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Osteogenic PTHs and Vascular Ossification—Is There a Danger for Osteoporotics?

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Abstract Inflammation in vascular (mostly arterial) walls and heart valves triggered by the trans-endothelial influx of LDL particles and the action of subsequently modified (e.g., by oxidation) LDL particles can trigger true bone formation by valvar fibroblasts, by a subpopulation of re-differentiation-competent VSMCs (vascular smooth muscle cells) or by vascular pericytes. Vascular ossification can lead to heart failure and death. Elderly osteoporotic women who need osteogenic drugs to restore their lost skeletal bone are paradoxically prone to vascular ossification—the ''calcification paradox.'' The recent introduction into the clinic of a potently osteogenic parathyroid hormone peptide, Lilly's rhPTH-(1–34)OH (Forteo^{TM}), to reverse skeletal bone loss raises the question of whether this and other potently osteogenic PTHs still in clinical trial might also stimulate vascular ossification in such osteoporotic women. Indeed the VSMCs in human and rat atherosclerotic lesions hyperexpress PTHrP and the PTHR1 (or PTH1R) receptor as do maturing osteoblasts. And the evidence indicates that endogenous PTHrP with its NLS (nuclear/nucleolar localization sequence) does stimulate VSMC proliferation (a prime prerequisite for atheroma formation and ossification) via intranuclear targets that inactivate pRb, the inhibitory G_1/S checkpoint regulator, by stimulating its hyperphosphorylation. But neither externally added full-length PTHrP nor the NLS-lacking PTHrP-(1–34)OH gets into the VSMC nucleus and instead they *inhibit* proliferation and calcification by only activating the cell's PTHR1 receptors. No PTH has an NLS and, as expected from the observations on the externally added PTHrPs, hPTH-(1–34)OH inhibits calcification by VSMCs and cannot stimulate vascular ossification in a diabetic mouse model. Encouraging though this may be for osteoporotics with their ''calcification paradox,'' more work is needed to be sure that the skeletally osteogenic PTHs do not promote vascular ossification with its cardiovascular consequences. J. Cell. Biochem. 95: 437–444, 2005. © 2005 Wiley-Liss, Inc.

Key words: atherosclerosis; atheroma; bisphosphonates; CVCs (calcifying vascular cells); cholesterolemia; LDL particles; macrophages; MCP-1 chemokine; monocytes; osteoblasts; osteoporosis; pericytes; PTH (parathyroid hormone); PTHrP (parathyroid hormone-related protein); PTH/PTHrP receptor; vascular endothelial cells; vascular ossification; vascular smooth muscle cells

During the first decade after menopause all women suffer an accelerating loss of bone, which can be severe enough to result in "spontaneous" crushing of vertebrae by normal spinal bending and the breaking of other bones by normal muscle pulling. This is osteoporosis. The accelerating microarchitectural deterioration and fragility of postmenopausal women's bones are caused by an estrogen shortage. The slower development of osteoporosis in aging men is also partly due to a dwindling supply of estrogen made by bone cells from circulating testosterone and is needed for bone maintenance just as it is in women.

The estrogen drop triggers a vicious cycle of a disproportionally rising resorptive arm of the coupled resorption → microcrack-repairing remodeling mechanism with a consequently rising fragility and increasing microfracturing [reviewed by Whitfield et al., 2002; Whitfield, 2005]. This vicious cycle is due to increasing generation, longevity, and activity of the microfracture-excavating osteoclasts in the growing numbers of crack-repairing osteoclast-osteoblast teams in the increasingly microcrack-prone bones. The accelerating generation of hyperactive osteoclasts can be stopped, or at least significantly reduced, by several different things such as the osteoclast-disabling/killing

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bisphosphonates [reviewed by Whitfield et al., 2002; Whitfield, 2005]. But these agents are resorption arrestors, *antiresorptives* not osteoblast-stimulating *bone anabolic*s [reviewed by Whitfield et al., 2002; Whitfield, 2005]. What the growing number of osteoporotics with their weakening bones have been needing are anabolic drugs to restore their deteriorating bone microstructure.

