1 真実を伝えることの重要性

A なぜ真実を伝えるのか

今日の医療では，インフォームドコンセントが重要視されている．これは患者の基本的人権を守り，患者の自主性を尊重した医療を実践するための基盤となるからである．治療方針を決定するためには，患者の意思を尊重して，家族と医療者がその決定を支持していくという考え方が重要である．最終的な治療方針を決定するには，そうした患者の意思だけでなく，医学的な適応，家族の思い，医療者の思いなども考慮する必要がある．

医療はめまぐるしいスピードで進歩している．一方でそのことは患者が選択する治療法が増えているということになる．そうしたなかで，患者が自主的に治療法を選択するためには，「理解できる説明を十分に受けたうえで，納得して選択する」というインフォームドコンセントが，ますます重要視されるようになってくるのである．「患者が理解しないままの同意」や「真実を告げないままの同意」は，インフォームドコンセントとはよべない．大切なことは，患者が医療者から説明を受けたかどうかではなく，患者がよく理解できているかどうかなのである．

真実を伝えるということは，決して癌だけが問題になるわけではない．癌以外にも，現在の医療で治癒できない致命的な疾患は数多く存在する．しかし，なかでも「癌」は２人に１人が罹患するほどその発生頻度が高く，また３人に１人が癌で亡くなり，わが国の死因の第１位を占め続けていることなどから，社会的にも大きな問題となっている．

医療の現場では，癌であることを含めた疾患の病名や病状，検査結果，治療効果，経過の予測などについて，厳しい病状やつらい事実を知らせる機会は多い．それだけに，「真実を伝える」ための技術を学ぶことは重要である．

近年，インターネットなどを用いて情報を容易に手に入れることができるようになってきた．また，癌体験者が増えていることや，テレビや新聞などでも，癌であっても病名を隠すことをしなくなってきたことも影響して，病名を隠すという対応から「いかに真実を伝えるか」という方向での対応に積極的に取り組むようになってきた．

B 真実を伝えることに関する倫理的問題

患者には自分の病気について知る権利とともに，知らないでいたいという権利もある．しかし，多くの患者は自分の身体に起こった事実を正確に知り，今後どのように対処していくのがよいのかを医療者と話し合いたいと願っている．

患者がわずらっている病気が何であるのか，そして今どのような状態にあるのか，治療にはどういう方法があるのか，さらには今後どのようになっていくのかといった重要な情報を，医療者の判断で操作して伝えることは倫理的に問題がある．

医療者の側に，悪い情報は患者にとって有害であるという意識があると，情報を伝えるときに事実がゆがめられたり曖昧になったり，治療効果が実際よりもオーバーに伝えられる可能性もある．患者の治りたいという気持ちが強く，効果が期待できない状況にもかかわらず負担の大きい治療を受ける結果を招き，外泊や退院の機会を逃すことにもなりかねない．正しく理解できないために正しい自己決定ができなくなる可能性があり，治療法の選択判断を誤ることにもなる．情報を伝える際には，伝える側の考え方や倫理観などが影響することは避けられないが，できるだけ誤解のないような伝え方を心がける必要がある．

患者の権利の尊重という視点に立ちさえすれば，悪い情報を伝えることによって生じる問題のすべてを解決することは困難である．これからは，「悪い情報を伝えるか否か」よりも，「いかにして伝えるか」さらには「いかにして情報を分かち合うべきか」の議論をしていく必要がある．

C 真実を伝える際の留意点

癌であることや，予後が悪いといった事実を伝えられて衝撃を受けない人はいないはずである．たとえ真実を知ることがつらくて残酷なものであっても，その伝え方が残酷なものでなく，患者が自己決定しやすいように配慮されたもので，医療者を中心としたチームがサポートすることを保証していけば，現実的な対応が可能になるはずである．つらい事実を伝えることは医師だけで行うものではない．常に医療チームとして取り組むことが大切であり，チーム一人ひとりが患者とその家族とどのように向き合うかが問われる問題でもある．

