

Forum for Collaborative HIV Research

Bone Metabolism and HIV Disease Meeting Report

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The Forum for Collaborative HIV Research, a project of the Center for Health Services Research and Policy at the George Washington University School of Public Health and Health Services, was founded in 1997. The goal of the Forum is to facilitate discussion regarding emerging issues in HIV clinical research and the transfer of research results into care.

The Forum is a coalition of government agencies, clinical researchers, health care providers, pharmaceutical companies, and patient advocates. The Forum is governed by an Executive Committee made up of representatives from each of the above named constituency groups. The Executive Committee determines the subject and scope of the Forum projects. The Forum brings these constituencies together to identify gaps and impediments in the understanding of the medical management of HIV disease and develops recommendations to fill those gaps. The Forum is a public/private partnership, which receives financial support from its governmental and industry members and with in-kind support from its membership within the academic research, patient care, and advocacy communities.

For more information about the Forum or to download reports from this meeting or prior ones, visit the Website at

www.hivforum.org

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Karen M. Eddleman did a wonderful job of writing up the notes from the meeting and prepared this report. Forum staff members Paul Oh and Houtan Movafagh handled all the meeting logistics with grace and patience.

David Barr – Executive Director

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HIV AND BONE METABOLISM: WHERE DO WE GO FROM HERE?

The Forum for Collaborative HIV Research met on August 29, 2000, in Washington, DC, for a discussion of bone metabolism and human immunodeficiency virus (HIV) disease. This was the first assembly of clinicians, basic scientists, and representatives of the pharmaceutical industry, the patient-advocate community, and regulatory agencies to discuss bone metabolism and HIV disease. The purpose of the meeting was to make recommendations about future research directions in light of the most recent research on the topic.

After eight presentations, the meeting culminated in a discussion that generated recommendations for future research. The recommended research will further our understanding about the nature of bone metabolism associated with HIV infection and/or the drugs used to treat HIV, including protease inhibitors (PIs), nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs, respectively).

Specific topics for discussion included:

- prevalence of bone problems in HIV disease
- pathophysiologic mechanisms of bone disorders in HIV disease
- role of the immune system and cytokines in bone dysfunction in HIV disease
- role of the HIV virus in bone metabolism
- current prevention, diagnostic, and therapeutic options
- next steps for research in bone dysfunction and HIV disease.

Research Recommendations

The Forum participants formulated three key questions to frame future research:

- How can we use epidemiological studies to elucidate the nature of bone metabolism dysfunction in HIV/AIDS?
- What can we determine about the specific effects of drugs used to treat HIV infection?

- Whither the cytokine search?

Elucidating the nature of bone metabolic dysfunctions in HIV infection. Controversies abound regarding the roles played by many factors—HIV itself, protease inhibitors (PI's), NRTIs, NNRTIs, combination therapies, host response, immune reconstitution, and other metabolic syndromes—in the bone metabolism dysfunction observed during the course of HIV infection and therapy. To pinpoint the causes of bone disorders observed in HIV infection, researchers must abandon preconceived notions and test all hypotheses in prospective epidemiological studies.

To conduct these studies, all Forum panelists agreed that new epidemiological methodologies are needed to generate good bone mineral density (BMD) data. Specific needs include turnover measurements and analysis of resorption and formation markers on archived sera. Some studies should include site-specific and total body dual-energy x-ray absorptiometry (DEXA) scans. Second, related to the need for BMD data is the requirement for good hormone data, including measurement of sex hormone binding globulin and determination of estrogen levels in men.

Research Recommendation 1:

Integrate bone mineral density measurements into new and ongoing epidemiological studies. Of particular interest are site-specific DEXA scans of the spine, femur, and hip.

Research Recommendation 2:

Researchers need valid hormone data, including levels of sex hormone binding globulin and measurements of estrogen concentrations in men.

The question then posed by the Forum panel was how feasible would these studies be within the framework of current epidemiological research? The panelists identified several studies that may accommodate site-specific DEXA scans either *ab initio* or as add-ons:

- *The Fat Redistribution and Metabolic Change in HIV Infection (FRAM) study*, a large, cross-sectional, multicenter study of metabolic and morphologic complications that may be related to PIs. Sixteen centers will be conducting total body DEXA and total body MRI studies for 1,200 randomly selected, HIV-infected patients. Although total body MRI will not pick up avascular necrosis (AVN) of the hip (which requires high-resolution studies), it can be used for bone marrow studies. The study will generate archived sera and, at some centers, urine.
- Other *prospective studies of the initiation of therapy* that are randomized according to the type of HAART therapy: NNRTI-based, PI-based, or a combination of the two. One AIDS Clinical Trial Group (ACTG) study has a metabolic subset with more than one third of the people receiving total body DEXA. Some studies have stored urine; all have banked sera; this study does not include site-specific DEXA.
- *New therapeutic trials* that are being undertaken as new PIs, NRTIs, and NNRTIs are brought to the market by pharmaceutical companies. The pharmaceutical companies could include studies of BMD and turnover biomarkers in the new protocols.
- *Gilead Science's study*. This company is studying one of its new drugs in conjunction with possible reductions in BMD; built into the protocols are regional DEXA scans. Gilead has one or two years' worth of data available from its initial study, which could be examined.
- *Cross-sectional study using whole body DEXA*. Serum and other biological samples will be preserved and possibly available for study.
- *Switch trials*. Examine the effects on bone metabolism and fat distribution as HIV patients switch from one therapy to another.
- *Randomized, long-term, double-blind studies* of high-risk individuals, some of whom have seroconverted over the years. Such studies likely have many samples of banked sera. Some patients may have embarked on therapies, so samples would be available before therapy was initiated and monthly samples thereafter. Many of these patients were probably rolled up into triple therapy subsequently.

- There is a proposal before the National Institutes of Health right now for supplemental funding for a *cross-sectional bone study utilizing the Women's Interagency HIV Study (WIHS) study population*, a large cohort of HIV-infected women in five large metropolitan areas (New York, Washington D.C., Chicago, Los Angeles, and San Francisco). Some of the women are on PIs, on other therapies, and others are HIV-negative controls. The proposal calls for total, hip, and spine DEXA studies and determinations of lipid status.

Research Recommendation 3:

Gather archived sera and urine obtained during the course of prior HIV-related studies and analyze for at least three measures of bone formation and two markers of bone resorption.

Some barriers exist to incorporating bone investigations into the aforementioned studies. For example, there is access to the FRAM multicenter study, but it may be difficult to increase DEXA scan time at certain centers. The ACTG study has ceased enrollment, so no baseline data are available. Nevertheless, one participant noted that bone turnover rates increase when therapy commences as the remodeling space expands, resulting in initial decreases in BMD. The real question is whether this phenomenon is progressive and whether the BMD is falling over time; therefore, the lack of baseline data, although not an ideal situation, might not preclude use of the ACTG data. In addition, initial decreases in BMD should not immediately raise a red flag for safety committees. Another barrier is that researchers will likely be reluctant to thaw biological samples for testing because such samples lose value when they are thawed and cannot be used for other testing. Furthermore, some samples may not have been stored at sufficiently low temperatures to preserve markers of bone turnover.

Specific effects of HIV therapies on bone metabolism. Is there progressive bone loss in patients who are doing comparatively well on therapy over a period of two to three years or longer? To answer this question, the Forum panel suggested a number of studies:

- Site-specific (hip and spine) densitometry studies would be an important *adjunct to the ACTG studies*.
- *Bone studies on healthy volunteers* receiving individual drugs.
- *Women's cross-sectional studies* with patients and controls matched for socio-economic factors and means of transmission may shed light on specific drug effects.
- *Studies to account for hormone effects* must be standardized according to the menstrual cycle of women; hormone measurements should be performed during early follicular stage. Further complicating the picture, some patients are self-treating with anabolic steroids and testosterone.
- *Two large, long-term studies* (each comprising more than 500 patients) are commencing. Total body DEXA scans will be performed, and sera will be stored on all patients. No biological differences are expected for two or three years due to potent antiviral therapies.
- *More animal studies* were called for because it is easy to measure bone density in mice, and the results are available in a few months. Some researchers believe that it is premature to be discussing big prospective studies on humans, that the focus for now should be on animal models.
- Another way to address the question of drug effects on bone metabolism is by *analyzing fracture data*. Prospective data could be worked into ongoing trials.

Pharmaceutical companies could look back at their data from clinical trials. An excellent source of fracture data is the Veterans Affairs (VA) medical system—the number one provider of health care to HIV-positive individuals. Some 19,000 HIV-positive patients are in the VA system. Researchers could assess fracture rates, age-adjust the data, and analyze via computer databases. Alternatively, they could do a retrospective on HIV patients by performing a computer analysis of admission records, looking for hip fractures over the past decade.

Research Recommendation 4:

To separate the effects of HIV from the effects of the drugs used to treat HIV, research must include bone studies performed on healthy volunteers who receive HIV drugs.

Research Recommendation 5:

Initiate long-term prospective studies to look at effect of highly active antiretroviral therapy (HAART) on bone metabolism. Such studies should take into account the effects of menopause, hormone therapy, and so forth.

Research Recommendation 6:

Although fractures do not seem to be a significant problem now among those receiving HAART, it appears that turnover markers and BMD indicate the potential for bone loss. We must identify data sources and monitor fracture rates because problems may not become evident for 5 or 10 years or perhaps longer.

One suggestion made by the Forum panel was to conduct a follow-up meeting to look at metabolic dysfunctions, some of which may appear over a long period of time. Because many of these dysfunctions do not occur in therapy-naïve patients early in the course of disease, we need long-term studies. Perhaps a working group could look 2 or 3 years ahead instead of waiting for 5 years to initiate studies.

Some researchers like the idea of starting to look prospectively now using long-term epidemiologic studies. If these bone loss effects are analogous to effects of menopause or steroid therapies, we should be seeing a bolus of cases soon. A number of excellent long-term prospective studies of osteoporosis—not necessarily of osteoporosis treatment, but of the disease itself—could serve as models for prospective studies of bone metabolism changes that occur concomitantly with HIV treatment. It is critically important to look at the underlying HIV infection and its impact on development of

osteoporosis over time. Studies should involve both newly diagnosed patients and patients who may have received a variety of drug treatments.

Research Recommendation 7:

Consider creating a working group to look at bone metabolism dysfunction. Because many of these problems do not occur in therapy-naïve patients early in the course of the disease, what is needed is a look ahead into the next 2 or 3 years instead of waiting for 5 years to initiate studies.

The panel recommended that pharmaceutical companies provide compounds for studies of bone loss associated with HIV disease and/or therapy. Clearly, there is a risk to the drug companies and their markets in embarking in such studies. Such studies are expensive; we cannot push entire cost and responsibility unto drug companies. The support of government will be needed. And, all companies must be treated equally because there is a risk to companies doing such studies. Such studies should be entered into with a spirit of collaboration in keeping with the mission of the Forum.

Research Recommendation 8:

Procure government support for conducting studies on biological samples archived by pharmaceutical companies during the course of earlier HIV drug studies. These studies are expensive; the pharmaceutical industry cannot—nor should it have to—bear the entire cost and responsibility for these studies. The government’s support will be needed.

One panelist suggested that the Forum should consider making some recommendations to the FDA around these issues, asking the FDA to address possible bone toxicity issues for new drugs in development and during post-marketing evaluation.

