG63 cells with 10 nM or 50 nM sCT for 24 h increased cell growth by 16 and 40%, respectively compared to untreated cells (Figure 1A).

Additionally, several studies indicate that members of the calcitonin family induce cell growth through activation of signaling pathways that include MAPKs and PKB (14). We examined activation of ERK1/2 MAPKs and PKB in MG63 cells treated with 10 nM sCT for different time intervals ranging from 0 to 30 min. Western blot analysis of total cell lysates indicated that treatment of MG63 cells with 10 nM sCT did not induce phosphorylation of ERK1/2, whereas it did induce phosphorylation of PKB at Ser473, 5 min following stimulation (Figure 1B and C, respectively).

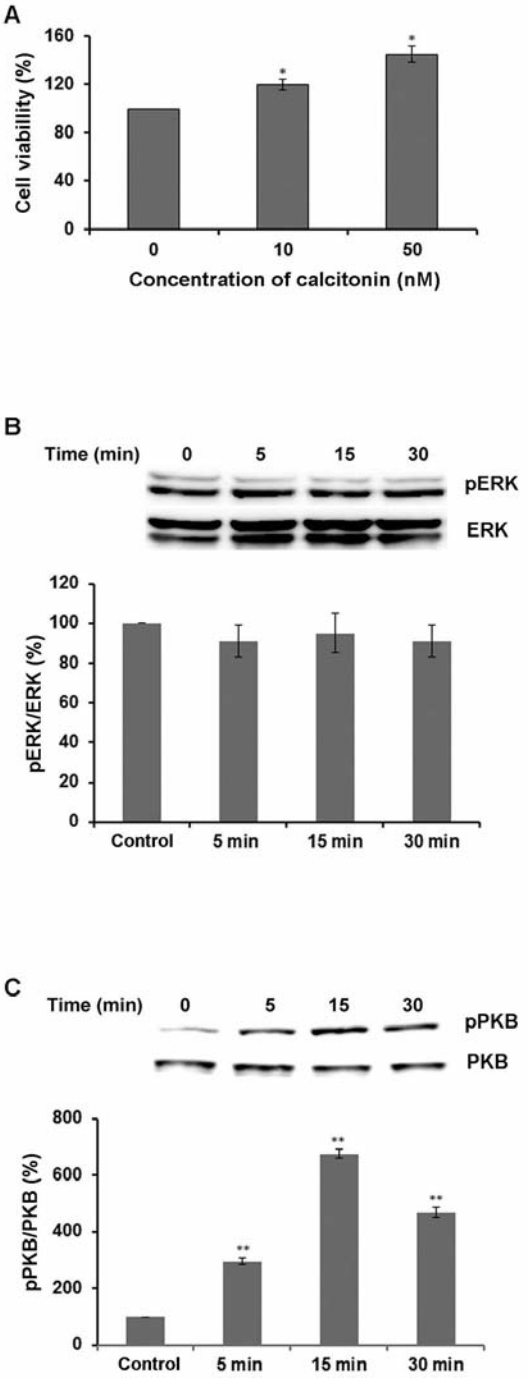

Figure 1. *Calcitonin increases growth of MG63 cells through PKB activation. A: Cell growth in the presence of 10 or 50 nM salmon calcitonin (sCT) for 24 h was estimated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data represent the mean±SD from three independent experiments. Cells were treated with 10 nM sCT for 5, 15 and 30 min, then activation of ERK1/2 (B) and PKB (C) kinases was determined by western blot analysis with antibodies against their phosphorylated forms. For normalization of the results, the membranes were re-probed with antibodies against the non phosphorylated proteins. Data represent the mean±SD from three independent experiments. Differences from the control were significant at *p<0.05.*