また真実を伝えていくには，患者が自ら抱いている疑問を表現することができているか，患者が自分の感情を医療チームに十分伝えることができ

ているかといった点に配慮する必要がある．患者と医療チームがお互いの考えを十分に伝え合えて，話し合いが患者家族にとってよい方向に向いているかといったプロセスが重要である．真実を伝えるためには，以下のような点に配慮しながら実践することが大切である．

1 | 話し合いの環境について

大切な話し合いをするためには，十分な時間を確保する必要がある．話し合いが呼び出しなどで中断することがないように準備することや，話し合いをナースステーションの片隅でしたり，廊下で立ち話として伝えるようなことをしてはならない．患者のプライバシーが十分に守られる空間を用意して，ゆったりとした気持ちで座って話し合うことができるようにする．

また，話し合う内容について事前に再確認することも大切である．話し合いが始まってから検査結果などについて事実確認するようでは，信頼を得にくくなってしまう．

2 | 患者の理解していることの確認

患者の考えや思いは，病気の状況や治療経過とともに変化していく．「今の状況を，どのように理解しているか」について再確認しながら話し合いを始める必要がある．患者との信頼関係を築くためには，医療者が多くのことを話したり説明したりするよりも，患者の言葉を「聞く」ことのほうが大切である．何をどこまで伝えるかについては，あくまで患者の知りたいと思っていることに応じて対応していく．

話し合いが進むなかで「病状はかなり厳しい状況です．病気のことについて，さらに詳しく知っておきたいとお考えですか」とか，「厳しい状況でも全部聞きたいとお考えですか」「悪いことはあまり聞きたくないとしたら，どなたと話し合ったらよいですか」と確認する．

また，説明内容が十分に理解されたかどうか，説明内容を聞いてどのように感じたかなどを確かめるためにも，看護師が同席できるように準備して話し合うことも重要なポイントである．看護師は医師がどのような内容を伝えるのかを確認するだけでなく，そのときの患者とその家族の反応がどうであるかをアセスメントしていく．看護師が同席することによって，その後の患者とその家族のケアの方向性について大きくかかわることになるので，できるだけチーム内で協力して時間をつくって同席できるように努める必要がある．

3｜質問の促し

日本人には自分の思いを素直に表現することが苦手な人が多いといわれる．それはたとえ死への不安がある場合でも，その不安を言葉に出せないでいるという実態からも理解できる．不安を感じていても言葉に出さないのは，「恐怖心」や「あきらめ」というよりも，日本人特有の「家族への思いやり」や「医療者への遠慮」であることも多い．患者から「わかりました．よろしくお願いします」という言葉があっても，それを言葉どおりに受け止めるのではなく，まだ十分な理解ができていないと考える必要もある．したがって，そのようなときには，話し合いに同席した看護師は，質問しやすいように声をかけることが必要である．しかし，それでも声に出せない場合も多いので，「いつでも聞きたいことがあったら言ってください．医師には直接聞きにくいようでしたら，看護師でも構いません．お聞きする用意はありますから」と伝えることも大切である．

4｜感情への対応

つらい事実を聞いて，長い時間沈黙が続いたり，泣き出したり，怒りの気持ちを表出したりといった反応を示すことがある．そのような場合には，いずれも「自然な反応であって，異常なことではない」ことをしっかり伝えたり，「つらい話でしたね」「まだ信じられないという気持ちでしょうね」といった共感的な対応をする必要がある．

さらに患者の気持ちに踏み込んで，「いろいろなことが頭をよぎっていると思いますが，よければ聞かせていただけますか」など，患者の気持ちを言葉にできるように促すような援助をする必要もある．

5｜家族から反対されたときの対応

従来の癌医療では，癌と診断がついたら，まず家族をよんで事実を伝えていた．そうすると，多くの家族は「本人がショックを受けるから」とか「気が小さくて，癌と知ったら自殺するかもしれないから」と言って事実を伝えることに反対してきた．