Another barrier involves the interpretation of animal studies. Small-animal models are interesting but are not always true predictors of human effects. For one thing, animal models beg the question of disease effects on bone loss because the most frequently used animals are not infected by HIV. What we need is to understand the effect of the drugs, the effect of HIV disease in the absence of drugs, and the effects of the drug plus the disease. Most animal models, albeit important, cannot address the latter two scenarios. The crux of the problem is this: It is difficult to identify a valid animal model when we do not know what is going on in humans. We need an animal model of pathogenesis and treatment, but can we develop one that is a predictor of the situation in humans?

Research Recommendation 9:

We need a good, validated animal model of pathogenesis and treatment, one that is a true predictor of the situation in humans. Consider establishing a preclinical or animal working group that involves all the drug companies in the design of animal studies to help target future investigations of specific effects in humans.

The panel was especially concerned about the public response to reports of bone loss that may be associated with HAART. Every precaution should be taken to avoid demonizing drugs that overall offer much greater benefits than risks for the people who take them. If HIV patients begin to live into their 60s and 70s, we may see a big change in fracture rates. Nevertheless, the publication of data that show lifesaving drugs in an unfavorable light is untenable to some researchers who caution that good drugs may be withdrawn, and, as a consequence, patients may suffer.

Whither the cytokine search? Large screening technologies may be useful in the cytokine search, because researchers must cast a wide net to find relevant cytokines. One central issue is the need to better characterize the phases of T-cell function and commensurate increases or decreases in bone resorption. Are the bone effects different in AIDS patients and in patients receiving HAART? To answer these questions, we need

to harvest and characterize T-cells and their products from HIV/AIDS patients and recovering patients who are receiving HAART.

Research Recommendation 10:

A need that is central to cytokine research is better characterization of the different phases of T-cell function and how these phases affect bone resorption. Do T-cells respond differently in AIDS patients? Do they respond differently in recovering patients receiving HAART? The answer to these questions may lie in studies that involve harvesting T-cells from patients undergoing different therapies.

Cytokine research is fraught with difficulties. For one thing, T-cells from HIV-infected patients are very short-lived and lose function quickly. Another difficulty stems from the fact that cytokine reactions are primarily local, so it is challenging to study them in any meaningful way in the whole body. From an HIV perspective, we need to recognize that one of the best models of CD4+ T-lymphocyte depletion is AIDS itself, but we have little evidence aside from biochemical markers that AIDS is associated in any significant way with major bone disease (with the possible exception of the wasting syndrome.) Therefore, it is important to pursue a fundamental understanding of the mechanism of HIV disease.

Would careful histology be useful in these studies? Although bone resorption is not well measured by biopsy, use of serial, small-needle biopsies before and after treatment may indicate activation frequency and bone formation rate. Gene expression and protein expression may also be measurable in the future. Bone resorption can be measured, at least roughly, through biochemical markers. The amount of bone is best measured by bone densitometry. The only reliable way to measure bone formation is through quantitation of tetracycline-based indices on serial histological samples taken over a period of time.

Research Recommendation 11:

Small-needle biopsies taken before and after treatment could yield valuable information about activation frequency and bone formation rates. Data from bone biopsy and biomarker analyses taken together can yield a snapshot of bone formation and resorption

In the absence of a mechanism, it is difficult to propose candidate cytokines for study. Some recent research indicates that perhaps TNF, an inflammatory cytokine, should be the subject of additional inquiry. Historically we have been able to identify candidates when we make progress on elucidating mechanisms. We need a better understanding of the disease and the drugs before we can study cytokines in a meaningful way.

Implications for patient care. The Forum panel agreed that when and if antiretroviral therapy is positively correlated with increased turnover and, consequently, a reduction in bone mass, it will be important to remember that osteoporosis is a manageable condition. We can monitor patients and treat them appropriately and effectively. We must not lose sight of the seriousness of HIV disease, and we need to treat it as the threat to life that it is. Certainly, we must not risk taking useful drugs off the market because of a clinically manageable condition—osteoporosis—that may be a side effect of those lifesaving drugs.

While the research continues, we need to know what to tell patients who are concerned about their quality of life while living with HIV, perhaps for decades with effective therapy. We must consider these patients as individuals by monitoring their BMDs and treating them appropriately. We must take these possible skeletal effects seriously but avoid demonizing these otherwise effective drugs.

Research Recommendation 12:

Understanding HIV disease apart from the drugs used to treat it is important. For example, the apparent differences between drugs within the same class points out

the critical importance of doing mechanistic studies early in the course of investigations. We continue to need appropriate clinical studies to learn about the fundamentals of the disease.

By incorporating the research recommendations from the participants of this meeting into future research efforts, we can clarify the issues around bone metabolism and HIV disease and /or therapy and develop therapies to use in a new era in which HIV-infected individuals can live long, productive lives.

Presentations

Bone disease and HIV

William Powderly, M.D., Washington University School of Medicine

Dr. Powderly adopted a clinical perspective to describe the current understanding of the association of HIV and highly active antiretroviral therapy (HAART) with bone disorders. Until the mid-1990s, acquired immune deficiency syndrome (AIDS) was a fatal disease with a median survival of 18 months after diagnosis of an AIDS-defining illness. Development of therapies extended that figure to about two years, but it was the advent of more effective antiretroviral therapy—protease inhibitors, combination therapies, and other potent drugs—that led to dramatic increases in survival.

Balancing HAART benefits and risks. Once the patient and physician have decided to initiate antiretroviral therapy, treatment should be aggressive with the goal of suppressing the plasma viral load to undetectable levels (*Guidelines for the Use of Antiretroviral Agents in HIV-Infected Adults and Adolescents*, U.S. Department of Health and Human Services). With these effective treatments, however, came some complications associated with long-term antiretroviral therapy. These fall into three general categories: toxic effects of nucleoside analogs, metabolic complications, and the potential for bone disorders. The toxic effects of nucleoside analogs include neuropathy, myopathy, pancreatitis, hepatic steatosis, lactic acidosis, and, perhaps, lipodystrophy. Metabolic complications include fat redistribution, insulin resistance, and hyperlipidemia. Under the category of bone disorders are avascular necrosis (AVN) and osteoporosis.

Avascular necrosis has been documented mainly in case reports, some of which predated antiretroviral therapy. It appears that AVN is increasing in the era of more potent therapy, although the link with treatment is uncertain. More recently, reports of osteopenia are cropping up in the literature, and some anecdotal reports indicate that

osteopenia may be progressing to clinically significant osteoporosis, leading to compression fractures of the lumbar spine.

When deciding on a time to commence therapy, doctors and patients must balance the benefits of lifesaving therapy against long-term morbidity and, as may be the case with some metabolic side effects, mortality. Should asymptomatic patients with high CD4+ counts receive combination therapy immediately or should treatment wait until a time when the clinical benefits will outweigh the potential risks of drug side effects?

Osteoporosis defined. Bone mineral density (BMD) as measured by DEXA is usually normalized to an age of 30 years (*t*-score) or to an age-matched population (*z*-score):

$t\text{-score} = (\text{measured BMD} - \text{mean BMD at age 30 years}) / (\text{standard deviation of BMD at age 30 years})$
$z\text{-score} = (\text{measured BMD} - \text{population mean BMD for same age subject}) / (\text{standard deviation of BMD at same age})$

According to the World Health Organization, osteopenia and osteoporosis can be defined thus:

Table 1. Osteopenia and osteoporosis as defined by *t*-score and *z*-score.

Clinical Status	<i>t</i>-score	<i>z</i>-score
Normal	> -1	-
Osteopenic	-1 to -2.5	-
Osteoporotic	< -2.5	< -2

Source: World Health Organization.

It is important to realize that, based on this definition, a substantial number of “normal” persons will have osteopenia.

Sorting out the causes of bone disorders in HIV infection. The question is: Are the bone changes observed in some HIV-positive individuals attributable to the virus, to the chronic disease associated with HIV infection, or to the therapy? To address the

putative association of bone disorders and/or HIV and HAART, Dr. Powderly reviewed findings from several studies, which are outlined below.

First, he described a few studies published during the era preceding HAART. One study looked at 22 patients, none of whom were receiving treatment at the time (Serrano et al., *Bone* 16:185, 1995). They found no alterations in bone densitometry by DEXA scanning but did find lower osteocalcin levels, especially in patients with more advanced disease. Histomorphometric studies suggested decreased rates of bone formation and turnover, especially among those with more advanced disease.

Another study (Paton et al., *Calcified Tissue International* 61:30-2, 1997) compared 45 HIV-infected men with sex-, age-, and weight-matched controls. Compared to controls, these men had marginally lower BMDs at the lumbar spine but no significant difference in total body or hip BMD as measured by DEXA. On longitudinal follow-up (mean of 15 months in 21 patients), small decreases in total body BMD were observed. None of the patients exhibited BMD levels that would be associated with a diagnosis of osteoporosis. The data did not suggest any correlation of BMD with HIV disease stage.

Tebas (unpublished data) at Washington University, St. Louis, recently looked at 20 therapy-naïve patients. The mean *t*-score in 20 patients naïve to therapy was $-0.29 + 1.51$, not significantly different from the general population. No significant correlation was observed between *z*-score and the severity of the illness as defined by CD4+ counts or by viral load.

Haug et al. (*J Clin Endocrinol Metab* 93:3832, 1998) found that 29 of 54 HIV patients had a deficiency of 1, 25-hydroxy vitamin D₃. Eighteen had undetectable levels; these cases were associated with advanced HIV disease and high serum levels of tumor necrosis factor (TNF)-alpha. Levels of parathyroid hormone (PTH), calcium, and 25-hydroxy vitamin D₂ were normal. Although not specifically addressed by this study, the impression is given that no evidence of clinical bone disease was found in this cohort.

These studies of therapy-naïve patients answer in part the question posed by Dr. Powderly. Taken alone, HIV infection cannot account for the osteopenia and

osteoporosis observed in some HIV-infected individuals receiving HAART. It seems that BMD changes attributable to HIV infection are few and trivial, although there is some biochemical evidence that HIV alters bone metabolism. What then, is the effect of HAART on bone metabolism?

Tebas et al. (*AIDS*, 14:F63, 2000) published the observations that initially prompted greater interest in bone disease and bone metabolic perturbations in patients receiving antiretroviral therapy. They conducted a cross-sectional cohort study of 122 subjects and found that osteopenia linked to PI-based treatment was significant and potentially alarming. Fifty percent of the patients receiving PI containing antiretroviral therapy had osteopenia, and 21% had osteoporosis; this was significantly more than patients who did not receive PI-based treatment or normal controls. This study also revealed that fat redistribution is not associated with osteoporosis, that these problems occur independently. Furthermore, these patients were not hypogonadal; their testosterone levels were normal.

Hoy et al. (7th CROI, 2000) looked at osteopenia and antiretroviral therapy in an Australian cohort of HIV-positive men with lipodystrophy who were taking PIs. By *t*-score, 28.4% were osteopenic, and 8.6% were osteoporotic. Switching from PIs made no difference at 24 weeks.

In patients with very advanced HIV disease, Aukrust et al. (*J Clin Endocrinol Metab*; 94:145, 1999) found decreased serum levels of osteocalcin and increased levels of *c*-telopeptide. After HAART was initiated, the researchers monitored bone markers in 16 patients over 24 months. They found significant and sustained increases in osteocalcin to normal levels and an insignificant rise in *c*-telopeptide and PTH.

