Kidney International, Vol. 50 (1996), pp. 1476–1482

LABORATORY INVESTIGATION

Selective effect of thiazides on the human osteoblast-like cell line MG-63

R. AUBIN, P. MÉNARD, and DANIEL LAJEUNESSE

Hôpital Maisonneuve-Rosemont, Montréal, Québec, Canada

Selective effect of thiazides on the human osteoblast-like cell line MG-63. Thiazide diuretics have been shown to decrease bone-loss rate and to improve bone mineral density in patients using this medication. However, the exact role of thiazides on bone cells is still debated. In the present work, we studied whether thiazides could affect the normal features of osteoblasts using the human model cell line MG-63. Hydrochlorothiazide (HCTZ) did not affect cell growth nor DNA synthesis in these cells, yet slightly increased alkaline phosphatase activity in these cells at pharmacologically relevant concentrations. Under similar conditions, HCTZ dose-dependently inhibited $1,25(OH)_2D_3$-induced osteocalcin secretion by these cells (maximal effect, −40 to 50%, $P < 0.005$). However, HCTZ did not inhibit the basal production of osteocalcin in MG-63 cells (without $1,25(OH)_2D_3$ induction), which was very low to undectable. Two different thiazide derivatives, chlorothiazide and cyclothiazide, and two structurally related sulfonamides with selective inhibition of carbonic anhydrase (Acetazolamide) or hyperglycemic effects (Diazoxide) were also tested. Chlorothiazide (1000 μM) inhibited osteocalcin secretion (−42 ± 12.7%) at doses 10-fold higher than HCTZ (100 μM) while cyclothiazide was effective at doses of 1 μM (−27 ± 3.6%), and hence 100-fold lower than HCTZ, compatible with the relative natriuretic effect *in vivo* of these compounds. Acetazolamide (10 μM) poorly affected osteocalcin secretion at doses 100-fold higher than those needed *in vivo* to inhibit carbonic anhydrase. Likewise, Diazoxide (100 μM) poorly affected osteocalcin secretion at doses known to promote its biological effect. Higher doses of acetazolamide and diazoxide induced cell death. Neither Acetazolamide nor Diazoxide affected alkaline phosphatase, whereas chlorothiazide had a weak positive effect on this enzymatic activity. The production of macrophage colony-stimulating factor (M-CSF) was stimulated in the presence of $1,25(OH)_2D_3$ (50 nM), TNF-α (2 ng/ml) or both in MG-63 cells. HCTZ (25 μM, 24 hr of preincubation) did not modify basal M-CSF production and did not reduce the response to $1,25(OH)_2D_3$ alone. In contrast, HCTZ inhibited the response to TNF-α alone ($P < 0.05$), and also reduced the response to a combination of $1,25(OH)_2D_3$ and TNF-α ($P < 0.01$). In conclusion, these results indicate that thiazide diuretics show a selective inhibion of osteocalcin secretion and M-CSF production by MG-63 cells unlike structurally related drugs. Therefore, these features may explain, in part, the positive effect of thiazides on bone mineral density.

In renal cells, the action of thiazides was first believed to be indirect. This was based on the lack of correlation between the degree of tubular secretion of thiazides and their natriuretic effect when infused in the renal portal system of the hen using the Sperber technique [1]. Supporting this hypothesis of a peritubular rather than a luminal action of thiazides, Dan and Gemba [2] reported that hydrochlorothiazide (HCTZ) significantly stimulated ATP-dependent Ca^{2+} uptake by microsomes. However, HCTZ was only effective at a concentration of 0.5 mM and the authors failed to clearly demonstrate the exact nature of their "microsomal" preparations.

In contrast, Costanzo and Windhager [3] and Costanzo [4] showed, with a microperfusion technique *in vivo*, that chlorothiazide (CTZ) was acting from the lumen of the tubule. CTZ increased net Ca^{2+} and reduced net sodium (Na^+) reabsorption. Therefore the mechanism of action of thiazides was still unclear. In elegant binding experiments, Beaumont, Vaughn and Fanestil [5] showed that [^{3}H]-metolazone, a diuretic with a thiazide-like mechanism of action, labels a site in rat kidney membranes that has characteristics of the thiazide-sensitive ion transporter. The binding of [^{3}H]-metholazone was later found only in the luminal membrane of distal tubules by the same group [6]. *In vitro*, thiazides act directly on the luminal membrane of the distal tubule to increase calcium uptake [7, 8], whereas luminal membrane of the proximal tubules are insensitive to the treatment [8].

Recent data also suggest a positive role of thiazides on bone [9]. Wasnich et al [10, 11] reported an increase in bone mineral density (by single photon absorptiometry) in patients treated with thiazides alone or in combination with estrogens. The long-term use of thiazides also results in a low prevalence of bone fractures in aged patients [12, 13], an effect not observed by Adland-Davenport et al [14]. However, a small number of patients was used in this study group (58 women) compared with the studies of Wasnich et al (993 women [11], 1368 men [10]) or those from Cauley and Cumming (9707 women) [12] and could explain these discrepancies. In contrast, a longitudinal study (5 years) of 325 men showed a decrease in bone-loss rate with thiazides [15]. In this study, men using antihypertensive medications other than thiazide showed an increase in bone-loss rate, providing more evidence that thiazides reduce fracture risk by preserving bone mass. LaCroix et al also concluded that, with a cohort of 9518 men and women, the use of thiazide diuretics reduced the risk of hip fracture by approximately one third [16]. Lemann et al [17] also reported that hydrochlorothiazide reduced bone resorption in healthy men despite elevated $1,25(OH)_2D_3$ levels. Thus, these studies point to a direct action of thiazides on bone metabolism. Moreover, a recent meta-analysis of a number of clinical studies suggests that thiazides should be considered as part of an approach to osteoporotic fracture prevention, particularly in hypertensive subjects [18].