まず家族に伝えた場合に，なぜ多くの家族が真実を伝えたくないと思うのかについて考えてみると，「患者自身が衝撃を受けることが心配」というのが理由の一つであるかもしれないし，「患者と接していかねばならない家族としての自分自身のことが心配」であることから発せられた言葉であるとも考えられる．また，十分な信頼関係が築けているとはいえない担当医師が，上手く伝えてくれるかどうかについての不安も理由の一つであろう．そうした不安を感じていることにも配慮したうえで，患者と家族が

表3-1 ● 末期状態を伝える際に考慮すべき状況

①告知の目的がはっきりしていること
②患者・家族に受容能力があること
③医師およびその他の医療従事者と，患者・家族との関係がよいこと
④告知後の患者の精神的ケアや支援ができること

出典／厚生労働省「末期医療に関するケアのあり方の検討会」より引用

真実を共有することによって，家族との語らいが患者にとって治療以上に慰めになることを伝えることも重要である．

さらに患者に真実を伝えることをためらう理由として，本人に悪い事実を伝えると落ち込んだり，ショックを受けて悪い状況になるという「思い込み」や，伝えたあとの状況が予測できない「恐怖心」，伝えたあとのかかわりに対する漠然とした「不安」「自信のなさ」なども考えられる．

治癒することが困難であるといった厳しい真実であっても，表3-1のようなことにも配慮しながら，事実を伝えることが患者自身にとって役に立つことなのかという視点を忘れないようにしながら取り組む必要がある．

2 コミュニケーションの重要性

A 意思決定のためのコミュニケーションの重要性

どのような状況においても，患者が意思決定するうえで重要なのは，患者とその家族そして医療者とが，互いの思いを知るためのコミュニケーションである．また，良好なコミュニケーションを築くためには，医療チームのメンバーがコミュニケーションの重要性を理解して，チーム医療を実践することの大切さを知っていることである．そうしたコミュニケーションが築かれてこそ，たとえ残された時間が限られているという厳しい状況であっても患者の思いが尊重され，最善の治療方針が決定できるのである．

「病名を告げると治療がスムーズに進む」ことなどを理由にして病状説明を行ったりすると，治療を行うことが優先されて患者とその家族の気持ちへの配慮が欠けてしまうことになる．本来医療は人間と人間とのかかわりによって展開されるものである．しかし，それにもかかわらず，医療者は病気や疾患に関心が向きがちになり，病気を体験している患者や家族への関心が薄くなる傾向がある．よいコミュニケーションを築くためには，表3-2に示すようなことにも配慮しながら，真実を告げた患者家族と向き合うことや，医療者一人ひとりが「豊かな感性をもった人間」になること，そして医療者自身が「人生」「生きること」「死ぬこと」などについて，

表3-2 コミュニケーションの基本

①座ってゆっくり話す
②患者の言葉に耳を傾ける
③患者の感情に焦点をあてて話をする
④専門用語を使用しないように，わかりやすい言葉で話をする
⑤できるだけ開かれた質問をする
⑥常に援助者であることを保証する

どう考えているかによって患者とその家族との向き合い方も変わってくる．医療者が自己の感性を磨いて，人間の尊厳を大切にしようとする姿勢をもち続けて，誠実さや正直さのなかで患者とその家族との結びつきを築く努力をしていくことがケアの原点といえる．

コミュニケーションを阻害する要因の一つに，真実を隠すことがある．真実を伝えないことによってオープンなコミュニケーションが阻害されてしまい，患者自身の意思が尊重されなくなってしまうからである．看護師は病状説明が十分になされていない患者と接する際には，オープンなコミュニケーションがとれないために，患者との信頼関係が築きにくいと感じている．そのため看護師が患者のニーズに応えることができず，不満足感や後ろめたさを感じる傾向もある．