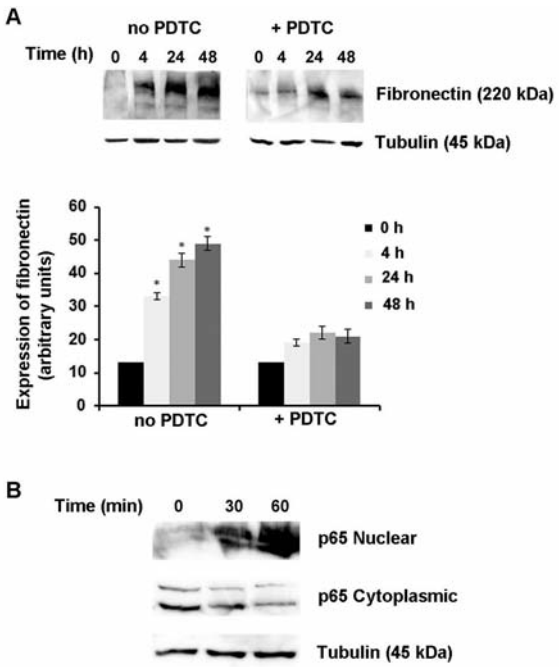

Figure 2. *Calcitonin induces fibronectin expression in a nuclear factor-kappa B (NF-κB)-dependent manner. Cells were treated with 10 nM salmon calcitonin (sCT) in the presence or absence of 100 μM pyrrolidine dithiocarbamate (PDTC). A: At different time intervals, cells were lysed and equal amounts of total cell lysates were analyzed on sodium dodecyl sulfate (SDS) gels and immunoblotted with antibody against fibronectin. For normalization, membranes were re-probed against tubulin. Data represent the mean±SD from three independent experiments. B: To confirm activation of NF-κB in the nucleus, the cells were treated as described above for 0-60 min, lysed and fractionated as described in the Materials and Methods. Cytoplasmic and nuclear extracts were immunoblotted with anti-p65 NF-κB. To ensure equal loading membranes carrying the cytoplasmic fraction were re-probed against tubulin. Data represent the mean±SD from three independent experiments. Differences from the control were significant at *p<0.05.*

sCT induces fibronectin expression in an NF-κB-dependent manner. We investigated the effect of sCT on the expression of fibronectin, since it is a major constituent of bone extracellular matrix. As shown in Figure 2A (left panel), treatment of MG63 cells with 10 nM sCT induced up-regulation of fibronectin expression, as early as 4 h after treatment in a time-dependent manner. NF-κB activation has been reported to be involved in fibronectin induction in several cell types including osteoblasts (6). To examine whether NF-κB activation is involved in the signal transduction pathway resulting in fibronectin up-regulation in calcitonin-treated MG63 cells, we used PDTC, an NF-κB inhibitor. Western blot analysis of total cell lysates showed that in the presence of 100 μM PDTC, sCT-induced up-regulation of fibronectin was abrogated (Figure 2A, right panel). Consequently, increased fibronectin expression in sCT-treated MG63 cells partly involved NF-κB activation. To

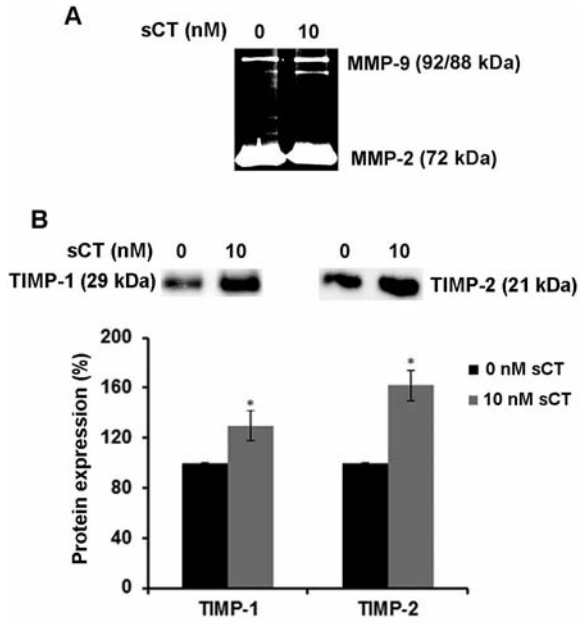

Figure 3. *The effect of salmon calcitonin (sCT) on the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Cells were treated with 10 nM sCT for 24 h. Conditioned culture supernatants were used to detect changes in the enzymatic activity of secreted MMPs by gelatin zymography (A). To determine secreted TIMP-1 and TIMP-2 levels, samples of conditioned culture supernatants were analyzed on 10% sodium dodecyl sulfate (SDS) gels and immunoblotted with polyclonal antibodies against TIMP-1 and TIMP-2 (B). The volume of conditioned medium loaded per lane was adjusted according to the cell number obtained at harvest. Data represent mean±SD from three independent experiments. Differences from the control were significant at *p<0.05.*