Dr. Powderly went on to describe another cross-sectional study by Tebas et al. (2nd International Workshop on adverse Drug Reactions and Lipodystrophy in HIV, Abstract 029, Toronto, 2000). They examined serum and urine markers of bone metabolism and performed DEXA scans of the spine and hip in 73 HIV patients receiving PIs. Bone alkaline phosphatase, osteocalcin, and urinary pyridinolines were increased in a significant number of patients. Urinary calcium was high-normal. Bone

mineral density, bone alkaline phosphatase, and urinary pyridinolines were slightly correlated. Urinary calcium was high-normal. Levels of 25-hydroxy vitamin D₂ levels were low-normal, levels of 1,25-dihydroxy vitamin D₃ were normal, and PTH levels were high-normal.

Conclusions. Dr. Powderly's review of the literature led him to three conclusions:

- HIV disease itself is associated with little overt bone disease.
- HAART is associated with some biochemical changes of bone formation and remodeling as evidenced by turnover biomarkers and bone demineralization as evidenced by DEXA scanning.
- The clinical significance of these findings is unknown, as are the specific drug interactions and mechanisms. It is also not known if these bone changes are the result of potent therapy and resultant reversal of HIV-associated effects or if these bone changes are specific complications of certain drugs.

Overview of the local regulation of bone

Graham Russell, M.D., Sheffield Medical School

Dr. Russell's presentation was an overview of how bone turnover is regulated. The focus was on aspects of bone regulation that may be relevant to the pathophysiology associated with HIV infection and/or HAART. He listed a number of conditions—Paget's disease, multiple myeloma, bone metastases, osteoporosis—that involve increased bone destruction. As an aside, he mentioned that Paget's disease is now believed to be a viral disease of bone, perhaps associated with respiratory syncytial virus or measles. One characteristic of Paget's disease is the presence of inclusion bodies within the OCs. No causative agent has been isolated, and the theory of viral causation is still deemed controversial.

Osteoporosis—a disease of our times. The most common fracture zones in osteoporotic individuals are the hip, vertebrae, and wrist. It is important to bear in mind the changing pattern

of bone mass and metabolism throughout life, for example, how bone loss accelerates after menopause because of estrogen loss.

Osteoporosis affects two compartments in bone: cortical and trabecular bone. Cortical bone becomes thinner and more porotic in osteoporosis. In trabecular bone, the trabeculae become thinner and perforated so they become disconnected and lose mechanical strength.

Dr. Russell showed a slide of bone anatomy and pointed out two components of particular relevance:

- blood supply, which is compromised in AVN
- neural supply—a topic of heightened interest since it has been shown that neuropeptides affect bone metabolism.

The cellular components of bone fall into several categories:

- osteoclasts (OCs): multinucleated cells that cause bone resorption
- osteoblasts (OBs): polarized cells that are responsible for bone formation
- osteocytes: the least-studied cells in bones because of their inaccessibility; they are embedded within the bone and form a cellular network, much like neural networks to mediate responses to mechanical loading via signaling pathways involving nitric oxide, glutamate (as in the central nervous system), estrogens, and specific prostanoids, such as prostacycline
- the marrow cavity: location of the marrow fat, precursor cells that become OBs and OCs lineages, and cells of the immune system, which can communicate by shed or cell-presented cytokines, which, in turn, affect differentiation processes.

The lineages of bone cells have been well defined; OCs and OBs derive from separate lineages. OCs, which are of hematopoietic origin, are distantly related to monocytes and macrophages. Osteoblasts arise from the stromal cell system. Precursors of OBs and OCs give rise to other, diverse types of cells, including adipocytes, fibroblasts, chondrocytes, and myocytes, among others, depending upon which differentiation factors are present. There are good cell systems now for studying these. By adding certain agents, OCs can be developed from peripheral blood precursors. Similarly, stromal cells can be derived from marrow and induced to differentiate along these different pathways in response to addition of various agents.

Turning to remodeling in cortical and cancellous bone, Dr. Russell commented that approximately 10% of the skeleton is replaced each year. Chronic loss of bone later in life,

especially in women, must result from an imbalance of the remodeling process. Although cortical and trabecular bone look different histologically in remodeling processes, they do share some characteristics. On trabecular surfaces, the OCs are activated and then dig a hole, the depth of which is relatively constant. It is not presently known what the termination signal is. The process is somewhat different in cortical bone. Here, the OCs drill out a tunnel, and the OBs follow behind and fill it in.

If the individual is in bone balance, the OBs replace the exact same amount of bone over the next few weeks. If a state of imbalance exists, the OBs replace less than the amount of bone removed. This linkage between the amount removed and the amount rebuilt is sometimes termed *coupling*, the mechanism of which is not fully known.

In osteoporotic cortical bone, the in-filling of the tunnels by the OBs is less than complete. In trabecular bone, three phenomena likely contribute to remodeling imbalance in trabecular bone, according to Dr. Russell:

- more remodeling sites are activated
- the OCs may dig a little deeper, removing more bone than normal
- the OBs may replace a little less bone than they should.

Research hot zones. Dr. Russell went on to list several areas of fast-moving research, which may be relevant to the search for a relationship, if one exists, between bone disease and HIV and/or HAART:

- master genes regulating bone development
- regulatory systems governing cell differentiation, especially leptins and the system of RANK (receptor activator of NF- κ B) and RANK ligand (a member of the TNF family)
- pharmacology and drug action
- apoptosis, or programmed cell death, which features prominently in bone biology discussions these days. Many systemic and local hormones either shorten or prolong the life of bone cells.

Genetic control of bone development of OB lineage. Of great interest presently are the genes that control the differentiation of stromal cell lineages. One such gene is Cbfa1. It is also important in T-cell differentiation and is absolutely essential for development of osteoblasts. Research by Karsenty et al. (1998) showed that deletion of the Cbfa1 gene causes defective

skeletal development in mice. The *Cbfa1* knock-out mice lack skeletons, fail to breathe at birth, and die. Another hot area of research is the peroxisome proliferator-activated receptor (PPAR) system, related to lipid metabolism and differentiation of adipocytes. Some of the agents that act on PPAR can affect the relative differentiation down the adipocyte versus osteoblast pathways.

Regulation of cell differentiation. Dr. Russell highlighted several key research areas regarding the relationship between fat and bone:

- Histomorphometric studies have demonstrated that adipocytes accumulate in osteopenic bone.
- Does switching between the adipocyte and osteoblast lineages contribute to the increased number of adipocytes in osteoporotic bone?
- Can this switching be manipulated pharmacologically?

Leptins and components of the PPAR system may be agents that induce such switching between adipocyte and osteoblast lineages. This switching can be done quite simply in vitro by adding any of a number of agents: vitamin D metabolites, cytokines (including TNF), and certain prostanoids and other lipids, in particular, prostaglandin J2 (PGJ2).

There is recent interest in the potential role of leptin in bone formation. Ducy, Karsenty, et al. (2000) studied mice that were leptin deficient (*ob/ob*) or leptin-receptor deficient (*db/db*). They found, particularly in the *ob/ob* mice, increased bone mass relative to their expectations despite the mice being hypogonadal and having excess glucocorticoid levels. Their hypothesis was that leptin derived from the periphery works through the hypothalamus to create an inhibitory efferent signal that depresses bone formation. The nature of this signal remains unclear, although it is possible that the signal emanates from neural pathways and neuropeptides. These findings establish another interesting link between fat and bone.

Osteoblasts make the bone matrix. Several of their products are used biochemically to monitor bone formation in vivo. The best known of such markers is alkaline phosphatase, but osteocalcin is also a marker of bone formation. Dr. Russell suggested that as bone formation markers go, the best are the propeptides derived from type-1 collagen, the major collagen found in bone. In particular, the N-terminal peptide is a good marker of bone formation. Different agents affect these markers in different ways. For example, glucocorticoids suppress osteocalcin without necessarily affecting other markers of bone formation. Vitamin D and vitamin K status also affects osteocalcin levels.

In lieu of invasive testing on patients, investigators can now measure quite specifically not only these markers of bone formation, but also markers of resorption. Among the products of bone resorption present in urine or blood are type-1 collagen cross-links and C-terminal telopeptides.

Dr. Russell showed a now-classic electron micrograph that depicts the features of multinucleated OCs sitting on the mineralized surface. At the cell-bone juncture is a ruffled border beneath which is a cavity with a low pH—as low as pH 4—created by an ATP-driven proton pump. OC function depends on several critical elements. Any change in the acidification process will interfere with bone resorption, as will any alteration of the sealing of the OC to the bone surface. The enzyme repertoire is also very important. The list of proteases critical to OC function includes cathepsin K, which has received a good deal of attention recently. In humans, a deficiency of cathepsin K leads to pyknodysostosis (Toulouse Lautrec's disease). The effect is similar in animals; they develop osteopetrosis.

These regulatory systems offer several opportunities for pharmacological intervention against osteoporosis. For example, several targets within the enzyme repertoire may be very important. Researchers are investigating inhibitors of proteinases, not just for their relevance in the HIV field but also for their putative roles in arthritis and invasive cancer. Increasingly selective inhibitors are being developed, some of which have been tested in clinical trials. Other targets for pharmacological intervention include the ATP-driven proton pump, inhibitors of cathepsins B, L, and K, inhibitors of the matrix metalloendopeptidases, and cytokines.

Dr. Russell discussed several cytokines that are important for OC differentiation and function. The difficulty is that many cytokines influence bone cell systems in experimental situations—and the problem is how to sort out which are significant. The most important drivers of bone resorption (physiological stimulators) are:

- RANK ligand (RANKL), also called osteoclast differentiation factor (ODF)
- macrophage (or monocyte) colony-stimulating factor (M-CSF), which is essential for producing active OCs
- miscellaneous cytokines (e.g., interleukin-1 and TNF) that are significant in pathological circumstances but may not be essential for producing normal OCs.

The most important inhibitors of OC differentiation and function are:

- some T-cell derived cytokines
- osteoprotegerin (OPG), a soluble molecule related to the TNF receptor family, which is a decoy receptor for RANKL and, therefore, an antagonist to RANKL (a stimulator of bone resorption)
- interleukin 1 receptor antagonist (IL-1RA), which is probably important in relation to estrogen action on bone.

Dr. Russell went on to describe other cytokine regulatory mechanisms. For example:

- osteoclast-activating factors (OAFs) derived from lymphocytes, and now identified as individual cytokines from cells of the immune system or from cancer cells may stimulate bone resorption.
- cytokines from myeloma cells (e.g., interleukins 1 and 6, TNF, RANKL) include some that may be involved in bone resorption and regulation.

The principal regulatory system for bone resorption works through the receptor activator for NF κ B and its ligand, which constitute the RANK/RANKL system. The ligand was discovered independently by three different groups; several taxonomies—RANK ligand, TRANCE, ODF, and osteoprotegerin ligand (OPGL)—have sprung up, but all these terms refer to the same agonist. Interestingly, this system is relevant both to T-cell biology and bone metabolism. The RANK/RANKL system is important physiologically, but it is likely that it is also activated in some pathological states, for example, in the rheumatoid joint. In this case, synovial cells can express RANKL, driving OC development and increasing localized bone resorption. M-CSF also interacts with RANKL in osteoclast generation.

For some time, it has been known that the differentiation of OCs in culture systems can be enhanced by having OBs or stromal cells present in culture. These days investigators can substitute for these cells by using the active principal of cell stimulation of this pathway, RANKL. This phenomenon is even more interesting because it unifies some old themes in bone biology. Many of the systemic hormones, such as PTH and vitamin D metabolites, are known to stimulate bone resorption, probably by increasing the expression of RANKL by OBs and stromal cells. RANKL, then, may be the link between systemic hormones and local bone resorption mechanisms.