Received for publication January 11, 1996
and in revised form June 3, 1996
Accepted for publication June 3, 1996

In the present study, we examined whether thiazides may have a positive role on bone mineral density by acting on the proliferation of osteoblasts, the differentiation of osteoblasts or on the release of mediators of bone resorption. We used the human osteosarcoma cell model, MG-63, which reproduces some of the features of osteoblast-like cells *in vitro*. With this model cell line we observed that thiazides seem to act directly on parameters that *in vivo* would regulate the activity of osteoclasts, which may explain their positive role on bone mineral density.

Methods

Cell culture and DNA synthesis

Human osteosarcoma MG-63 cells were obtained through the American Type Culture Collection (Rockville, MD, USA). They were grown in HAMF12/DMEM media containing 10% charcoal-stripped fetal bovine serum (FBS) prepared as previously described [19], 1% penicillin-streptomycin mixture (PS, Gibco) and 50 μg/ml ascorbic acid. The cells were cultured in an incubator gassed with 5% CO_2 at 37°C. Culture medium was changed twice a week. Cells were split once a week at a ratio of 1:9 with Trypsin 0.025%-EDTA 0.01% in phosphate buffered saline (PBS) pH 7.4 (Trypsin, EDTA). Confluent cells were treated with hydrochlorothiazide (HCTZ, 0.01 to 100 μM) for 24 to 72 hours in a mixture of HAMF12/DMEM (1:1) containing 2% FBS and 50 μg/ml ascorbic acid. Where appropriate, the cells were incubated in parallel with either $1,25(OH)_2D_3$ (50 nM), TNF-α or their combination. In another set of experiments, chlorothiazide (1000 μM), cyclothiazide (1 μM), acetazolamide (10 μM) and Diazoxide (100 μM) were incubated in parallel to HCTZ for 48 hours in the presence of $1,25(OH)_2D_3$ (50 nM). MG-63 cells were then treated as usual for the determination of alkaline phosphatase and osteocalcin secretion.

The effect of hydrochlorothiazide on cellular growth was evaluated by the incorporation of ^{3}H-thymidine in DNA following the procedure described by Puzas and Brand [20]. Briefly, cells treated with increasing doses of HCTZ or the vehicle for 48 hours were incubated for the last three hours of culture in the presence of ^{3}H-thymidine. After three washings with PBS, cells were solubilized and aliquots counted in a β-counter. Cells treated in parallel with the vehicle or HCTZ at the same final concentrations were used to evaluate total cell number. Cell death was also evaluated by the trypan blue exclusion method. Cells treated with HCTZ, chlorothiazide or acetazolamide as above were incubated with 1% trypan blue for 30 minutes at 37°C in culture media. After washing the cells twice with phosphate buffered saline, the cells were split with Trypsin. EDTA as above, and cells were counted with a haemocytometer. Results are given in terms of % cells not taking the dye per total number of cells counted.

Alkaline phosphatase

Alkaline phosphatase (ALPase) activity was determined as the release of p-nitrophenol hydrolyzed from p-nitrophenyl phosphate (12.5 mM) at 37°C for 30 minutes as previously described [21–24]. Briefly, cells were first solubilized in ALPase buffer (100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 10.5) with agitation for 60 minutes at 5°C. All samples were then sonicated for 5 seconds with an Ultrasonic Sonifier cell disruptor at a setting of 5.

Osteocalcin determination

Confluent cells in 35 mm petri dishes were washed twice with Hank's buffered salt solution (HBSS) containing: $CaCl_2$, 1.26 mM; KCl, 5.37 mM; KH_2PO_4, 0.44 mM; $MgCl_2$, 0.49 mM; $MgSO_4$, 0.41 mM; NaCl, 136.9 mM; $NaHCO_3$, 25 mM; Na_2HPO_4, 0.336 mM; D-glucose, 5.55 mM, pH 7.4. The cells were then covered with 1 ml of HAMF12/DMEM media containing 2% charcoal-stripped FBS, 1% PS, 50 μg/ml ascorbic acid and 10^{-8} menadione vitamin K_3 (Sigma, St. Louis, MO, USA), with or without $1,25(OH)_2D_3$ and increasing doses of HCTZ (1 to 100 μM). The media was removed at the end of the incubation and frozen at −80°C. Cells were washed again twice with HBSS, and either used to evaluate final cell density, alkaline phosphatase (see above), or protein determination by the method of Lowry et al [21].

Osteocalcin determinations were done by RIA (Biomedical Technologies Inc., Stoughton, MA, USA) with antibodies raised against bovine osteocalcin that cross-react with human osteocalcin. Osteocalcin was also determined on aliquots of the media used for the incubations to correct for bovine osteocalcin contamination, which was very low with the procedure used (< 0.1 ng/ml with the final dilution) [19, 22].

Measurement of macrophage colony-stimulating factor

Confluent cells, plated on 35 mm petri dishes, were washed twice with HBSS pH 7.4 and covered with 1 ml of HAMF12/DMEM (1:1) media containing 2% FBS, 50 μg/ml ascorbic acid and 2 ng/ml TNF-α, 50 nM $1,25(OH)_2D_3$ or their combination, in the presence or absence of hydrochlorothiazide (25 μM). After 48 hours of incubation at 37°C, the supernatant was recuperated and aliquots were taken to measure M-CSF by an in-house ELISA. Briefly, 96 wells plates were coated with 10 μg/ml of a goat anti-human M-CSF antibody (R & D Systems, Minneapolis, MN, USA) for two hours at 37°C. This was followed by the coating of non-specific sites by a treatment with 5% gelatin in a carbonate buffer pH 9.5 for two hours at 37°C. A standard curve was then established with known amounts of recombinant human M-CSF (Genzyme, Markham, Ontario, Canada) by incubating the cytokine for one hour at 37°C. Cell culture media from both cell cultures, at the appropriate dilution, were also incubated in parallel with the standard curve. After washing the plates, a polyclonal rabbit anti-human M-CSF (Genzyme) was added for one hour at 37°C. The detection of this second antibody was performed by the addition of a goat anti-rabbit antibody coupled to a peroxydase (Organon Technika, Scarborough, Ontario, USA). The detection of the peroxydase was evaluated by the hydrolysis of 0.05% ABTS substrate (Organon Technika) for 30 minutes at 25°C in the presence of 0.1% H_2O_2. Plates were then read on a plate reader at 405 nM and values determined against the standard curve established in parallel. The validation of the ELISA shows intra-assay variations of 6% and inter-assay variations of 8%.