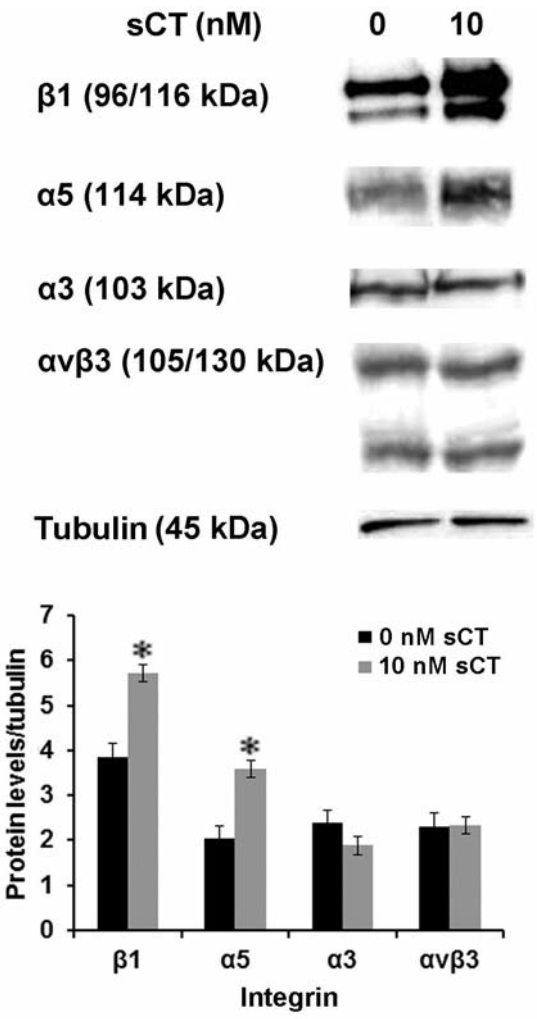

Figure 4. *The effect of salmon calcitonin (sCT) on integrin expression. Cells were treated with 10 nM sCT for 24 h, lysed and equal amounts of total cell lysates were analyzed on sodium dodecyl sulfate (SDS) gels and immunoblotted with monocloclonal antibodies against α5, α3 αvβ3 and β1 integrins. For normalization of the results the membranes were re-probed with antibodies against tubulin. Data represent the mean±SD from three independent experiments. Differences from the control were significant at *p<0.05.*

further evaluate NF-κB activation in MG63 cells treated with 10 nM sCT for 30 and 60 min, we performed western blot analysis of cytosolic and nuclear extracts. As shown in Figure 2B, treatment of MG63 cells with sCT resulted in a marked translocation of the p65 NF-κB subunit from the cytosol to the nucleus as early as 30 min after treatment.

Effect of sCT on the expression of ECM-related molecules. Calcitonin has been reported both to enhance and inhibit the expression/activity of MMPs in various cell types (1, 5). To examine the effect of calcitonin on the enzymatic activity of MMPs, we performed gelatin zymography using conditioned culture supernatants of MG63 cells treated with 10 nM sCT for 24 h. Our results show that treatment of MG63 cells with 10 nM sCT for 24 h increased the activated form of MMP-9, whereas MMP-2 was not altered (Figure3A). The same experimental conditions led to changes in protein expression of TIMPs, specific MMP inhibitors regulating the activity of MMPs; this expression was also examined by western blot analysis of conditioned culture supernatants. Our results show that in the presence of sCT, the expression of TIMP-1 and -2 was increased. More specifically, expression of TIMP-1, the specific inhibitor of MMP-9, was increased by 30% and that of TIMP-2, the specific inhibitor of MMP-2, was increased by 60% in the presence of 10 nM of sCT (Figure 3B).

Recent data suggest that fibronectin-induced MMP-9 expression involves both α5β1 and αvβ3 integrins (15). Thus, we examined integrin expression of MG63 cells treated with 10 nM sCT for 24 h. Western blot analysis of total cell lysates indicated up-regulation of β1 integrin by 48% and that of α5 integrin by approximately 80%, whereas the expression of α3 and αvβ3 integrins remained unchanged (Figure 4).