Dr. Russell summarized an important hypothesis of Suda: Under physiological conditions, RANKL and M-CSF may be the key drivers of osteoclastogenesis, and, under pathological conditions, other players, such as interleukin-1 (IL-1) and TNF may come to the fore. This notion is not universally accepted, but it provides a useful concept for research.

Dr. Russell cited several putative interactions between tumor cells and bone as evidence of intercellular signaling in myeloma, breast cancer, and prostate cancer. Such signaling mechanisms may produce augmentation cycles. In the case of breast cancer with increased resorption, it is postulated that the bone produces transforming growth factor (TGF)-beta, which acts on breast cancer cells to increase production of PTH-related peptide, thereby stimulating bone resorption. An analogous system exists in myeloma probably through the interplay of IL-1, TNF, and IL-6. In prostate cancer, it may be endothelin, for example, that plays a role.

Pharmacology and drug action. Dr. Russell suggested a number of targets for therapeutic intervention on OCs. Pharmacologic attack at the receptor level, the cell attachment level, or the intracellular signaling level could interfere with OC function and slow bone resorption, restoring bone mass balance. Dr. Russell showed several slides of osteopetrosis that was induced in animal models by knocking out genes that code for cytokines, receptors, intracellular signaling molecules, or enzymes.

The Amgen company is developing the soluble decoy receptor, OPG, as a potential therapeutic. If one administers by injection OPG chimerized to the Fc portion of immunoglobulin (to prolong biological circulating life), bone resorption can be suppressed for up to one month. This finding was presented at last year's American Bone and Mineral meeting.

Most drugs used to treat osteoporosis today act by reducing the number of remodeling sites in bone turnover. Drugs used in osteoporosis as inhibitors of bone resorption include estradiol, selective estrogen receptor modulators (SERMs), calcitonin and bisphosphonates.

Estradiol and raloxifene, an estrogen-like molecule used to treat osteoporosis, compete for the same estrogen receptor site. There is a great deal of interest in developing new SERMs because they avoid the risks of breast cancer and endometrial stimulation.

Dr. Russell described two major pathways by which bisphosphonates work:

- Simple bisphosphonates are incorporated into intracellular analogs of ATP by reversing *t*-RNA synthetase reactions

- nitrogen-containing biphosphonates selectively inhibit prenylation—the attachment of isoprenoid groups to GTP-binding proteins, which are required for OC formation, function, and survival. If protein prenylation is inhibited in OCs, they cease to function.

Statins are the subject of much interest. These compounds lower plasma cholesterol, by inhibiting HMG CoA reductase. Their putative mechanism of action is the induction of BMP2 (bone morphogenetic protein 2, a member of the TGF-beta family). This is an important topic because it opens up a whole new area of bone pharmacology. These compounds mimic nitrogen-containing biphosphonates and, at least experimentally, inhibit bone resorption, increase bone formation, and, according to several recent reports, may reduce bone loss and prevent hip fractures in humans.

Also of great interest is PTH, which is a bone anabolic agent in rats and in man. Given intermittently, it has been shown to increase bone mass and to prevent fractures in patients.

Prostaglandins are also important to consider in any inflammatory situation. Because some inflammatory cytokine action is probably mediated through induction of cyclooxygenase-2 (COX-2), and because prostaglandin production may be involved in biochemical responses to bone, the use of COX-2 inhibitors may be important. Prostaglandins given by injection can augment new bone formation.

Nitric oxide systems exist in bone and are certainly involved in bone resorption and possibly in bone formation. Glucocorticoids may interfere with this signaling system because they inhibit the cytokine-inducible nitric oxide pathway.

Finally, Dr. Russell suggested that researchers will need to consider nutritive approaches for ameliorating bone disorders.. For example, the elderly are frequently deficient in vitamin K, and this deficiency is associated with hip fractures. Vitamin K has a known effect on osteocalcin. Phytoestrogens from soy and other sources have also piqued the interest of clinicians and researchers. Dr. Russell also described a Swiss study that showed a remarkable, positive effect of onions (active constituent unknown) and other vegetables on bone mass in rats.

Apoptosis. It is in the context of osteoporosis therapy that the issue of programmed cell death, or apoptosis, comes to the fore. Estrogens and biphosphonates affect apoptosis of bone cells. In the case of OCs, estrogens may induce apoptosis (as reported by Mundy's group) using

TGF-beta as an intermediate and thereby inhibit bone resorption.. Biphosphonates adsorb to the bone surface and are ingested by osteoclasts which eventually undergoes apoptosis.

Osteocytes also undergo apoptosis. Some clinical, histological data show that osteocyte numbers are reduced in the region of hip fractures. Loss of these cells may be associated with aseptic necrosis and hip fracture. Animal and cell system studies have shown that glucocorticoids may induce apoptosis in osteocytes, and that biphosphonates may prevent this effect.

Genetic variants that may be relevant to HIV and bone research. Susceptibility to bone effects is likely influenced by genetic factors that affect peak bone mass or rates of bone loss. Dr. Russell listed several such factors: vitamin D receptors, estrogen receptors, and a transcriptionally activated site in type-1 collagen for which there is a polymorphism that alters rates of collagen synthesis from individual to individual. He emphasized the potential importance of this genetic variant, noting that it is associated with osteoporotic fractures.

In the area of cytokine gene research, Dr. Russell suggested that further investigation of interleukin 1 and IL-1-RA, for which polymorphisms are associated with osteoporosis, TGF-beta, and the PTH receptor may be fruitful.

Crosstalk: Bone and the immune system

Josef Penninger, M.D., Amgen Research Institute

Dr. Penninger set the scene for his presentation with some facts that reveal the impact of osteoporosis. Each year, osteoporosis costs the United States \$50 billion. In Canada 90 percent of elderly patients who are in home care are there because of osteoporosis; they can no longer get out of bed. The body must maintain a balance between osteoblasts forming bone and the OCs eating away the bone. Osteoporosis—too little bone mass—is the result of an imbalance where too much bone is resorbed or too little new bone is formed. If the imbalance is toward too much bone mass, resulting from too little bone being resorbed or too much bone being formed, osteopetrosis is the result. Those afflicted with osteopetrosis become blind and deaf because their bone is so dense.

Dr. Penninger outlined his goals:

- to describe the system of RANKL and OPG (figure 1), a very beautifully simple system
- to highlight the relevance of this system in infectious diseases, in particular, the role of activated T-cells in modulating bone resorption
- to impart new insights about the RANKL and OPG system
- to reveal some surprising findings from an evolutionary point of view.

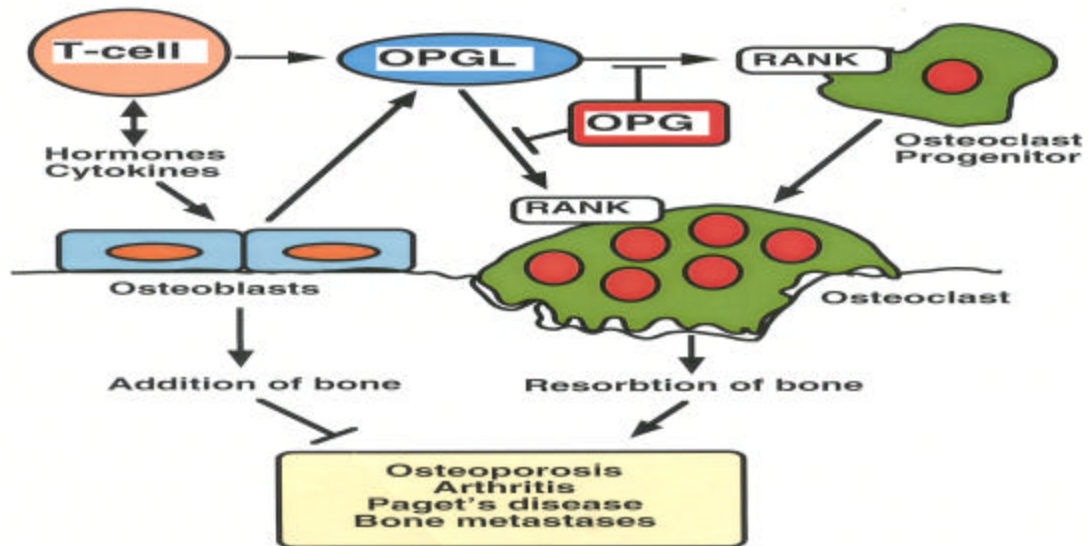


Figure 1. The interaction of activated T-cells with the bone regulatory system. RANK is the receptor for OPGL (also called RANKL); osteoprotegerin (OPG) is the soluble decoy receptor for OPGL.

Source: Josef Penninger, M.D.

A protector of bones, a destroyer of bones. The story starts with Dr. Bill Boyle, a researcher at Amgen in Los Angeles who worked on transgenic (knock-out) mice, in which a particular gene has been functionally inactivated by gene targeting. He had embarked on an ambitious project, taking every protein he could find, sequencing it, and introducing it into transgenic mice to see what would happen. He did this with one protein and found that the mouse had very strong bones compared to controls, so he called this molecule osteoprotegerin, “the protector of the bones.”

The race was on to find the ligand because it was hoped that the molecule that binds to OPG could modulate bone turnover. Four groups independently found the ligand (OPGL), also known as RANKL, ODF, or TRANCE. It is a TNF-family molecule, which is made inside the cell and transported to the cell surface. An enzyme then cleaves off some of the OPGL, releasing it into circulation. Therefore, some OPGL is cell-associated, and some of it is circulating freely. If recombinant OPGL is added to

blood, then bone-resorbing OCs form. This phenomenon enabled the development of an in vitro system to turn on OC development.

At the same time, a paper came out in *Nature* that was puzzling. Researchers discovered that activated T-cells, the target cells of HIV infection, were expressing a certain molecule on their surface. That molecule was the same one—OPGL—that activates OCs. Dr. Penninger and his colleagues were very interested in learning more about this curious connection between the immune system and bone physiology. Is this connection significant?

Dr. Penninger's group found that if the OPGL gene was mutated in mice, they exhibited osteopetrosis. They were very tiny, and their bones were completely solid with no marrow cavity. They had not a single OC. They had teeth, but their jawbones were too solid for the teeth to emerge, because OCs are necessary to allow teeth to erupt.

It appeared that Dr. Penninger's group had found the essential molecule—OPGL—that regulates OC differentiation in the whole organism. OPGL serves a dual role in bone turnover:

- It binds to the RANK receptors on OCs and tells them to start working.
- It affects RANK receptors on OC precursors, stimulating formation of more OC cells.

If no OPGL is present, as in the case of the OPGL knock-out mice, then OCs can neither be generated nor stimulated. The RANK/OPGL system is the essential one for maintaining bone balance. If it is out of balance, the result can be osteoporosis and bone loss. In Dr. Penninger's experience, no cytokine—including prostaglandins, vitamin D₃, and inflammatory cytokines—has an effect without working through the RANK/OPGL system. In addition, OPGL is an essential molecule for organizing the whole immune system. For example, if the OPGL gene in mice is mutated, a curious phenotype results: a mouse without a single lymph node in its body.