The currently leading anabolic agents are the potent 84-amino acid parathyroid hormone (PTH) and certain of its 31- and 34-amino-acid fragments, which are in clinical trial, or, as in the case of recombinant hPTH-[1–34]OH, actually being used to treat osteoporosis [reviewed by Whitfield et al., 2002; Whitfield, 2005]. There are four potently osteogenic PTHs either on the market or being trialed. One of these is the full-length, recombinant(r) human (h) rhPTH-(1–84)OH which has completed its phase III clinical trial and is called Preos™ by its manufacturer NPS pharmaceuticals (Salt Lake City, UT) [reviewed by Whitfield et al., 2002; Whitfield, 2005]. The next in order of size is Lilly's rhPTH-(1–34)OH (also known as teriparatide) which is being used under the name Forteo™ [reviewed by Whitfield et al., 2002; Whitfield, 2005]. Then there are the smaller, potently osteogenic peptides of the Ostabolin family, Ostabolin™ (hPTH- $(1–31)NH_2$) and Ostabolin-C™ (i.e., cyclized Ostabolin™—[Leu^{27}]*cyclo* Glu^{22}-Lys^{26} hPTH-$(1–31)NH_2$) [Whitfield et al., 1996, 1997; Barbier et al., 1997].

These four peptides strongly stimulate the growth of skeletal bone in ovariectomized rats, cynomolgus monkeys and osteoporotic postmenopausal women when injected subcutaneously once daily [reviewed by Whitfield et al., 2002; Whitfield, 2005]. They appear to stimulate skeletal bone formation in three ways: (1) by stimulating mature non-proliferating PTHR1-bearing osteoblasts to make paracrine agents, such as FGF-2 and IGFs-1 and -2, which stimulate the proliferation of osteoprogenitor cells that are not mature enough to have PTHR1 receptors; (2) extending osteoblasts' working lives by preventing them from initiating apoptosis; and (3) by stimulating the reversible reversion of bone-lining cells to working osteoblasts [reviewed by Whitfield et al., 2002; Whitfield, 2005].

But there are osteogenic cells which don't live in the skeleton. Instead they build true bone, not just an amorphous Ca-phosphate precipitate, in aortic atherosclerotic plaques, aortic valves, coronary arteries, and in other arteries but far less often in veins [Wallin et al., 2001; Demer and Tintut, 2003; Rajamannan et al., 2003; Doherty et al., 2004; Vattikuti and Towler, 2004]. These are the so-called CVCs (calcifying vascular cells), that are a subpopulation of re-differentiated arterial VSMCs (vascular smooth muscle cells), and vascular pericytes [Boström, 2000; Wallin et al., 2001; Doherty et al., 2004; Vattikuti and Towler, 2004].

Presumably accumulation of vascular bone should not be a problem for osteoporotics who are losing skeletal bone. They should also lose vascular bone, shouldn't they? But they don't. Elderly osteoporotic post-menopausal women, the main consumers of the osteogenic PTHs, are, surprisingly, prone to ossifying their arteries while de-ossifying their skeletons—the "calcification paradox" [Parhami et al., 1997; Wallin et al., 2001; Demer, 2002; Demer and Tintut, 2003; Iba et al., 2004; Rubin and Silverberg, 2004; Schultz et al., 2004]. Indeed according to Schultz et al. [2004], "aortic calcifications are a strong predictor of low bone density and fragility fractures." Parhami et al. [1997] have suggested that the reason for the "calcification paradox" is that subendothelial minimally modified LDLs (low-density lipoproteins) stimulate the re-differentiation of arterial VSMCs into osteovascular CVCs, but they inhibit the differentiation of skeletal preosteoblasts. Therefore, if the osteogenic PTHs stimulate vascular bone formation as strongly as they stimulate skeletal bone formation they could promote aortic valve disease, heart attacks, and stroke in osteoporotic postmenopausal women.