Discussion

Calcitonin has been used extensively as a therapeutic tool in the regulation of bone remodeling, even though recent concerns have been raised that associate its use with an increased risk of cancer (16). Recent studies report that calcitonin is involved in several cellular functions, including cell invasion, migration, and survival (1, 2, 17). Moreover, calcitonin plays a major role in cell growth and has been suggested to have either mitogenic or inhibitory effect on cell proliferation depending on the cell type and experimental conditions (18). In particular, calcitonin has an anabolic effect in bone through inhibition of osteoclast activity and promotion of growth of osteoblastic cells, including osteosarcoma cell lines SaOS-2 and TE-85 (13). In accordance with this, our results demonstrate that sCT increased cell growth of MG63 osteosarcoma cells.

Calcitonin family members have been shown to activate several kinases; the calcitonin gene-related peptide was reported to activate MAPKs and regulate proliferation of several cell types positively or negatively (19, 20); adrenomedullin activates both PKB and MAPKs, promotes migration and proliferation, and results in resistance to apoptosis in several cell types, including osteoblastic and osteocytic cell lines (21, 22). In the present study, we showed that calcitonin activates PKB kinase, similarly to previous studies demonstrating that calcitonin induced a rapid but sustained increase in PKB activation in prostate cancer cells (23). On the contrary, we did not observe phosphorylation of ERK1/2 in the presence of sCT; calcitonin was proposed to reduce ERK1/2 phosphorylation in MDA-MB-231 cells with constitutive phosphorylation of ERK1/2 (24). Furthermore, another calciotrophic polypeptide hormone, parathyroid hormone was shown to induce cell growth and increase both collagen and fibronectin in chondrocytes (25). In this study, we demonstrated, as far as we are concerned, for the first time, that sCT, induces fibronectin expression in a time-dependent manner, suggesting that calcitonin plays significant role in the regulation of cell matrix interactions and the expression of ECM components (5).

Expression of fibronectin is induced by growth factors, glucose and cytokines in a PKB-dependent manner and is regulated by various transcription factors including NF-κB, AP-1 Sp1, depending on the stimulus and the cell type used (6, 26). According to our results, sCT-induced NF-κB activation contributes to up-regulation of fibronectin expression in the MG63 osteosarcoma cell line, since in the presence of the p65 inhibitor PDTC, up-regulation of fibronectin was abrogated. This is consistent with previous studies indicating the critical role of NF-κB in induction of fibronectin in osteoblasts (6).

Recent reports attribute a pro-oncogenic role of fibronectin due to its promotion of cell growth and proliferation, resistance to apoptosis, control of cell-cycle progression, and stimulation of the invasive and metastatic potential of cancer cells (7, 27). Such a pro-oncogenic effect has been attributed in part to its regulation of expression of MMPs and TIMPs (9). In our system, we showed that sCT-induced up-regulation of fibronectin expression was accompanied by enhanced enzymatic activity of MMP-9. Previous reports have suggested that changes in ECM composition/structure alter the expression and activity of MMPs in various cell types (15, 28). Moreover, it has been shown that calcitonin either induces increased enzymatic activity of gelatinases (MMP-2 and -9) or inhibits the expression and activity of MMPs (1, 5). Consequently, increased enzymatic activity of MMP-9 could involve either calcitonin itself or calcitonin-induced fibronectin up-regulation. Furthermore, the effect of sCT on the expression of both TIMP-1 and TIMP-2 was also examined. We documented that sCT induced increased expression of both TIMP-1 and TIMP-2, which have additional roles apart from MMP inhibition. More specifically, TIMP-1 and TIMP-2 have been implicated in increased cell growth and survival of MG63 cells, as well as regulation of cell migration and angiogenesis in several cell lines (29). These data indicate an important role for MMP-9 and TIMPs in several physiological and pathological cellular processes.

The stimulation of MMP-9 by sCT was paralleled by an increase in protein expression of the α5 integrin subunit, in agreement with a recent report suggesting that interactions of integrin receptor α5β1 with fibronectin induce MMP-9 expression (15, 30). In addition, the effect of calcitonin on integrin expression has been previously reported (31).

In conclusion, our results suggest that the anabolic effect of calcitonin in bone includes increased cell growth, activation of NF-κB, and increased expression of fibronectin, an ECM component with pro-oncogenic effect. This effect could be related to increased MMP-9 enzymatic activity and increased expression of integrin receptors including α5β1. In this phenomenon, increased expression of TIMP-1 and TIMP-2 seem to be involved. These findings raise serious concerns with regard to the use of calcitonin in clinical practice as a treatment for osteoporosis.

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