Statistics

Statistical differences were evaluated by analysis of variance in dose-response experiments and by two-tailed Student's *t*-tests. In each case, the statistical test used is indicated and the number of experiments is stated individually in the legend of each Figure. Further analysis of dose-response experiments were performed by the multiple comparison analysis of Bonferroni/Dunn with a

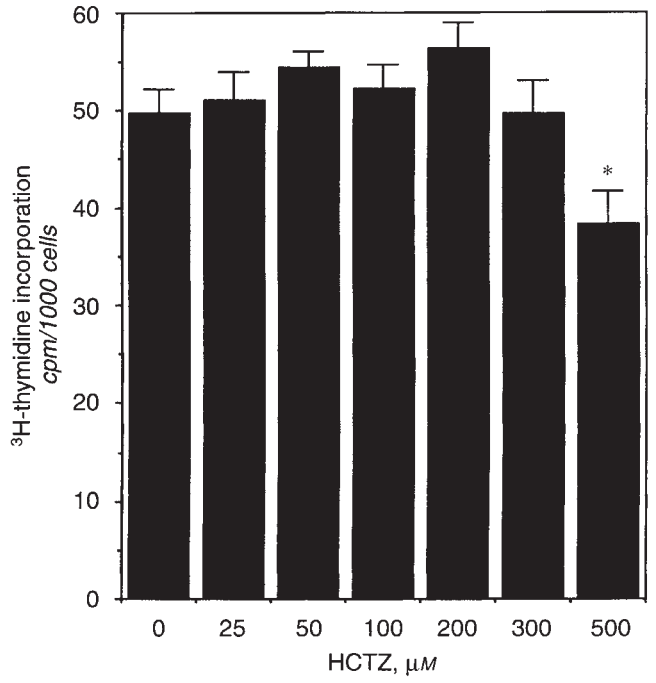

Fig. 1. *Effect of hydrochlorothiazide on DNA synthesis by MG-63 cells.* Cells were pretreated for 48 hours with increasing doses of HCTZ in culture media containing 2% FBS. ^{3}H-thymidine was added for the last three hours of incubation, and incorporation into DNA was evaluated on aliquots after the solubilization of cells in 1 N NaOH overnight at 4°C. The results are the mean ± SEM of 7 experiments with triplicate dishes per condition. *$P < 0.025$.

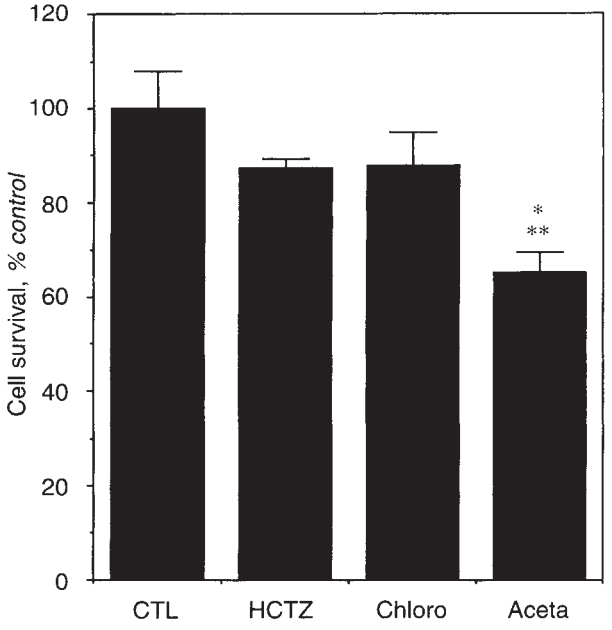

Fig. 2. *Effect of HCTZ, chlorothiazide (Chloro) and acetazolamide (Aceta) on cell growth by MG-63 cells.* Cells were incubated for 48 hours with 100 μM HCTZ, 1000 μM Chloro, 1 μM Aceta or the vehicle (CTL) in culture media containing 2% FBS. At the end of this incubation, cells were stained with 1% trypan blue for 30 minutes at 37°C, split by Trypsin. EDTA treatment and counted. Values represent the relative percentage of viable cells over total viable cells counted in controls. Results are expressed as mean ± SEM of four separate experiments with three dishes per conditions tested. *$P < 0.005$ versus CTL and HCTZ, **$P < 0.05$ versus Chloro.

computer assisted program (StatView 4.0 from MacIntosh). A level of 5% for the Bonferroni/Dunn analysis was chosen as significant.

Results

Cellular proliferation

Cellular proliferation was evaluated by the incorporation of ^{3}H-thymidine in MG-63 cells in response to increasing doses of hydrochlorothiazide (Fig. 1). The diuretic (up to 300 μM) failed to modify DNA synthesis by MG-63 cells. Higher concentrations (500 μM) of HCTZ produced slight reductions in DNA synthesis, that could be due to a toxic effect of this diuretic at these high doses. Indeed, cellular death was evaluated in response to the different thiazide diuretics used and to acetazolamide by the trypan blue exclusion method. Figure 2 shows that HCTZ (100 μM) and chlorothiazide (1000 μM) did not induce significant cell death at these maximal concentrations as compared to controls, whereas acetazolamide induced cell death at doses higher than 10 μM as compared to controls, HCTZ and chlorothiazide.