VASCULAR OSTEOGENESIS

"The emerging view is that plaque calcification represents a meeting of bone biology with chronic plaque inflammation."—T.M. Doherty et al. [2003a]

"...we see ossification declare itself in precisely the same manner as when an osteophyte forms on the surface of bone ...the osteophytes of the inner table of the skull ...follow the same course of development as the ossifying plates of the internal coat of the aorta and even of the veins ..."—R. Virchow [1863/1971].

Vascular ossification has been regarded until recently as just a passive precipitation of Ca-phosphates in advanced atherosclerotic plaques.

But that has now radically changed; vascular ossification is far from passive [Wallin et al., 2001; Fitzpatrick et al., 2003; Doherty et al., 2003a, 2004; Vattikuti and Towler, 2004]. It is now known to be the product of the same cellular machinery that makes bone in the skeleton. In fact, there are two kinds of vascular bone formation just as there are two kinds of skeletal bone formation. The first resembles the direct, cartilage-independent, intramembranous skeletal bone formation and operates in heart valves and ossifies arteries and veins of types 1 and 2 diabetics [though see Qiao et al., 2003; Doherty et al., 2004; Vattikuti and Towler, 2004]. The second resembles endochondral bone formation which involves a preliminary formation of vascular cartilage which is invaded by neoangiogenic microvessels, chewed up by chondroclasts, and replaced with bone by the osteoblast-like CVCs [Fitzpatrick et al., 2003; Doherty et al., 2004; Vattikuti and Towler, 2004].

Vascular ossification is not rare: it has been seen in 15% of carotid atherosclerotic plaque specimens and in 60% of restenotic aortical valve specimens after balloon angioplasty [Abedin et al., 2004]. Moreover, 75%–95% of men and women have been found to have coronary calcification regardless of the cause of death [Wallin et al., 2001]. It is a major problem which, like osteoporosis, is increasing as the population ages.

Nor are its consequences trivial. Ossification of the aorta, for example, increases the risk of myocardial infarction, heart failure, and death by impairing coronary blood flow in both men and women [Boström and Demer, 2000; Rubin and Silverberg, 2004; Speer and Giachelli, 2004; Trion and van der Laarse, 2004]. Coronary blood flow is normally driven by the diastolic elastic recoil of the aorta from its systolic stretch, which, like a second heart operating by the so-called "Windkessel" mechanism, pumps blood into the coronary vascular network. But the inelastic wall of an ossified aorta cannot balloon like a normal aorta during systole and, when recoiling, pump blood into the coronary vessels [Boström and Demer, 2000; Demer, 2002; O'Rourke et al., 2002].

Another problem created by ossified vascular plaques is the biomechanically unstable interfaces between hard ossified and soft parts of the plaque. These interfaces are sites of high shear stress which in a pulsing blood vessel are prone to rupture and produce a clot that blocks the blood vessel. The risk of rupture peaks along with the number of ossified plaques and the hard/soft plaque interface area, but it then drops as the plaques coalesce [Abedin et al., 2004]. Therefore the risk of plaque rupture is biphasic and the coalescence of ossified plaques into a boney sheet or plate may actually stabilize a blood vessel. This could explain, for example, why Hunt et al. [2002] found that patients with heavily calcified carotid plaques had fewer strokes and transient ischemic attacks (TIAs). In other words, a few ossified plaques might rupture in one of your carotids and cause a stroke or TIA, but if you survive until the plaques are numerous enough and big enough to fuse and reduce the strain-prone interfaces, you will have solid, armor-plated carotid blood vessels, but because of your stiff, recoilless aorta the blood flow through your coronary blood vessel may be falling toward infarct and heart attack.