OPG, the soluble decoy receptor, is the “brake” on the system. Dr. Penninger indicated that OPG is a good target for drug development and is now in phase I clinical trials. If osteoporosis is induced in female rats by removing their ovaries, within weeks they exhibit severe bone loss. If OPG is supplied, their bones return to completely normal within 3-4 weeks.

Others have shown that when a T-cell is activated via the T-cell receptor, OPGL messenger RNA expression is turned on, inducing OPGL formation on the cell surface of CD4+ and CD8+ cells and the secretion of OPGL. Dr. Penninger then queried: If T-cells can make OPGL, and OPGL is the central factor for turning on OCs, then is it possible that activated T-cells can induce osteoclastogenesis via this RANK/OPGL system?

Activated T-cells can stimulate differentiation of OCs via secreted and T-cell-bound OPGL, thereby creating even more OCs. This phenomenon has been demonstrated in cell cultures developed from permanently turned-on T-cells from mutated mice, and it has been demonstrated in whole animal models. Clearly, OPGL secreted by activated T-cells can cause differentiation of OCs from osteoprogenitors.

OPGL and local bone loss. Turning to the clinical picture, Dr. Penninger then asked if an activated immune system can affect bone homeostasis. Patients with rheumatoid arthritis, autoimmune diseases, chronic infection (e.g., hepatitis C), and leukemia exhibit osteoporosis. The controlling principle in all these diseases, is that when T-cells are activated, the activated T-cells produce OPGL, and the OPGL increases bone resorption via OCs. The effect is magnified because T-cell activation stimulates production of massive amounts of cytokines, including TNF, IL-1, and some 20 others, which stimulate a host of tissues to produce OPGL. In rheumatoid arthritis, for example, inflammatory cells move into the joints, and activated T-cells express OPGL and release cytokines into the inflamed joint, initiating the cascade of OC formation and stimulation that leads to local bone and cartilage degeneration.

Dr. Penninger’s group studied the rat model for rheumatoid arthritis. They injected rats with bacterial extracts to induce arthritis. The rats’ joints swelled and 4–5

days later, their joints were destroyed, and the rats became completely crippled. If, however, when the joint swelling started the rats were injected with OPG, they were completely protected from developing arthritis. Cytological studies showed that inflammatory cells crept into the joint space (i.e., the inflammatory response was unaffected), but the rats did not develop arthritis and were able to move without problem. The OPG inhibited OC activity and formation, preventing destruction of bone and cartilage. Other groups have reproduced these findings in other models of arthritis. It appears, then, that OPG is the master regulator that protects bone and cartilage against arthritis.

These findings may have implications in periodontal disease and tooth loss because teeth are embedded in a bony matrix. For example, 10% of diabetics lose their teeth, and other patients with severe infections are prone to tooth loss. The situation may be similar with immunodeficiency patients. Dr. Penninger's group looked at tooth loss in mice as a response to environmental pathogens. They collected T-cells from patients with periodontal disease. They introduced these T-cells into immunodeficient mice and then infected the mice with the bacteria that had caused the patients' periodontal disease and tooth loss. The T-cells moved to the site of the inflammation and produced OPGL, leading to in situ bone resorption and, eventually, tooth loss in the mice. If the mice were given OPG, tooth loss was prevented. Inflammation still occurred, but the OCs were not activated, so the teeth did not fall out (*J Clin Invest*, Sept 2000).

Which is the culprit—HIV or HAART? Dr. Penninger then addressed the question of whether it is HIV or HAART that may lead to osteoporosis in HIV patients. He proposed setting up in vitro culture systems or measuring expression of OPG and OPGL to see if HAART or HIV turns on OPG or OPGL. Similar test systems could be used to see whether HIV-infected T-cells express OPGL and turn on OC differentiation. The systems to answer these questions are simple and readily available, according to Dr. Penninger.

As an aside, Dr. Penninger mentioned another finding that is perhaps relevant to HIV patients taking PIs. If the OPG gene is mutated in mice or if high-dose OPGL is administered, calcium leaches from the bone via OC activity, resulting in high plasma levels of calcium. (99% of calcium is in bone.) The arteries of these animals spontaneously calcify. Patients on PIs may be prone to this sort of calcification. Dr. Penninger hypothesized that this phenomenon, if it occurs, may correlate clinically with hypertension and stroke.

Evolutionary implications of sex bias in osteoporosis. Dr. Penninger posed a host of intriguing questions: Why is this system regulated by sex hormones? Does this phenomenon make sense in an evolutionary context? Why is bone loss tied to sex hormones? Dr. Penninger's group (*Cell*, September 29, 2000) found that every mouse pup born to a mutated-OPGL or mutated-RANK-receptor mouse died. They found that pregnancy hormones in mammals stimulate OPGL expression in the mammary gland. OPGL removes calcium from maternal bone for the lactating mammary gland to sustain the young. This scheme is essential for survival, and it provides molecular and evolutionary rationales for the sex bias in osteoporosis.

The big picture. Every time T-cells are turned on, they produce OPGL, stimulate osteoclastogenesis, and activate existing OCs. When the system is activated locally, as is the case in arthritis or periodontal disease, the result is local activation of OCs and local bone loss. Systemic OPGL production leads to systemic diseases, such as osteoporosis. In a larger context, OPGL is the central regulator, which acts by binding to its receptor, RANK. OPG, a soluble decoy receptor, can interfere with this process and, therefore, can and should be considered for treating bone loss associated with a broad range of conditions, including the bone loss associated with HIV infection and/or HAART.

Cytokine aspects of bone biology

Roberto Pacifici, M.D., Washington University

Dr. Pacifici addressed the role of cytokines in the regulation of bone remodeling in the context of estrogen depletion. Lack of estrogen is a major source of stress in bone,

stress that is mediated by the activation of a number of cytokines. Thus, estrogen deficiency is a good model to investigate how cytokines regulate bone turnover. He introduced the issue by discussing how estrogen regulates bone turnover.

Cytokines and estrogen in bone metabolism. Osteoclast differentiation, activation, and survival are affected by the interplay of cytokines in physiology and pathology. The previous speakers have emphasized the role of RANKL, but Dr. Pacifici reminded the group that RANKL only works in the presence of M-CSF. The same osteopetrotic phenotype induced by deficiency of RANKL is also induced by the deficiency of M-CSF. In estrogen depletion and inflammation, other cytokines (inflammatory cytokines) come into play, primarily Il-1 and TNF, which stimulate bone marrow stromal cells to produce two essential osteoclastogenic factors: RANKL and M-CSF. The concerted action of these two factors on OC precursors triggers the formation of OCs. Because many cells are involved in OC formation, there are many potential targets for estrogen therapy.

An extensive body of literature points to the roles of IL-1 and TNF in the mechanisms by which estrogen depletion causes bone loss. Bone marrow IL-1 and TNF levels are increased by ovariectomy and decreased by estrogen replacement. The functional block of IL-1 and TNF by specific antagonists, IL-1 receptor antagonist and TNF-binding protein, prevents ovariectomy-induced bone loss in mice and rats. Mice insensitive to TNF due to the overexpression of soluble TNF receptor-1 are also protected against ovariectomy-induced bone loss. Ovariectomy does not induce bone loss in mice lacking the expression of the IL-1 receptor type-1.

The molecular basis for estrogen action on bone regulation. It is not known how estrogen regulates the production of IL-1. In contrast, data demonstrates that estrogen decreases the activity of jun NH₂-terminal kinase (JNK). This kinase phosphorylates jun, which specifically binds to the jun promoter. Estrogen down-regulates cytokine-induced TNF gene expression by blocking JNK activation and the subsequent autostimulation of the jun gene, thus leading to lower production of c-jun and junD.

The resultant decreased levels of jun lead to diminished binding of jun to the TNF promoter and, thus, to decreased activation of the TNF gene.

An obvious question is: Does estrogen regulate the production of RANKL? The answer seems to be no. Dr. Pacifici's group and others have failed to find a regulatory effect on the production of these cytokines. This is consistent with the fact that the RANKL promoter does not have binding sites for the transcription factors that are either directly or indirectly regulated by estrogen. Dr. Pacifici and others, however, have shown that estrogen, regulates the ability of the OC precursors to respond to RANKL. It has been demonstrated that RAW 264.7 cells, monocytic line has the ability to differentiate into OCs when it is stimulated with OPGL only in the absence of M-CSF. In vitro estrogen treatment has been found to reduce the ability of OPGL to induce OC formation. This phenomenon, is a consequence of the ability of estrogen to regulate JNK. Thus, estrogen, by regulating JNK activity, also decreases the ability of OPGL to induce OC formation.

Estrogen's role in M-CSF production. M-CSF is one of the essential regulatory molecules for OC formation. Dr. Pacifici's group and others have shown that estrogen regulates the production of M-CSF by stromal cells. His findings showed that stromal cells from ovariectomized animals produced more soluble M-CSF. When estrogen was added to stromal cells in vitro, no regulatory effect of estrogen was evident. This inconsistency underscores the fact that the sex sterols very often produce different effects in vivo and in vitro. It is rare that in vitro and in vivo findings correlate in estrogen experiments.

They then wished to clarify the mechanism by which estrogen regulates M-CSF production. This effect is an indirect one. Estrogen ends up altering the differentiation of stromal cell precursors rather than directly affecting mature stromal cells. Specifically, in the bone microenvironment of ovariectomized animals, there are increased levels of IL-1 and TNF. Exposure of stromal cell precursors to these high levels of cytokines leads to the selection of a stromal cell population with the ability to produce more M-CSF. Because of this, these stromal cells induce the formation of a

larger number of OCs. Conversely, in the bone microenvironment of an estrogen-replete animal, the low levels of IL-1 and TNF lead to the selection of a population of stromal cells that makes less M-CSF.

The difference has to do with levels of a kinase (CK-II) present in the stromal cells. The stromal cells from estrogen-replete animals have low levels of CK-2. CK-2 phosphorylates a transcription factor, *egr-1*. When it is not phosphorylated, *egr-1* is capable of binding and sequestering another transcription factor, *sp-1*, which is a critical inducer of M-CSF gene expression. In cells from estrogen-replete animals, *egr-1* is not phosphorylated, and there are low levels of *sp-1* available for binding M-CSF promoter, resulting in low levels of M-CSF. Conversely, stromal cells from ovariectomized mice (estrogen-deficient) have high CK-2 activity, leading to phosphorylation of *egr-1* by CK-2 and, which when phosphorylated, can no longer bind to *sp-1*, leaving *sp-1* available to bind to the M-CSF promoter and thereby increase M-CSF gene expression.

How relevant is this regulatory mechanism? To answer this question, Dr. Pacifici's group looked at *egr-1* deficient animals. The prediction from this model is that stromal cells harvested from *egr-1* deficient animals, whether estrogen-replete or estrogen-deficient, should exhibit maximal levels of free *sp-1*, maximal levels of *sp-1* binding to the M-CSF promoter, and maximal production of M-CSF. That is exactly what happened in their experiments. The *egr-1*-deficient mice exhibited higher levels of M-CSF compared to wild-type controls and increased bone resorption as determined by urinary excretion of type-1 collagen cross-link and slightly decreased bone density. Because these animals cannot produce more M-CSF, ovariectomy did not lead to increased bone loss. Ovariectomy of the wild-type controls led to a profound loss of bone, which was preventable with estrogen replacement. The *egr-1* deficient mice were protected against ovariectomy-induced bone loss because they were unable to up-regulate production of M-CSF. In another, related experiment, treatment with a neutralizing anti-M-CSF antibody prevented ovariectomy-induced bone loss.