Alkaline phosphatase activity

In contrast to the absence of an effect on DNA synthesis, HCTZ slightly but dose-dependently stimulated $1,25(OH)_2D_3$-induced alkaline phosphatase activity in MG-63 cells (Fig. 3). The increase in alkaline phosphatase activity was gradual, and a significant effect of the diuretic was noted at doses as low as 1 μM ($P < 0.005$ by analysis of variance, Fig. 3). The Bonferroni/Dunn multiple analysis further indicated a significant difference ($P < 0.05$) between 0 (control) and 1 or 10 μM HCTZ, and between 0.1 μM and 10 μM, hence indicating that the effect saturated between 1 to 100 μM. Higher doses (up to 500 μM) caused a further increase in alkaline phosphatase activity while reducing DNA synthesis, but this was associated with a toxic effect of the diuretic as assessed by the trypan blue exclusion method (not shown).

Osteocalcin secretion

In contrast to the increase in alkaline phosphatase activity induced by HCTZ in MG-63 cells, osteocalcin secretion was strongly reduced by this diuretic (Fig. 4). Our results indicated that osteocalcin release in the absence of $1,25(OH)_2D_3$, which is very low in MG-63 cells, was not affected by HCTZ. However, HCTZ dose-dependently (1 to 100 μM) inhibited $1,25(OH)_2D_3$-induced osteocalcin secretion by these cells (Fig. 4, $P < 0.005$ by analysis of variance). The Bonferroni/Dunn multiple comparison analysis further indicated significant differences between 0 (control) and 10 and 100 μM HCTZ, and between 1 and 100 μM HCTZ ($P < 0.05$ for all comparisons). This inhibition ranged between 30 to 50% in the presence of 10 to 100 μM HCTZ.

Production of macrophage colony-stimulating factor

Using an in-house ELISA for the measure of M-CSF (range, 0.05 to 5 U/ml; recovery, 89.7 ± 4.4%), we evaluated that MG-63 cells can release constitutively M-CSF at a rate of 1.5 U/mg protein/48 hours. This basal release of M-CSF is stimulated in MG-63 cells about twofold by $1,25(OH)_2D_3$ ($P < 0.05$), TNF-α ($P < 0.005$) or a combination of both agents ($P < 0.005$). The

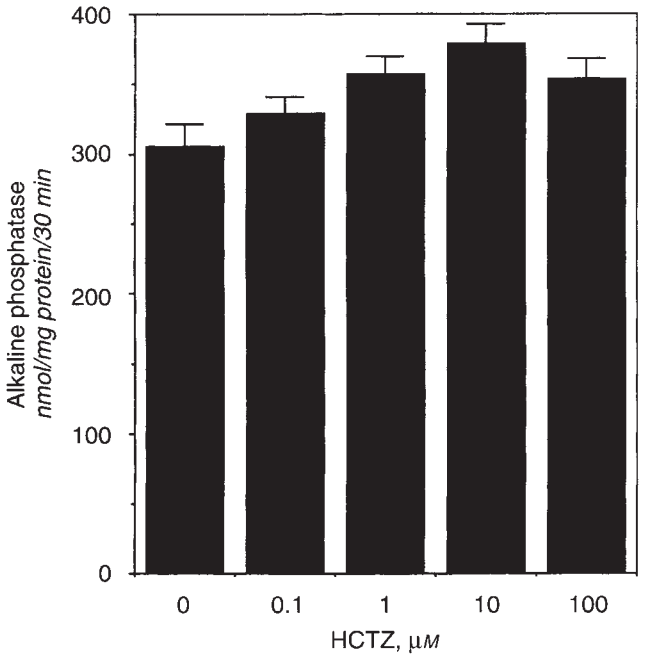

Fig. 3. *Dose-dependent effect of hydrochlorothiazide on alkaline phosphatase activity.* Cells were treated as described in Figure 1 with the addition of $1,25(OH)_2D_3$ (50 nM) for the last 48 hours of culture. They were then solubilized in ALPase buffer and enzymatic activity determined on aliquots. The results are the mean ± SEM of 6 preparations. $P < 0.005$ by analysis of variance.

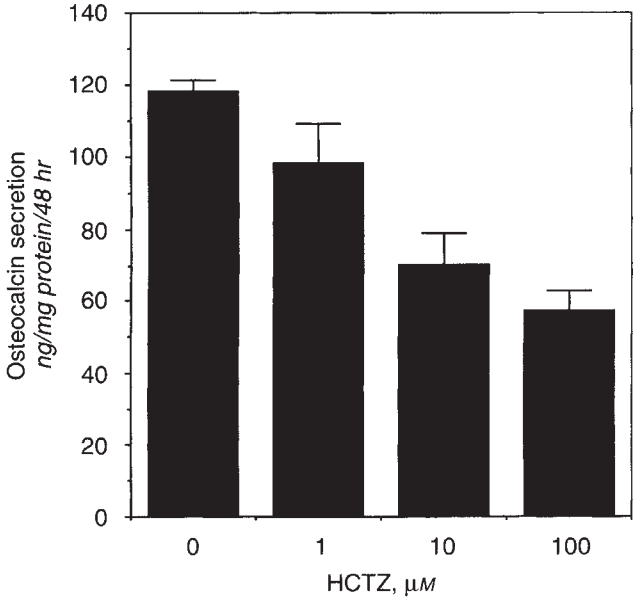

Fig. 4. *Dose-dependent effect of hydrochlorothiazide on osteocalcin release by MG-63 cells.* Cells were treated as in Figure 1, with the addition of $1,25(OH)_2D_3$ (50 nM). At the end of the incubation, aliquots of the supernatant were used to determined osteocalcin by RIA. The results are the mean ± SEM of 4 experiments. $P < 0.005$ by analysis of variance.

treatment of MG-63 cells in parallel with hydrochlorothiazide did not significantly inhibit the stimulating effect of $1,25(OH)_2D_3$, yet reduced the stimulating effect of TNF-α (−40%, $P < 0.05$) on this