PLAQUES AND ATHEROMAS—OSSIFICATION INCUBATORS

"*. . . I have . . . . felt no hesitation in siding with the old view . . . . . in admitting an inflammation of the inner arterial coat to be the starting point of the so-called atheromatous degeneration*"—R. Virchow [1863/1971].

"*. . . the atheroma . . . a graveyard of acellular lipid debris enrobed by a capsule of proliferated smooth muscle cells*"—P. Libby [2002].

The atherosclerosis story in a person with a high blood cholesterol level begins in a coronary artery, for example, at a lesion-prone region of the artery's wall where low density lipoprotein complexes (LDLs) accumulate beneath the endothelium in the intima where they are oxidized by the products of macrophages' myeloperoxidase, nitric oxide synthase-2 (iNOS), and 12/15-lipoxygenase [Glass and Witztum, 2001; Libby, 2002; Steinberg, 2002; Zhu et al., 2003; Stocker and Keaney, 2004]. The modified LDLs initiate the fatty streak/plaque/atheroma development by causing endothelial cells to display molecules such as E-selectin that grab passing monocytes and T-lymphocytes which collect on the vessel wall. The snared monocytes are then stimulated to move by diapedesis through the endothelium into the vascular intima when their CCR-2 receptors are activated by the chemokine (monocyte chemoattractant protein) MCP-1 [Reape and Groot,

1999; Libby, 2002] (Fig. 1). There the monocytes mature into macrophages which grab the altered LDLs with scavenger receptors and ingest them. While feasting on LDLs they become fat globule-loaded "foam cells" which produce pro-inflammatory cytokines, growth factors and chemokines such as MCP(monocyte chemoattractant protein)-1 that activate T-cells, amplify the trans-endothelial flow of monocytes and foam cell production, and stimulate VSMC proliferation [Glass and Witztum, 2001; Charo and Taubman, 2004] (Fig. 1). The proliferating VSMCs crawl up into the intima under the endothelium and lay down a matrix canopy over the growing nest of foam cells and T-cells. This is the initial plaque which the growing mass of foam cells and proliferating VSMCs eventually transform into an atheroma [Libby, 2002; Steinberg, 2002].

But along with the excited endothelial lining cells, LDLs and invading monocytes are the 10%–30% of the proliferating, migrating VSMCs that are capable of re-differentiating into CVCs [Broström et al. 1993]. If bombarded long