Estrogen's effect on osteoclastogenesis via T-cells. As previous speakers discussed, T-cells, through their ability to produce osteoclastogenic cytokines via RANKL and TNF,

can up-regulate the production of OCs under inflammatory conditions. Therefore, Dr. Pacifici's group addressed the question of whether estrogen affects OC formation through some effect on T-cells. For one thing, T-cells are known to express estrogen receptors. In addition, alterations in T-cell populations have been described in patients with osteoporosis. Dr. Pacifici looked at bone density of T-cell deficient nude mice compared to wild-type controls. Four weeks after ovariectomy, the bone density of the control animals decreased by 30% compared to estrogen-replete animals. In T-cell deficient animals, ovariectomy caused no bone loss. When T-cell populations were reconstituted by transplanting T-cells back into the ovariectomized, nude animals, bone resorption activity was restored and bone loss ensued. These findings demonstrated that T-cells have a critical role in the mechanisms by which estrogen regulates bone resorption.

How does the system work? To answer this question, Dr. Pacifici's group looked at the role of T-cells in OC formation in vitro. They used a well-established culture system for the induction of OCs: cultures of unfractionated bone marrow cells stimulated with 1,25-dihydroxy vitamin D₃. As previously observed by many groups, more OCs can be harvested from ovariectomized animals than from estrogen-replete controls. This phenomenon does not happen in ovariectomized, nude mice; they do not make an excessive number of OCs.

One could argue that these effects have to do with ability of estrogen to regulate the production of RANKL from T-cells themselves or the effect of T-cells on stromal cells. To explore this possibility, they used another culture system of nonadherent cells, which is really a mix of monocytes and lymphocytes stimulated with M-CSF and RANKL. Under these conditions, the nonadherent cells from ovariectomized animals produced an increased number of OCs in the presence of the maximally active levels of M-CSF and RANKL. No augmentation of OC formation occurred in nude mice. This T-cell-mediated augmentation of OC formation must have been caused by something other than M-CSF and RANKL. Dr. Pacifici concluded, therefore, that T-cells must regulate OC formation via different cytokines.

T-cells and TNF. At this point, the data suggested two possible regulatory mechanisms:

- Ovariectomy increases the T-cell production of an unknown cytokine that stimulates monocytes, which in the presence of RANKL and M-CSF, differentiate into OCs.
- T-cells produce an osteoclastogenic cytokine, the production of this cytokine is not stimulated by ovariectomy; rather ovariectomy increases the responsiveness of OC precursors to T-cell-produced osteoclastogenic factors (i.e., the response of the target cell is enhanced when estrogen is removed).

To eliminate one of these possibilities, Dr. Pacifici's group conducted a series of cross-culture experiments. They harvested bone marrow monocytes from ovariectomized and estrogen-replete animals and cultured them with T-cells harvested from sham-ovariectomized or ovariectomized animals. Regardless of which type of mouse was the donor for the monocytes, the addition of T-cells from an estrogen-deficient animal markedly increased the ability of RANKL and M-CSF to induce OC formation. They concluded that the T-cells from ovariectomized animals make a factor that potentiates the ability of M-CSF and RANKL to induce osteoclastogenesis. The addition of either TNF antibody or TNF-binding protein abolished the ability of the T-cells to augment OC formation, suggesting that the factor produced by the T-cells was TNF.

Dr. Pacifici went on to describe experiments that proved that T-cells make soluble TNF and that TNF acts on monocytes, potentiating the ability of M-CSF and RANKL to induce OC formation. They cultured T-cells harvested from a different group of mice with monocytes lacking either the p55 TNF receptor or the p75 TNF receptor. When T-cells were cultured with monocytes lacking the p55 receptor, there was no increase in OC formation. When the T-cells were cultured with wild-type monocytes or monocytes lacking the p75 receptor, OC formation increased. These data

established that soluble TNF produced by T-cells targets monocytes via the p55 receptor.

A natural corollary from that experiment was that T-cells from ovariectomized animals must produce more TNF. Dr. Pacifici's group measured TNF in purified T-cells from ovariectomized animals. The T-cells from ovariectomized animals made about three times the amount of TNF that was produced by T-cells from estrogen-replete animals.

In vivo, T-cells coexist with monocytes and other cells. Dr. Pacifici's group reconstituted their cultures to the normal ratio of T-cells to monocytes in the bone marrow in vivo. They found that reconstituted bone marrow from ovariectomized mice produced about 300 picograms TNF per milliliter, most of which came from the T-cells.

TNF's potentiating role in osteoclastogenesis. Dr. Pacifici and his colleagues then addressed the effects of TNF on OC formation. They took purified monocytes, stimulated them with RANKL and M-CSF, and then added increasing amounts of TNF. (TNF is capable of potentiating RANKL-mediated OC formation.) The dose-response curve was very steep. Several groups have confirmed these data. There is a very potent synergistic effect between TNF and RANKL. One way to up-regulate OC formation is through very small increases of TNF in the bone marrow. Even in the presence of low-level RANKL, there is a marked up-regulation of OC formation. In many circumstances, RANKL is present at minimal, constant levels, but the synergistic effect of other cytokines, including TNF, results in a sharp stimulation of OC formation. RANKL and TNF both use the same intracellular signaling molecule to activate JNK and NF- κ B.

When they added estrogen in vitro to T-cells, it did not suppress the production of TNF. Estrogen does not directly target mature T-cells but either targets T-cell precursors or works through another intermediary cell. The T-cells from ovariectomized mice appeared to be activated compared to the T-cells harvested from estrogen-replete animals. When they examined the classic markers of T-cell activation, they determined that the T-cells harvested from estrogen-deficient mice were activated. More important, when the ability of T-cells to proliferate was examined in vitro, the T-

cells from estrogen-deficient animals proliferated more rapidly than the T-cells from estrogen-replete mice, suggesting that estrogen deficiency may augment the number of T-cells capable of making TNF, rather than increasing the amount of TNF produced by each cell.

Summing up. There is an extensive body of literature suggesting that estrogen is capable of regulating the activity of antigen-presented cells. In many models of delayed hypersensitivity, estrogen has been shown to block TNF activation via an effect on antigen-presented cells. Estrogen may regulate the activity of T-cells and their ability to proliferate via target cells that are the equivalent of antigen-presented cells, perhaps, Dr. Pacifici hypothesized, stromal cells. Stromal cells may be the major target of estrogen.

Dr. Pacifici summed up his findings in terms of a model: Stromal cells may function as an alternative to antigen-presented cells. According to the model, stromal cells would then potentiate the ability of T-cells to proliferate and produce TNF, and TNF would synergistically act with RANKL and M-CSF to induce osteoclastogenesis.

The role of T-lymphocytes in regulating osteoclast formation in murine marrow cultures

Joseph Lorenzo, M.D., University of Connecticut Health Center

Dr. Lorenzo opened his talk with a review of T-cell development. T-cells begin their lives as precursor cells in the bone marrow. Unlike B-cells, which undergo most of their maturation in the bone marrow, T-cell precursors migrate to the thymus where they are influenced by a variety of thymic factors and eventually become naïve CD4+ and CD8+ T-lymphocytes. There are double-positive precursor stages that subsequently mature into single-positive cells. The single-positive T-cells leave the thymus and migrate throughout the organism, settling in the spleen and lymph. However, a few return to the bone marrow.

Dr. Lorenzo became interested in the T-cell's role in bone regulation for two main reasons:

- Inhibitors of T function (e.g., cyclosporin A) cause high-turnover osteoporosis in transplant patients receiving immunosuppressive therapy. This phenomenon is reproducible in rats.
- T-cells produce cytokines that regulate osteoclastogenesis.

Dr. Lorenzo's group has been using murine bone marrow cultures in the presence of stimulators of bone resorption. His studies have focused on using 1,25-dihydroxy vitamin D₃, the active metabolite of vitamin D, as a stimulator. The result of this process is bone-resorbing OCs. This is a standard model, having been used for about 15 years by many researchers.

Dr. Lorenzo described experiments in which they acutely depleted the mice of CD4⁺ and CD8⁺ cells. They injected the animals with complement-fixing CD4⁺ and CD8⁺ antibodies and, after 24 hours, removed the lymph nodes, spleen, and bone marrow cells. By flow cytometry, they found 99% depletion of CD4⁺ and CD8⁺ in the lymph nodes compared to control animals injected with nonimmune rat immunoglobulin. The findings were similar in spleen cells. Bone marrow contains few mature CD4⁺ and CD8⁺ cells, but the numbers of these cells also decreased after treatment with the complement-fixing antibodies.

What, then, is the effect of T-cell depletion on OC formation? Dr. Lorenzo said that they performed their standard marrow cultures on mice treated with CD4⁺ and/or CD8⁺ complement-fixing-antibodies. The cultures were stimulated with 1,25-dihydroxy vitamin D₃ and then, on day 7, fixed with glutaraldehyde. They performed tartrate-resistant acid phosphatase (TRAP) staining. TRAP-positive, multinucleated giant cells with more than four nuclei per cell were considered OCs. They found that (figure 2):

- CD4⁺ depletion doubled the number of OCs in culture.
- CD8⁺ depletion caused a 40% to 50% increase in OCs.
- CD4⁺ and CD8⁺ depletion caused a 250% increase in OCs.

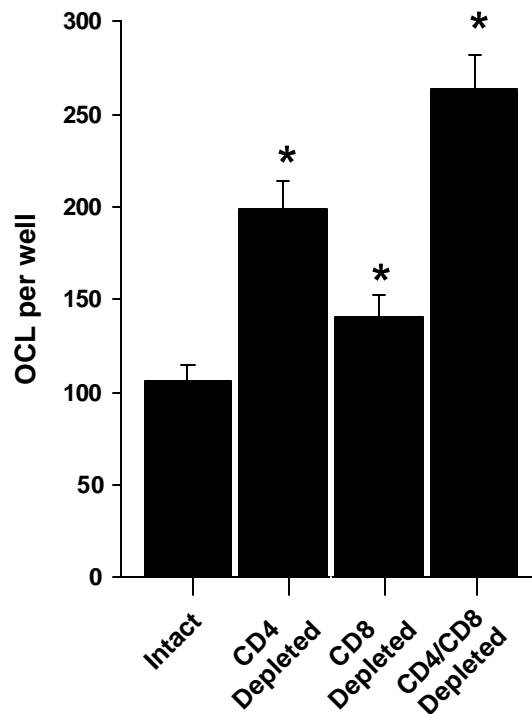


Figure 2. Effect of CD4⁺ and CD8⁺ depletion on osteoclast formation in murine bone marrow cultures. (OCL=osteoclast)

Source: Greecic, Lee, Marusic, and Lorenzo. *J Immunol.* 165:4231-4238, 2000.

Just 24 hours of CD4⁺ or CD8⁺ depletion markedly increased the ability of the marrow cultures to produce OCs in response to 1,25-dihydroxy vitamin D₃. They then performed a reverse time-course experiment and demonstrated a marked response in OC production after 3 days of culture with 1,25-dihydroxy vitamin D₃. The peak occurred at day 5 and began to diminish after 7 days. Calcitonin receptor expression on the cells was confirmed experimentally, showing that they have characteristics of authentic osteoclasts.