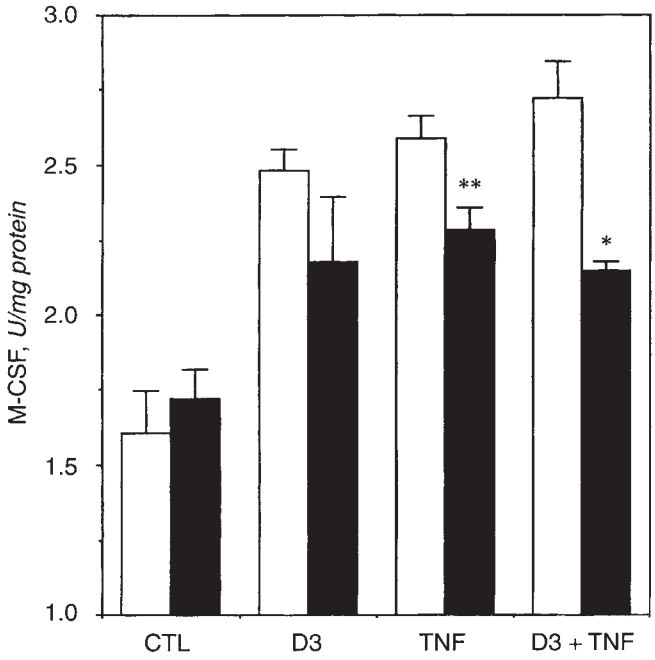

Fig. 5. *Effect of hydrochlorothiazide on macrophage colony-stimulating factor release by MG-63 cells.* Cells were incubated for 48 hours in the presence or absence of $1,25(OH)_2D_3$ (50 nM) alone, TNF-α (2 ng/ml) alone or their combination with (■) or without (□) HCTZ (25 μM). At the end of the incubation, aliquots of the supernatant were recuperated and M-CSF evaluated by ELISA as described in the **Methods** section. The results are the mean ± SEM of 4 to 6 experiments. $^*P < 0.01$, $^{**}P < 0.05$ versus same treatment without thiazides. $P < 0.001$ for $1,25(OH)_2D_3$, TNF-α and $1,25(OH)_2D_3$ + TNF-α versus CTL.

process (similar results were obtained for IL-1α, not shown). In contrast, the addition of HCTZ to a combination of $1,25(OH)_2D_3$ + TNF-α produced a significant inhibition of their combined effect on M-CSF release (50% inhibition, $P < 0.01$, Fig. 5).

Specificity of the thiazide effect of osteocalcin secretion

We next tested whether the inhibition of osteocalcin secretion by MG-63 cells was a thiazide-like selective effect or if this was related to their weak carbonic anhydrase inhibitory activity. As shown in Figure 6, chlorothiazide (1000 μM) reproduced the inhibitory effect of HCTZ (100 μM) on osteocalcin secretion (−42 ± 12.7%), albeit at a concentration 10 times that for HCTZ, while cyclothiazide induced a similar inhibition (−27 ± 3.6%) at a concentration of 1 μM. In contrast, the sulfonamide related agent acetazolamide, which is a selective carbonic anhydrase inhibitor, had no effect on this activity. Likewise, Diazoxide, a structurally related sulfonamide derivative very similar to thiazides, also showed very poor inhibition of osteocalcin secretion (Fig. 6). In comparison, PGE_2, which is one of the most potent inhibitor of osteocalcin secretion, reduced this activity by 69.5 ± 3.3% (Fig. 6).

Discussion

The exact mechanism of action for the positive effect of thiazide diuretics on bone mineral density in patients using this type of medication instead of other diuretics or antihypertensive agents has never been elucidated. Both a direct stimulating effect on

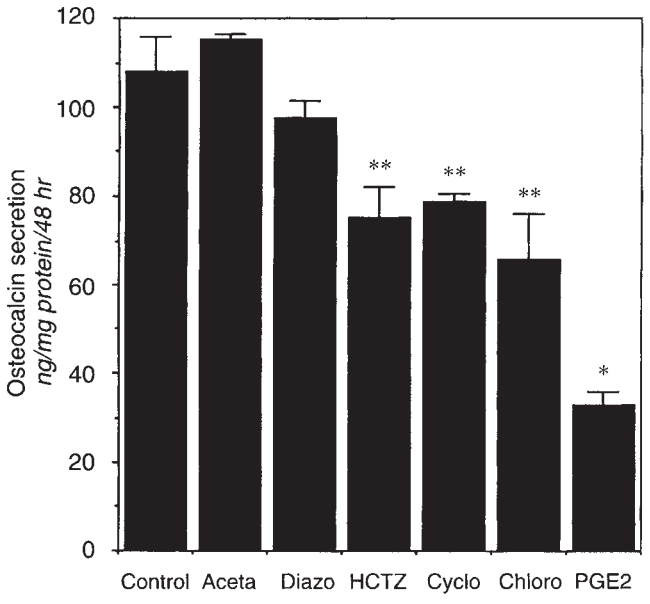

Fig. 6. *Specificity of thiazide derivatives on osteocalcin secretion.* Confluent cells were preincubated for 48 hours with either HCTZ (100 μM), chlorothiazide (Chloro, 1000 μM), cyclothiazide (Cyclo, 1 μM), Acetazolamide (Aceta, 1 μM) or Diazoxide (Diazo, 100 μM) in culture media containing 2% FBS and 50 nM $1,25(OH)_2D_3$. At the end of the incubation, osteocalcin release was determined on aliquots of cell culture media by RIA. The results are the mean ± SEM of 5 to 12 experiments run in triplicate. $^*P < 0.001$, $^{**}P < 0.005$ versus Control.

bone formation rate/osteoblast growth or an inhibiting effect on bone resorption (or both) could potentially explain this important clinical finding. Moreover, this effect may potentially directly affect the cells responsible for these mechanisms or yet may be indirect via the regulation of other effectors. The present study indicates that osteoblasts are targets for the action of thiazides, and that this action is specific for thiazide derivatives.