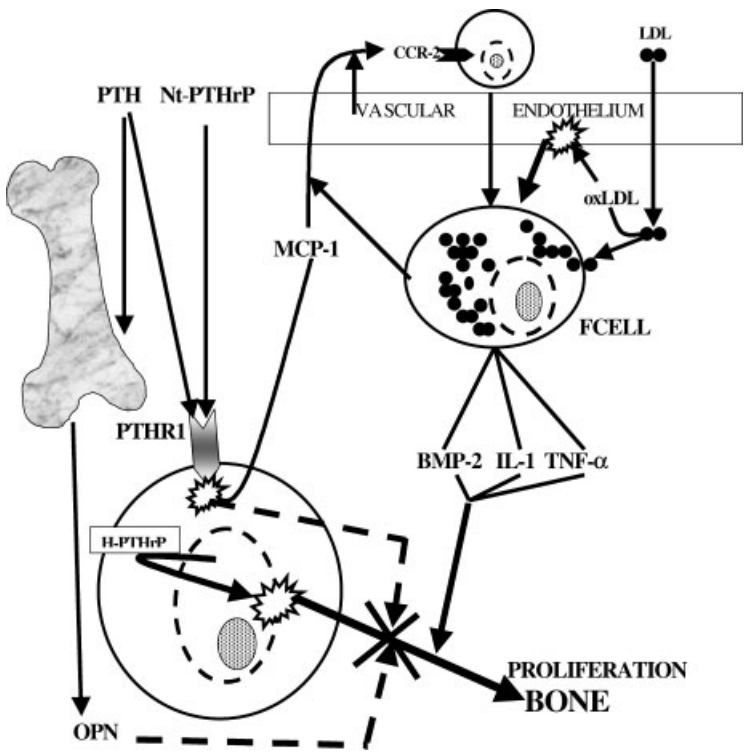

Fig. 1. The involvements of PTH, PTHrP, and their shared PTHR1 receptor in vascular ossification. Vascular bone formation starts in a hypercholesterolemic person with the influx of LDL particles through the endothelium into the intima of the lesion–prone region of an arterial wall to start an inflammatory process. There the LDLs are modified by oxidation (oxLDL) and cause the endothelial cells to express surface molecules that grab passing mononuclear cells. The snared mononuclear cells are induced to move across the endothelium by diapedesis when the MCP-1 chemoattractant chemokine binds to their CCR-2 receptors. In the subendothelial intima the mononuclear cells transform into macrophages that feed on LDLs, becoming foam cells (FCELL) in the process and producing several cytokines, chemokines (including MCP-1), and growth factors. One of the effects of the many factors from the endothelial cells and macrophages, particularly the osteogenic BMP-2, is to cause VSMCs (vascular smooth muscle cells) to express PTHrP and the PTHR1 (or PTH1R) receptor. The full length PTHrP (H[holo]-PTHrP) made by the cells gets into the cell's nucleus via its NLS (nuclear/nucleolar loacalization signal sequence). There it stimulates VSMC proliferation, migration of the proliferating cells from the media to the intima to thicken the intima and form the plaque and ultimately an atheroma. However, among the PTHrP-driven, proliferating VSMCs are some that can discard their muscle phenotype and retool themselves to become osteoblast-mimicking CVCs (calcifying vascular cells) and make bone. However, a PTH such as hPTH-(1–34)OH (Forteo^{TM}) that is being used to grow skeletal bone in osteoporotics or an Nt(N-terminal)-PTHrP peptide without an NLS [e.g., hPTHrP-(1–34)OH] only activates the PTHR1 receptor, the signals from which inhibit proliferation and vascular bone formation (broken line). However, the PTHR1 signals (specifically cyclic AMP and the cyclicAMP-dependent protein kinase A) also stimulate the expression MCP-1 which would further drive the migration of mononuclear cells into the vascular wall. The PTH also stimulates skeletal cells to make OPN (osteopontin) which, when released into the circulation, inhibits vascular ossification (broken line).

enough with various factors the potential CVCs shelve their smooth muscle phenotype and re-equip themselves with an osteoblast's bone-making tool box. Among their stimulators are the minimally modified LDLs, the above-mentioned agents of the osteoporotic women's "calcium paradox," [Parhami et al., 1997; Steitz et al., 2001]. When stimulated by proinflammatory cytokines such as IL-1β and TNF-α, the plaque's endothelial cells make osteogenic factors such as BMP-2 and -4. These factors stimulate the VSMC/CVCs to express the master osteoblast regulator Runx-2 (Cbfa-1) which induces the cells to become "osteovascular" cells by expressing the osteoblast package of genes for alkaline phosphatase, bone sialoprotein, collagen I, osteocalcin, and osteonectin and running the bone-making machinery which ends in the mineralization of the bone matrix by hydroxyapatite-loaded matrix vesicles released from apoptosing CVCs [Virchow, 1863/1971; Jakoby and Semenkovich, 2000; Proudfoot et al., 2000; Engelse et al., 2001; Steitz et al., 2001; Wallin et al., 2001; Doherty et al., 2002, 2003a,b, 2004; Demer and Tintut, 2003; Whitfield, 2005] (Fig. 1).