Effect of prostaglandins. Vitamin D treatment slightly increased prostaglandin levels in bone marrow cultures measured as prostaglandin E₂ (PGE₂) concentration in the medium of these cultures. Indomethacin, an inhibitor of cyclooxygenase, completely blocked the ability of 1,25-dihydroxy vitamin D₃ to stimulate prostaglandin production. Most strikingly, they found that, in CD4⁺/CD8⁺ depleted marrow cultures, that PGE₂ concentrations increased sixfold in

media to which 1,25-dihydroxy vitamin D₃ had been added and treatment with indomethacin reversed this effect. Indomethacin also blocked the enhancing effect of T-cell depletion on the numbers of OCs that formed in the vitamin D-stimulated cultures (i.e., indomethacin abrogated the PGE₂ effect). Hence, the effect of T-cell depletion on vitamin D-stimulated OC formation in the cultures appeared to be prostaglandin-dependent.

Effect of RANKL and M-CSF. They also looked at the ability of RANKL plus M-CSF to stimulate OC formation in these cultures. Addition of RANKL and M-CSF enhanced OC formation equally in the CD4⁺/CD8⁺ depleted mice and the controls. This result implies that T cell depletion probably does not cause a change in the number of OC precursors, which are the cells that differentiate into OCs.

Dr. Lorenzo's group obtained additional data that corroborated these findings. They performed a similar experiment, in spleen-cell cultures, which lack stromal cells. They treated the spleen-cell cultures with RANKL and M-CSF and found no difference in the number of OC that formed in cultures from CD4⁺/CD8⁺ depleted mice and controls. These data were interpreted to further demonstrate that, there is no difference in the number of OC precursors in mice that can be differentiated into OCs as a result of acute (24-hour) CD4⁺/CD8⁺ depletion.

Dr. Lorenzo described their systems for looking at RANKL mRNA expression in the bone marrow cultures. They used RT-PCR analysis of the bone marrow cells to quantitate mRNA expression. 1,25-dihydroxy vitamin D₃ treatment increased RANKL mRNA expression twofold in intact (control) animals. The CD4⁺/CD8⁺ depleted animals had a twofold increase in RANKL mRNA expression in both the basal state, and after 1,25-dihydroxy vitamin D₃ stimulation compared to the intact animals.

In these bone marrow systems, levels of OPG mRNA are completely inhibited by a number of stimulators of bone resorption, including 1,25-dihydroxy vitamin D₃, PTH, and PGE₂. When murine bone marrow cultures were treated with 1,25-dihydroxy vitamin D₃, OPG mRNA became undetectable. This was a rapid effect, which occurred within 12 hours. It is likely that few OCs formed in the basal state in both intact and T-cell depleted cultures because OPG levels were high. However, when OPG mRNA expression was inhibited by 1,25-dihydroxy vitamin D₃ treatment, then roughly twice the number of OCs formed in response to 1,25-dihydroxy vitamin D₃ stimulation in T-cell depleted cultures, presumably because RANKL mRNA levels were twice as high.

Effect of interleukin-1. Dr. Lorenzo was also interested in IL-1-alpha production in CD4+/CD8+ depleted animals. They found that the levels of IL-1 mRNA increased about 20% in T-cell depleted bone marrow cultures compared to cultures from intact mice. These data led Dr. Lorenzo's group to perform additional experiments. IL-1 receptor knock-out mice that were T-cell depleted demonstrated increased OC production compared to IL-1 receptor-knock out mice that had intact T-cells. These results imply that the effect of T-cell depletion did not depend on whether the cultures could respond to IL-1. Therefore it is unlikely that IL-1 is involved in the effects of T-cell depletion on OC formation.

Dr. Lorenzo also recited a list of other cytokines that they have investigated in the marrow cultures. GM-CSF, interferon-gamma, IL-4, and IL-13 were not modulated by any of these manipulations. Furthermore, neutralizing antibodies to most of these cytokines did not modulate the effect of T-cell depletion on OC formation .

Conclusions. These findings imply that T-cell depletion up-regulates OC formation in vitro by increasing prostaglandin production, which, in turn, increases RANKL and decreased OPG expression. These results suggest that T-cells influence osteoclastogenesis by altering bone marrow stromal cell function.

Dr. Lorenzo summed up his presentation with several conclusions:

- Depletion of T-cells enhances osteoclastogenesis in murine bone marrow cultures.
- This effect was mediated by increased prostaglandin synthesis in the cultures.
- T-cells (in the resting state) modulate the bone marrow microenvironment and influence osteoclastogenesis.

Dr. Lorenzo further speculated that:

- T-cells in normal bone marrow produce cytokines that inhibit osteoclastogenesis.
- The effect of T depletion on osteoclastogenesis is mediated by enhanced prostaglandin production in stromal cells and OBs.

Effect of HIV protease inhibitors on osteoblast and osteoclast differentiation and function

Steven Teitelbaum, M.D., Washington University

Dr. Teitelbaum became interested in the effect of PIs on OB and OC recruitment and function when he learned about the clinical data of Dr. Powderly and Dr. Tebas, which show that HIV patients receiving PIs lose bone mass. Dr. Teitelbaum reminded the group that, regardless of the cause, osteoporosis always reflects a balance between the bone-resorbing activity of OCs and the bone-forming activity of OBs. The studies described by Dr. Teitelbaum dealt with two basic processes:

- the recruitment of cells, both OBs and OCs
- the capacity of these cells to resorb and synthesize bone.

The common paradigm is that, in any circumstance of osteoporosis, there is more resorption than there is formation. Both can be suppressed, both can be enhanced, but the net effect is that resorption is greater than formation. Dr. Teitelbaum first addressed the question of whether PIs affect OB and OC activity in vitro and in vivo, and he described some rather puzzling findings that may undermine the common paradigm about the root cause of osteoporosis.

Osteoblast mineralization assay. For this assay, Dr. Teitelbaum and his colleagues isolated OBs in a situation in which they would form bone nodules, an indicator of OB mineralization activity. They digested neonatal murine calvariae with collagenase to isolate OB precursors and plated the cells in mineralization media for 28 days. To identify mineralizing bone nodules in culture, they stained the cultures with Alizarin red. (Alizarin-red-positive colonies represented the OB colony-forming unit count.) They found that indinavir inhibited in vitro bone nodule formation (mineralization). This effect was not observed with ritonavir or nelfinavir. Indinavir appeared to selectively suppress bone nodule formation in cell culture.

They performed additional experiments to see if indinavir was toxic to the cell cultures; no toxic effects for OBs were noted. It seemed that indinavir prevented OB precursor cells from differentiating into mature OBs.

Dr. Teitelbaum then addressed the question of whether this effect was time-dependent. Presumably, if this were an effect on cell differentiation, the effect would be evident early on. They added indinavir throughout the culture period, during the first two weeks of the culture period, or during the latter two weeks of the 4-week culture period. They found that when indinavir was present throughout the culture period, virtually no mineralized nodules formed. They observed the same thing if indinavir was present during first 14 days of culture. If, however, indinavir was present only during the latter two weeks of the culture period, there was no effect on mineralized bone nodule formation. Dr. Teitelbaum concluded from this study that indinavir exerts its effect during the first half of the culture period, consistent with an effect on cell differentiation.

In vivo osteoblastogenesis assay. Dr. Teitelbaum continued by discussing some in vivo work—actually an ex vivo osteoblastogenesis assay—in which they injected mice with indinavir for 2 weeks, harvested their bones, and established osteoblastogenic culture systems in mineralization medium. They performed alkaline phosphatase and Alizarin red stains after 28 days of culture to identify colony-forming units (CFUs) that have the capacity to form OBs. They found that indinavir markedly diminished OB numbers. Clearly, there was a marked suppression in vivo of the capacity of cells to form OB precursors, which have the capacity to differentiate into bone-forming OBs ex vivo.

Calvarial osteoblast system. Dr. Teitelbaum's group took calvariae from newborn mice and placed them in culture, using the basic calvarial OB culture system as originally described by Boyce. This process yields an organ culture system, which is more complex than tissue culture and more representative of conditions in the whole organism. They looked for growth, or thickening, of the calvariae histologically. When they added indinavir, it inhibited calvarial thickening and OB formation.

They then followed up on Mundy's observation (published in *Science* last year) that statins can stimulate bone formation. Dr. Teitelbaum found that in this organ culture system, lovastatin can stimulate bone formation, yielding an abundance of so-called active OBs. In a corollary experiment, they found that indinavir exhibited dose-dependent inhibition of the lovastatin effect and, hence, inhibited new bone formation in organ culture. Dr. Teitelbaum and his associates concluded that indinavir inhibits osteoblastogenesis *in vitro*, *in vivo*, and *ex vivo*.

A look at osteoclastogenesis. They next turned their attention to OCs, the bone-resorbing cells of hematopoietic origin, which are members of the monocyte-macrophage family. These cells originate in the bone marrow and then become circulating mononuclear precursors. The precursor cells differentiate by attaching to bone, undergoing multinucleation, and then assuming the OC phenotype. As discussed by previous speakers, the basis for this differentiation process is the RANKL and M-CSF system. RANKL interacts with RANK (receptor) and M-CSF to induce the macrophages to become OCs.

OC recruitment studies. The idea behind these studies was to isolate pure macrophages, culture them with RANKL and M-CSF, thereby obtaining pure populations of OCs and their committed precursors. That was the system described by Dr. Teitelbaum for studying the effects of PIs on osteoclastogenesis. First, they isolated murine bone marrow from the femur and tibia. They maintained the marrow with preservative M-CSF. The mononuclear cells were purified, and the macrophages were separated on a Ficoll-Hypaque gradient. They cultured the macrophages with M-CSF and RANKL. The TRAP stain was used to distinguish OCs, and TRAP solution assay served to quantitate commitment to the OC phenotype. No inhibition of osteoclastogenesis was observed with indinavir treatment; therefore, they concluded that indinavir specifically targets osteoblastogenesis. Ritonavir, however, substantially inhibited osteoclastogenesis.

Their next step was to look for a concentration-dependent effect of ritonavir on osteoclastogenesis. They did find a concentration-dependent effect, which became apparent at 5–10 mcg/mL, within the likely pharmacological range.

Next, they looked at the time course of this effect using the TRAP solution assay. Virtually complete inhibition of osteoclastogenesis was observed by day 6 or 7 in the presence of ritonavir.

Was this effect reversible? They withdrew the drug at various time points and checked to see if osteoclastogenesis would resume. Osteoclastogenesis was inhibited as long as the drug was maintained in culture but resumed when the drug was removed. Dr. Teitelbaum was able to conclude from these studies that ritonavir reversibly inhibits osteoclastogenesis by blocking the differentiation of OC precursors into mature OCs, not through a toxic effect but through a direct effect on the cells.

OC function studies. The studies described above dealt with cell recruitment. Dr. Teitelbaum next described the group's studies of the function of recruited cells. For these studies, they employed an intriguing assay system based on formation of resorption pits on thin sections of whale teeth. When OCs are placed on the whale tooth sections, they form pits in the dentin. They cultured OCs on the whale dentin slices, allowed the OCs to mature (4 days), added PIs, and then performed TRAP staining of the sections. Despite an abundance of OCs in the culture, Ritonavir completely arrested resorption pitting in dentin. The control and indinavir-treated cells showed resorption pitting. The OCs were viable, but nonfunctioning. They found that ritonavir had no effect on cell survival although it abrogated resorption activity.