Indeed, using the human osteoblast-like cell line MG-63, we were able to demonstrate that hydrochlorothiazide, a diuretic often prescribed to hypertensive patients, influences different pathways in these cells. The diuretic was without effect on DNA synthesis and cell growth by these cells *in vitro*, however, whether this is also the case *in vivo* remains to be investigated. Hall and Schaueblin obtained similar results with rat osteosarcoma UMR cells [23], whereas Song and Wergedal presented data showing that at 1 μM HCTZ, ^{3}H-thymidine incorporation was increased in human primary osteoblasts [24]. This discrepancy between the results of Song and Wergedal and ours may be related to the length of exposition of cells to HCTZ (only two days in our experimental protocol as opposed to seven days in theirs) or to the use of primary human osteoblasts in the former study. The slight increase in alkaline phosphatase activity measured in MG-63 cells *in vitro* in response to HCTZ may reflect an increased differentiation of these cells with thiazides, as these were the only drugs tested that reproduced that effect, and because alkaline phosphatase is a good indicator of osteoblast-like cell differentiation. However, serum alkaline phosphatase levels do not seem to be influenced by thiazides *in vivo* [25-27], although none of these studies were performed on large numbers of patients, and one lasted only for a few days [27], inconsistent with the long-term action of thiazides on bone metabolic parameters *in vivo* [9–13].

In addition, HCTZ influenced pathways involved in the triggering/recruitment of osteoclasts. The diuretic inhibited the release of osteocalcin, a protein proposed to influence bone resorption [28]. This inhibitory effect was observed in a range of concentration for HCTZ that is compatible with the doses obtained in patients using this medication [29]. Hence, this *in vitro* inhibition of osteocalcin release may also be present in these patients *in vivo*, which may retard bone resorption. The direct inhibitory effect of HCTZ on the release of M-CSF also indicates that this diuretic can retard resorption. Indeed, the local release of M-CSF by osteoblasts *in vivo* participates in the induction of bone resorption either by the recruitment of preosteoclasts or by directly promoting the differentiation of osteoclasts [30]. It is noteworthy that HCTZ was more effective in the presence of TNF-α than in the presence of $1,25(OH)_2D_3$, since TNF-α is a more potent inducer of bone resorption. Hence, thiazides could be more effective in regulating the inhibitory action of TNF-α on bone mineral density.

The effect of hydrochlorothiazide is specific to this class of diuretics and does not result from secondary effects of these drugs for several reasons. Chlorothiazide reproduced the effects of HCTZ on both alkaline phosphatase activity and osteocalcin secretion, albeit at doses 10-fold higher, compatible with their *in vivo* natriuretic effects [31]. Cyclothiazide, a more potent thiazide diuretic than HCTZ, also inhibited osteocalcin release at doses 100-fold lower than HCTZ compatible with its *in vivo* effect. This inhibitory effect is probably not related to their carbonic anhydrase inhibitory capacities since HCTZ (100 μM) inhibited osteocalcin secretion as efficiently as chlorothiazide (1000 μM), hence at 10-fold lower doses, whereas chlorothiazide is a much more potent inhibitor of CA than HCTZ (15-fold) [31]. Moreover, acetazolamide, a selective CA inhibitor, was unable to reproduce the effect of HCTZ even at doses in the μM range while *in vivo* its maximal effect is observed at 10^{-8} M [32]. Only at 100 μM did it slightly reduce osteocalcin secretion, a dose that also induced cellular damage and death. Thiazides may also show phosphodiesterase inhibition, a situation that can reduce osteocalcin secretion in MG-63 cells due to an elevation of intracellular cAMP [22]. However, the three thiazide diuretics used all inhibited osteocalcin secretion to similar levels whereas chlorothiazide should be the most potent phosphodiesterase inhibitor, about 10- to 20-fold as compared to HCTZ and cyclothiazide [33]. Finally, a structurally related sulfonamide drug, Diazoxide, with known inhibitory effects on insulin secretion [34], was unable to reproduce the full effects of thiazides at effective doses. Indeed, its effect was inconsistent and much weaker than with thiazides or PGE_2. In contrast, PGE_2, used at maximal effective doses, was shown to be the most potent inhibitor of osteocalcin secretion under our experimental setting, a situation directly related to its stimulation of cAMP synthesis in MG-63 cells [22]. This result with PGE_2 also indicates that osteocalcin secretion could not be inhibited by more than 70% under the present experimental conditions, hence suggesting that the inhibition observed in the presence of thiazide derivatives (30 to 50%) is very potent as well.

In a rat model mimicking, in many ways, the events of human urinary stone disease [35], McKee, Nanci and Khan [36] have

shown an intense immunolabeling for two "bone" proteins, osteocalcin and osteopontin. Osteocalcin is strictly produced by osteoblasts [19, 22, 28], whereas osteopontin is synthesized by renal cells as well [37]. Since osteopontin and osteocalcin can form supramolecular complexes *in vitro* [38], the combination of both proteins may contribute to the organic backbone of kidney stones as observed by McKee et al *in vivo* [36]. Hence, the observation that *in vitro*, thiazides inhibited osteocalcin release by osteoblast-like cells suggest that this could participate in the reduction of stone formation, but also in the reduction of bone resorption. It is noteworthy that alendronate, a bisphosphonate used to reduce bone resorption, reduces stone formation during immobilization or bedrest [39], whereas this type of drug also reduces osteocalcin release by osteoblasts *in vitro* [40]. Moreover, long-term use of thiazides reduces serum parathyroid hormone and osteocalcin levels in both aged men and women [41], and postmenopausal women [42]. Hence, since osteocalcin is only synthesized by osteoblasts [28], these results also strongly suggest that thiazides reduce osteocalcin synthesis and/or release *in vivo*, a situation that we were able to show *in vitro* with a human osteoblast-like cell model (MG-63 cells) using three types of thiazide diuretics. It also indicates that this observed reduction in osteocalcin secretion by MG-63 cells in response to thiazides is not due to culture conditions nor to the cell type used.