INVOLVEMENT OF PTHrP AND PTHs IN VASCULAR OSSIFICATION

The expressions of PTHrP and the PTHR1 (or PTH1R) receptor are features of the maturation program of skeletal osteoblasts, with the P3 promoter of the *PTHR1* gene being one of the targets of Runx-2/Cbfa-1 [Karperien et al., 2000; Aubin and Triffitt, 2002; Whitfield et al., 2002; Whitfield, 2005]. PTHrP and PTHR1 appear to function together in osteoblast maturation by shutting off proliferation by turning off the cyclin D1-CDK 4/CDK 6 protein kinases that would otherwise start the chain of events leading up to DNA replication [Datta et al., 2004].

PTHrP and the PTHR1 receptor are also made by VSMCs in human and rat atherosclerotic lesions and an upsurge of their expression is involved in the restenosis following injury from balloon angioplasty [Nakayama et al., 1994; Ozeki et al., 1996; Ishikawa et al., 2000; Martin-Ventura et al., 2003]. The VSMCs in the shoulder region—the inflammation action center—of human carotid atherosclerotic plaques also overexpress PTHrP and the PTHR1 receptor [Martin-Ventura et al., 2003]. The cyclic AMP and the cyclic AMP-dependent PKA (protein kinase A) signals from the PTHR1 receptors activated by externally added PTHrP stimulate the VSMCs to make the MCP-1 chemokine to further amplify the migration of circulating monocytes through the vascular endothelium and consequently increase the foam cell population in the vascular intima [Martin-Ventura et al., 2003; Charo and Taubman, 2004] (Fig. 1). Thus, the VSMCs' overexpressed endogenous PTHrP loads the plaque's intima with proinflammatory and osteoinductive factors from accumulating macrophages. But the endogenous PTHrP does much more.

The full-length PTHrP made by the VSMCs in the plaque shoulder has something in its 86–106 region that no PTH has-an NLS (nuclear/nucleolar localizing signal) that enables some emerging full-length PTHrPs to be shipped from their synthesis site in the VSMC's endoplasmic reticulum to targets in the nucleus [Massfelder et al., 1997; De Miguel et al., 2001; Fiaschi-Taesch et al., 2004]. A full-length PTHrP translation product with a traditional upstream signal sequence is fed into the cell's secretion machinery, secreted and then with its N-terminal 1–36 region activates the producer cell's or a neighboring cells' PTHR1 receptors [Massfelder et al., 1997; De Miguel et al., 2001; Fiaschi-Taesch et al., 2004; Whitfield, 2005]. But there are other translation initiation sites on the transcript that disrupt the secretion signal sequence which prevents the emerging protein from being grabbed by the secretory mechanism [Massfelder et al., 1997; De Miguel et al., 2001; Fiaschi-Taesch et al., 2004; Whitfield, 2005]. The emerging full-length PTHrP's NLS is grabbed by β1-importin and shipped into the nucleus where it stimulates proliferation by a process that requires its C-terminal 112–139 region and hyperphosphorylates and switches off the inhibitory cell cycle G_1/S checkpoint regulator pRb (retinoblastoma protein) [Fiaschi-Taesch et al., 2004] (Fig. 1). VSMCs engineered to overexpress an NLS-deleted PTHrP fragment, which cannot get into the nucleus to trigger pRB hyperphosphorylation, secrete the peptide which stimulates the PTHR1 receptors, the signals from which inhibit proliferation, carotid intimal thickening and probably vascular ossification [Massfelder et al., 1997, 1998; Massfelder and Helwig, 2003; Fiaschi-Taesch et al., 2004] (Fig. 1). In other words, preventing PTHrP from getting into the

nucleus and stimulating the proliferogenic mechanism converts it from a stimulator to a PTHR1-activating inhibitor of VSMC proliferation [Massfelder et al., 1997, 1998; Massfelder and Helwig, 2003].