In vivo osteoclastogenesis assay. Dr. Teitelbaum described their method for an in vivo osteoclastogenesis assay, which was based on a system initially described by Crane. The question addressed by this experiment was: Will ritonavir block osteoclastogenesis both in the basal state and with PTH stimulation? Dr. Teitelbaum described the experimental protocol: Mice received subcutaneous calvarial PTH injection 4 times per day for 3 days to stimulate osteoclastogenesis. The calvariae were sectioned through the midline suture and TRAP-stained. They found that the PTH-

treated animals had many more OCs than the control animals did. They then repeated the experiment, except that the mice received ritonavir intraperitoneally. If the OCs were not stimulated with PTH, the ritonavir made no difference in the number of OCs. If they stimulated the calvariae with PTH, they found three- to fourfold increases in OC numbers. If they treated the animals systemically with ritonavir, the number of OCs came down to baseline levels. Therefore, Dr. Teitelbaum concluded that ritonavir blocked the PTH stimulation.

How do these findings fit into the bone remodeling picture? Dr. Teitelbaum acknowledged that these data are quite puzzling. The findings for the OB experiments are what would be expected, but the OC data run counter to the current paradigm of bone regulation.

By way of explanation, he reminded the group that both OBs and OCs participate in the remodeling process. In acquired osteoporosis, the OCs dig out holes that are only incompletely filled by the OBs. In fact, there are circumstances in which both OC and OB activity is suppressed, but the result is still profound osteoporosis. He first discussed an experimental form of this phenomenon and then the human form of this disease, so-called low-turnover osteoporosis.

He described an IL-4 transgenic mouse, which overexpresses IL-4 and exhibits severe osteoporosis. As expected, there are reduced numbers of OBs as determined by alkaline phosphatase staining (a marker of osteoblastogenesis), but there are also reduced numbers of OCs. Such suppression of bone remodeling—both formation and resorption—is associated with severe osteoporosis. Dr. Teitelbaum mentioned a recent literature review by Manolagas (*Endocr Rev* 2000 Apr;21(2):115–37), which discussed various forms of osteoporosis and showed that in many forms of acquired osteoporosis, both OBs and OCs are affected. Table 2 provides a few highlights from the review.

Table 2. Status of osteoblastogenesis and osteoclastogenesis in various forms of acquired osteoporosis.

Form of osteoporosis	Bone regulatory status
Sex steroid deficiency	<ul style="list-style-type: none"> • increased osteoblastogenesis • increased osteoclastogenesis • increased lifespan of OCs • decreased lifespan of OBs
Senescence	<ul style="list-style-type: none"> • decreased osteoblastogenesis • decreased osteoclastogenesis • increased adipogenesis • decreased lifespan of osteocytes
Glucocorticoid excess	<ul style="list-style-type: none"> • decreased osteoblastogenesis • decreased osteoclastogenesis • increased adipogenesis • increased lifespan of OCs • decreased lifespan of OBs • decreased lifespan of osteocytes

Source: Based on Manolagas, SC. 2000. *Endocr Rev* Apr;21(2):115-37.

The data presented by Dr. Teitelbaum indicate that PIs have a direct effect on OCs and OBs in vitro and in vivo. It appears that PI-associated osteoporosis is a condition that diminishes both osteoblastogenesis and osteoclastogenesis. To interpret these findings in a clinical context, investigators must ferret out which HIV patients are on which PIs and which PIs are associated with osteoporosis.

Increased prevalence of avascular necrosis in HIV-infected adults

Judith Falloon, M.D., NIAID, National Institutes of Health

Dr. Falloon presented data from a study conducted at the NIH, which was presented by Dr. Henry Masur at the 38th annual meeting of the Infectious Diseases Society of America (Masur, et. al., 38th Annual IDSA Meeting Abstracts, Abstract 15, New Orleans, 2000).

AVN has rarely been reported in the setting of HIV infection, however within a 10-month period, 4 patients were diagnosed AVN of the hip in the NIH clinic population. There was concern that HIV infected patients might be at higher risk for developing AVN than previously recognized. Therefore, a MRI-based study of

asymptomatic HIV-infected patients was conducted to determine the prevalence of hip AVN.

Over a seven-month period in 1999, patients who were enrolled in studies at NIH were recruited to participate. Coronal T1- and fat-suppressed T2-weighted images were obtained. Participants completed a questionnaire and received a screening MRI.

Fifteen (4.4%) of 339 patients had evidence of AVN by MRI: 9 had unilateral and 6 had bilateral involvement. None had evidence of joint abnormality on standard x-rays. None of 118 HIV negative volunteers had MRI evidence of AVN. Prospectively performed physical examinations did not differentiate HIV-infected patients with AVN from those without. Patients with osteonecrosis were more likely to have used lipid-lowering agents ($P=0.004$), testosterone ($P=0.01$), and systemic corticosteroids ($P=0.02$). They were also more likely to exercise routinely by bodybuilding ($P=0.03$). The presence of anticardiolipin antibodies was highly associated with the development of osteonecrosis ($P=0.004$), however triglyceride ($P=0.07$) and cholesterol levels ($P=0.08$) were marginally associated.

HIV-infected patients are at much higher risk of AVN of the hip than previously recognized. Frequently patients are asymptomatic without any abnormalities on physical examination or routine radiographs. Multiple risk factors associated with HIV infection or its therapy may in fact come together to produce this increased risk.

Bone metabolism in HIV disease: New and old paradigms

Steven Grinspoon, M.D., Massachusetts General Hospital

Dr. Grinspoon opened his presentation by saying that what is known about bone disease and HIV infection is breaking into two paradigms: an old paradigm and a new one. His presentation was structured along those lines and touched on the following topics:

- evidence for altered bone metabolism in the era prior to HAART and lipodystrophy
- relationship of altered bone metabolism to wasting syndrome

- relationship of altered bone metabolism to HAART and lipodystrophy.

He went on to identify several potential mechanisms of abnormal calcium and bone homeostasis in HIV disease, namely:

- malabsorption
- drug effects
- relative hypoparathyroidism
- 1,25-dihydroxy vitamin D₃ deficiency.

Dr. Grinspoon briefly reviewed some literature on the correlation of abnormal bone remodeling with activation of the immune system. The best study to date on this topic is the one by Aukrust et al. (*J Clin Endocrinol Metab*; 94:145, 1999). In the study, they examined 73 HIV patients, looking at various bone turnover markers and indicators of disease severity, including D4+, RNA, TNF-alpha, p 55 and p75 TNF receptors, 1, 25-dihydroxy vitamin D₃, PTH, and baseline response to HAART. The HAART regimen for treated patients consisted of indinavir, AZT, and 3TC. They showed a significant systemic elevation of TNF-alpha in asymptomatic HIV patients. Compared to asymptomatic controls, osteocalcin levels were significantly reduced in patients symptomatic with AIDS. They also demonstrated an inverse correlation with p55 TNF receptors and osteocalcin levels. Osteocalcin levels increased in patients treated with indinavir.

Turning to the histomorphometry of bone disease in HIV infection, Dr. Grinspoon described the findings of Serrano et al. (*Bone*, 1995). They studied 22 HIV-infected patients and found that osteocalcin levels decreased with increasing disease severity. They also found a positive correlation of osteocalcin levels and CD4+ counts. They also demonstrated reduced bone formation rates, reduced surface activation frequencies, and reduced osteoclast indices in conjunction with increased disease severity.

Dr. Grinspoon also addressed the issue of reduced bone density in HIV-infected patients by discussing the findings of Paton et al. (*Calcif Tissue Intl*, 1997). In their study of 45 HIV-infected men with a relatively low mean body-mass index of 21.5 kg/m²) and a mean CD4+ count of 90, they found an average of 1.6% bone loss over 16 months in longitudinal follow-up. They also demonstrated a 3% difference in lumbar spine bone density ($P = .04$) and no difference in hip bone density compared to age-and weight-matched controls. Similarly, Hoy et al. presented data at the 7th Annual Retrovirus Conference on 80 HIV-infected patients, of whom 28% exhibited osteopenia and 10% had osteoporosis.

The old paradigm. Dr. Grinspoon characterized the “old paradigm” in these terms:

- The effect of PIs on *t*-score was not statistically different.
- Significant reduction in bone density occurred in men with AIDS wasting syndrome.
- Reduced bone density was associated with decreased BMI especially at the hip.

The New Paradigm. Starting in about 1997, Dr. Grinspoon noticed changes in the HIV population. He was asked to work up four HIV patients for what seemed to be Cushing’s “buffalo humps.” The patients shared a couple of other characteristics: loss of subcutaneous fat and increased belly girth. However, these patients did not have increased serum free cortisol levels. There were no other signs of Cushing’s disease. This new paradigm, ushered in during the HAART era, is characterized by an enormous increase in visceral fat, decreased subcutaneous fat, and some metabolic abnormalities (2-hour glucose more than 140, cholesterol in excess of 200, triglycerides more than 200, and so forth).

Tebas et al. (*AIDS*, 2000) examined bone density in HAART-treated patients via a cross-sectional comparison of 112 HIV-infected males receiving PIs. They found that:

- The incidence of osteopenia was higher in men taking PIs, with a relative risk of 2.19 ($P = .02$).

- Men receiving PIs were centrally obese, but the central:appendicular fat ratios did not correlate with bone density.
- Increased trunk:appendicular adipose tissue ratio is a marker for HIV wasting.

Dr. Grinspoon described an ongoing study that explores the relationships between bone density, fat distribution, and HAART. The study involved 41 HIV-infected patients, prospectively recruited and categorized as being lipodystrophic or not, based on loss of body fat in face or extremities or a gain in truncal fat.

Based on a review of the literature, Dr. Grinspoon concluded that abnormalities in bone turnover and metabolism occur in HIV-infected patients. Further studies to determine the effects of fat distribution per se and to elucidate drug effects on bone density and bone turnover indices in HIV lipodystrophy are needed. Moreover, studies are needed to determine the relationship between cytokines and bone density, as well as increased marrow fat and bone density in the HIV lipodystrophy syndrome.

PROGRAM AGENDA

Tuesday, August 29, 2000

7:30 – 8:00 A.M. Arrival/Continental Breakfast

8:00 A.M. *Introduction*

Facilitator: June Bray, Ph.D.

Forum Director: David Barr

Co-Chairs: Lawrence Raisz, M.D.
Jane E. Aubin, Ph.D.

8:30 A.M. ***Bone Disease and HIV***

William Powderly, M.D.

9:00 A.M. ***Overview of the Local Regulation of Bone***

Graham Russell, M.D.

9:40 A.M. ***Crosstalk: Bone and the Immune System***

Josepf Penninger, M.D.

10:10- 10:30 A.M. Coffee Break

10:30 A.M. ***Cytokine Aspects of Bone Biology***

Roberto Pacifici, M.D.

- 11:00 A.M. ***The Role of T-lymphocytes in Regulating Osteoclast Formation in Murine Marrow Cultures***
Joseph Lorenzo, M.D.
- 11:30 AM ***Affect of HIV Protease Inhibitors on Osteoblast and Osteoclast Differentiation and Function***
Steven Teitelbaum, M.D.
- 12:00- 1:00 Lunch
- 1:00 P.M. ***Increased Prevalence of Osteonecrosis in HIV-infected Adults***
Judith Faloon, M.D.
- 1:30 P.M. ***Bone Metabolism in HIV disease: New and Old Paradigms***
Steven Grinspoon, M.D.
- 2:00-2:15 *Coffee Break*
- 2:15-4:30 P.M. ***Discussion Session***

Forum for Collaborative HIV Research

Bone Metabolism and HIV Disease Meeting

August 28, 2000

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