Peripheral blood monocytes (PBMC) from calcium stone formers with idiopathic hypercalciuria release large amounts of IL-1β and GM-CSF [43], and IL-1β can stimulate the release of M-CSF and IL-6 by osteoblasts. M-CSF and IL-6 are potent inducers of bone resorption, the first one through recruitment of precursor cells for osteoclasts and/or stimulation of the differentiation of these cells, whereas IL-6 would only enhance the second pathway. The reduction of M-CSF production by osteoblast-like MG-63 cells with HCTZ *in vitro* would then suggest that *in vivo* thiazides may also contribute to the reduction of bone resorption via this pathway. In addition, Hall and Schaueblin reported that HCTZ, at doses of 30 to 100 μM, directly inhibits bone resorption by isolated neonatal rat osteoclasts *in vitro* [23]. Hence our results and theirs suggest that thiazides may affect bone resorption via two pathways: directly on osteoclasts, and indirectly via the release from osteoblasts of cytokines/proteins triggering osteoclasts.

In conclusion, thiazide derivatives like hydrochlorothiazide act directly on osteoblast-like cells to inhibit the release of bone resorbing factors, namely osteocalcin and M-CSF, while not affecting the proliferation of osteoblast-like cells. This effect is specific to thiazide-like structures, as related compounds did not reproduce these effects.

Acknowledgment

This work was supported by a grant from the Kidney Foundation of Canada to D. Lajeunesse.

Reprint requests to Daniel Lajeunesse, Ph.D., Centre de Recherche L.C. Simard, Unité des maladies rhumatismales, Hôpital Notre-Dame, 1560, rue Sherbrooke Est, Montréal, Québec, Canada, H2L 4M1.

References

1. ODLIND B, LONNERHOLM G: Renal tubular secretion and effects of chlorothiazide, hydrochlorothiazide and clopamide: A study in the avian kidney. *Acta Pharmacol Toxicol* 51:187–197, 1982
2. DAN T, GEMBA M: Effects of diuretics on calcium uptake and release in renal microsomes. *Biochem Pharmacol* 29:2339–2343, 1980
3. COSTANZO LS, WINDHAGER EE: Calcium and sodium transport by the distal convoluted tubule of the rat. *Am J Physiol* 235:F492–F506, 1978
4. COSTANZO LS: Localization of diuretic action in microperfused rat distal tubules: Ca and Na transport. *Am J Physiol* 17:F527–F535, 1985
5. BEAUMONT K, VAUGHN DA, FANESTIL DD: Thiazide diuretic receptors in rat kidney: Identification with [^{3}H]metalozone. *Proc Natl Acad Sci USA* 85:2311–2314, 1988
6. BEAUMONT K, VAUGHN DA, HEALY DP: Thiazide diuretic receptors: autoradiographic localization in rat kidney with [^{3}H]Metolazone. *J Pharmacol Exp Ther* 250:414–419, 1989
7. BRUNETTE MG, HARVEY N, MAILLOUX J, BOUHTIAUY I, LAJEUNESSE D: The hypocalciuric effect of thiazides: study of the mechanisms, in *Proceedings of the Third International Conference on Diuretics. Diuretics III. Chemistry, Pharmacology and Clinical Applications*, edited by PUSCHETT JB, GREENBERG A, New York, Elsevier Science Publishing Co., 1989, pp 225–227
8. LAJEUNESSE D, BRUNETTE MG: The hypocalciuric effect of thiazides: Subcellular localization of the action. *Pflügers Arch* 417:454–462, 1991
9. COE FL, PARKS JH, BUSHINSHY DA, LANGMAN DB, FAVUS MJ: Chlorthalidone promotes mineral retention in patients with idiopathic hypercalciuria. *Kidney Int* 33:1140–1146, 1988
10. WASNICH RD, BENFANTE RJ, YANO K, HEILBRUN L, VOGEL JM: Thiazide effect on the mineral content of bone. *N Engl J Med* 309:344–347, 1983
11. WASNICH RD, ROSS PD, HEILBRUN LK, VOGEL JM, YANO K, BENFANTE RJ: Differential effects of thiazide and estrogen upon bone mineral content and fracture prevalence. *Obstetrics Gynecol* 67:457–462, 1986
12. CAULEY JA, CUMMINGS SR: Thiazide diuretics preserve bone mass and reduce the risk of fractures in elderly women: A prospective study. (abstract) *J Bone Miner Res* 4(Suppl 1):216, 1989
13. RAY WA, GRIFIN MR, DOWNEY W, MELTON LJ: Long-term use of thiazide diuretics and risk of hip fracture. *Lancet* 1:687–690, 1989
14. ADLAND-DAVENPORT P, MCKENZIE MW, NOLFLOFITZ M, MCKENZIE LC, PENDERGAST JF: Thiazide diuretics and bone mineral content in postmenopausal women. *Am J Obstet Gynecol* 152:630–634, 1985
15. WASNICH RD, DAVIS JW, ROSS PD, VOGEL JM: Thiazides reduce bone loss rate among men. A longitudinal study. (abstract) *J Bone Miner Res* 5(Suppl 1):157, 1990
16. LACROIX AZ, WIENPAHL J, WHITE LR, WALLACE RB, SCHERR PA, GEORGE LK, CORNONI-HUNTLEY J, OSTFELD AM: Thiazide diuretic agents and the incidence of hip fracture. *N Engl J Med* 322:286–290, 1990
17. LEMANN J JR, GRAY RW, MAIERHOFER WJ, CHEUNG HS: Hydrochlorothiazide inhibits bone resorption in men despite experimentally elevated serum 1,25-dihydroxyvitamin D concentrations. *Kidney Int* 28:951–958, 1985
18. JONES G, NGUYEN T, SAMBROOK PN, EISMAN JA: Thiazide diuretics and fractures: Can meta-analysis help? *J Bone Miner Res* 10:106–111, 1995
19. LAJEUNESSE D, FRONDOZA C, SCHOFFIELD B, SACKTOR B: Osteocalcin secretion by the human osteosarcoma cell line MG-63. *J Bone Miner Res* 5:915–922, 1990
20. PUZAS JE, BRAND JS: The effect of bone cell stimulatory factors can be measured with thymidine incorporation only under specific conditions. *Calcif Tissue Int* 39:104–108, 1986
21. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
22. LAJEUNESSE D, KIEBZAK GM, FRONDOZA C, SACKTOR B: Regulation of osteocalcin secretion by human primary bone cells and by the human osteosarcoma cell line MG-63. *Bone Miner* 14:237–250, 1991
23. HALL TJ, SCHAUEBLIN M: Hydrochlorothiazide inhibits osteoclastic bone resorption *in vitro*. *Calcif Tissue Int* 55:266–268, 1994
24. SONG X, WERGEDAL JE: Hydrochlorothiazide stimulates proliferation of human osteoblasts *in vitro* (abstract). *J Bone Miner Res* 8:S362, 1993
25. SAKHAEE K, NICAR MJ, GLASS K, ZERWEKH JE, PAK CYC: Reduction in intestinal calcium absorption by hydrochlorothiazide in postmenopausal osteoporosis. *J Clin Endocrinol Metab* 59:1037–1043, 1984
26. SAKHAEE K, ZISMAN A, POINDEXTER JR, ZERWEKH JE, PAK CYC: Metabolic effects of thiazide and 1,25(OH)$_2$-vitamin D in postmenopausal osteoporosis. *Osteoporosis Int* 3:209–214, 1993
27. PEH CA, HOROWITZ M, WISHART JM, NEED AG, MORRIS HA,