It follows from this that a skeletally osteogenic PTH such as hPTH-(1–34)OH or an NLS-lacking N-terminal PTHrP fragment such as PTHrP-(1–34)OH, should reduce vascular intimal thickening and ossification. Indeed Ishikawa et al. [2000] have shown that PTHrP-(1–34) prevented cuff placement from inducing intimal thickening in rat femoral artery. And Jono et al. [1997] reported that hPTH-(1–34)OH and the hPTHrP-(1–34)OH fragment inhibited calcification by bovine VSMCs which needed to stimulate both their adenylyl cyclase and protein kinase Cs to do so. Then Shao et al. [2003] reported that intermittent injections of hPTH-(1–34)OH into LDLR (low density lipoprotein receptor)–/– mice prevented a diabetogenic high-fat diet from ossifying aortas and heart valves as it did in untreated control animals. Shao et al. [2003] attributed this prevention to the peptide increasing the production of soluble osteopontin by skeletal cells but not by the vascular cells (Fig. 1). The osteopontin from the PTH-stimulated skeleton prevented the vascular osteoprogenitors from expressing their osteogenic *Msx2* genes [Shao et al., 2003] (Fig. 1). And according to Wada et al. [1999] the osteopontin from the PTH-stimulated skeleton could also have interfered with hydroxyapatite crystal formation by interacting directly with the apatite crystal surfaces.

SUMMARY AND FUTURE PROSPECTS

The uptake and accumulation of excessive numbers of oxidized LDL particles in the subendothelial intima of a lesion-prone region of an artery starts as a lipid streak which becomes a plaque which grows into an atheroma. The modified LDLs stimulate the overlying endothelial cells to snare mononuclear cells and induce them to migrate into the intima where they become macrophages that bind and ingest the LDLs and, in doing so, become foam cells. The feeding macrophages and excited endothelial cells produce various pro-inflammatory agents and set up a chronic inflammation that drives the growth of the atheroma.

Among the products of this chronic inflammation are the osteogenic BMPs which together with stimulation by the altered LDLs induce a subpopulation of VSMCs, the CVCs, to shelve their muscle cell machinery and retool themselves to make bone. The retooling program turned on in the CVCs by these agents is the same one that drives the maturation of skeletal osteoprogenitors into functional osteoblasts. In both skeleton and artery, this process includes the expression of PTHrP and the PTHR1 receptor.

Most of the PTHrPs emerging from the translation machinery are variously processed and secreted. But some of them are not fed into the secretory machinery and because of their NLSs are shipped into the nucleus where they stimulate a mechanism that starts the cell proliferating and so contributes to the thickening of the plaque/atheroma intima. PTHrPs without NLSs inhibit proliferation by just activating PTHR1 receptors. So intranuclearly operating full-length PTHrP is a mitogenic, proinflammatory agent that promotes atheroma growth.

Luckily for elderly atherosclerotic osteoporotics with their "calcification paradox" the potent, skeletally osteogenic PTHs, such as Lilly's Forteo^{TM} [rhPTH-(1–34)OH], do not have an NLS. Because of this they operate only through the PTHR1 receptor and therefore inhibit vascular ossification. But these PTHR1-activating PTHs would still be expected to stimulate MCP-1 expression and macrophage accumulation in existing vascular lesions. However, such existing bony vascular lesions might be reduced or eliminated by the osteoclast-disabling and killing bisphosphonates which osteoporotic patients may use to protect their new "PTH-bone" after finishing their PTH treatment [Whitfield et al., 2002; Ylitalo, 2002; Hashiba et al., 2004; Nitta et al., 2004; Whitfield, 2005]. At first sight promoting vascular bone loss with bisphosphonates is another paradox—a "bisphosphonate paradox"—because they prevent skeletal bone loss. The different response is likely due to the bisphosphonates binding avidly to blood vessel walls and killing the atheromatous lesion's osteoclast-related macrophages which produce the pro-inflammatory cytokines that promote vascular osteogenesis [Libby, 2002; Steinberg, 2002; Ylitao, 2002].

Reassuring though these early observations on mice and cultured bovine VSMCs may be, much more work must be done to be sure that

the PTHs that stimulate skeletal bone formation do not stimulate vascular ossification in osteoporotics with their "calcification paradox."

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