NORDIN BEC: The effect of chlorothiazide on bone-related biochemical variables in normal post-menopausal women. *J Amer Geriatric Soc* 41:513–516, 1993
28. HAUSCHKA PV, LIAN JB, COLE DEC, GUNDBERG CM: Osteocalcin and matrix Gla protein: Vitamin K-dependent proteins in bone. (Review) *Physiol Rev* 69:990–1047, 1989
29. BEERMANN B, GROSCHINSKY-GRIND M: Antihypertensive effect of various doses of hydrochlorothiazide and its relation to the plasma level of the drug. *Eur J Clin Pharmacol* 13:195–201, 1978
30. STANLEY ER: Action of the colony-stimulating factor, CSF-1, in *Biochemistry of Macrophages* (vol 118), New York, Ciba Foundation Symposium, Wiley J & Sons, 1986, pp 29–41
31. BEYER KH JR, BAER JE: The site and mode of action of some sulfonamide-derived diuretics. *Med Clin N Am* 59:735–750, 1975
32. MAREN TH, SANYAL G: A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. *J Pharm Exp Ther* 130:266–269, 1960
33. MOORE PF: The effects of diazoxide and benzothiadiazine diuretics upon phosphodiesterase. *Ann NY Acad Sci* 150:256–260, 1968
34. SENFT G, LOSERT W, SCHULTZ G, SITT R, BARTELHEIMER HK: Ursachen der Störungen im Kohlenhydratstoffwechsel unter dem Einfluss sulfonamidierter Diuretica. *Arch Exp Pathol Pharmakol* 255: 369–382, 1966
35. KHAN SR, HACKETT RL: Calcium oxalate urolithiasis in the rat: Is it a model for human stone disease? A review of recent literature. *Scanning EM* II:759–774, 1985
36. MCKEE MD, NANCI A, KHAN SR: Ultrastructural immunodetection of osteopontin and osteocalcin as major matrix components of urinary calculi. (abstract) *J Bone Miner Res* 9(Suppl 1):C190, 1994
37. NEMIR M, DEVOUGE MW, MUKHERJEE BB: Normal rat kidney cells secrete both phosphorylated and nonphosphorylated forms of osteopontin showing different physiological properties. *J Biol Chem* 264:18202–18208, 1989
38. RITTER NM, FARACH-CARSON MC, BUTLER WT: Evidence for the formation of a complex between osteopontin and osteocalcin. *J Bone Miner Res* 7:877–885, 1992
39. RUMLL LA, DUBOIS SK, ROBERTS ML, PAK CYC: The effect of alendronate on immobilization-induced bone loss and stone-forming propensity. (abstract) *J Bone Miner Res* 9(Suppl 1):B155, 1994
40. STRONSKI SA, BETTSCHEN-CAMIN L, WETTERWALD A, FELIX R, TRECHSEL U, FLEISCH H: Bisphosphonates inhibit 1,25-dihydroxyvitamin D_3-induced increase of osteocalcin in plasma of rats *in vivo* and in culture medium of rat calvaria in vitro. *Calcif Tissue Int* 42:248–254, 1988
41. PERRY HM 3 DAYS, JENSEN J, KAISER FE, HOROWITZ M, PERRY HM JR, MORLEY JE: The effects of thiazide diuretics on calcium metabolism in the aged. *J Am Geriatrics Soc* 41:818–822, 1993
42. DAWSON-HUGHES B, HARRIS S: Thiazides and seasonal bone change in healthy postmenopausal women. *Bone Miner* 21:41–51, 1993
43. GHAZALI A, BATAILLE P, FUENTES V, PRIN L, COHEN SOLAL ME, EL ESPER N, HUÉ P, FOURNIER A: Role of monokines in bone demineralization of calcium stone formers with idiopathic hypercalciuria. (abstract) *J Bone Miner Res* 9(Suppl 1):B468, 1994