最近は診断の段階で，癌という病名を伝えられることは増えてきている．しかし，治癒が困難な再発や転移を起こした場合には，その事実を伝えることが曖昧になる傾向がある．真実が伝えられないと，患者は病状が改善しないことに不安を抱き，家族や医療者が真実を隠しているのではないかと疑念を抱くようになる．また，だまされていると感じたり，怒りをぶつけるようなことも起こり，孤立感を感じるようになる．それでも隠し続けると，患者は「だれも気持ちをわかってくれない．自分を尊重してもらえていない」といった苦痛を感じるようになる．看護師も伝えていないことによる不自由さを感じるだけでなく，伝えたあとの対応に自信がもてないために不安を感じるようになってしまう．家族としても現状を理解したうえで仕事の整理をして欲しいとか，家族のあいだで確認しておきたいことがあるのにと感じるようになる．

こうした苦悩を解消するためには，医療者は患者とその家族との良好なコミュニケーションを築く努力をする必要がある．良好なコミュニケーションを築くことができれば，たとえ悪い情報であっても自然な形で共有できることが多い．悪い情報を伝えることは，患者だけでなく伝える医療者にとってもつらいことである．しかし，そうしたつらさも共有しながら患者とともに歩むという姿勢が必要である．

B コミュニケーションを築くための方法

1 目標設定の共有

病状についての患者の理解度を把握したうえで，今後の治療方法について選択肢を示すことになる．そのうえで最終的に何を目標として治療していくかを相談することになる．当面の目標などを共有するためにも，患者のこれまでのライフヒストリーを聞いて考え方を知ることも必要である．

当面の目標を設定したうえで，それを実現するための様々な治療法の選択肢を提示する．また，患者自身が人生最期のときをどのようにしたいと考えているのかを聞いたうえで，適切な治療法などを検討していくことになる．

2 医療者としての意見の提示

医療者は事実を伝える際には，説明を押しつける形にならないようにする必要がある．特に死が避けられないような厳しい事実を伝える際には，患者家族の感情に十分配慮する必要がある．病状を説明したあとで家族から予後期間について尋ねられることがある．そのような場合には，「どうして予後期間のことが気になるのか」を確認する．そのうえで「家族からみて，どのくらいの時間があると思いますか」と尋ねた後に，予後期間については2か月とか3か月というふうに数字で伝えるのではなく，「長めの月単位」「短めの月単位」とか「週単位」や「日単位」「時間単位」といった表現で伝えるほうがよい．

また，「大切なことは早めに確認したほうがよいと思います」とか「いつお別れがきても，おかしくない状態にある」といった表現で厳しい予後を伝えることがある．最終的には，本人の尊厳を守るためにも，むやみな蘇生術を実施しないという意思の確認（DNR：Do Not Resuscitate）をすることも必要である．また，死を身近で実際に体験したことがない人が増えていることから，病状が進行するとどのようなことが起こるのかということへの不安を感じていることも多い．そうした場合には，タイミングを見計らって表3-3のような資料を用いて説明することも勧められる．

厳しい予後を伝える場合にも，医療者として最期まで患者とその家族を支援し続けることを保証することが大切である．

3 真実を伝えたあとの看護援助

患者がつらい事実を受け入れるためには，ある程度の時間が必要であ

表3-3●家族に渡す死の説明書

家族の方へ
死の前後の患者さんの状態とその対処法

ここに書かれていることは，患者さんの死の前後にみられる身体の変化をあらかじめ知り，理解するために有用です．これらの変化は，すべてが見られるわけではなく，また必ずしも書いてある順序どおりに起こるわけでもありません．

大切なことは，ほとんどの変化が死にいたる自然の経過であり，ご本人にとっても苦痛なことではないということです．

それでも患者さんの状態で，何かわからないことがあったり，また患者さんが不快や苦痛を感じていると思われるときには，ご連絡ください．

(1) 死がさし迫ってきたときの徴候
①疲労と傾眠の傾向が強くなり，寝ていることが多くなる．
②食欲がさらに低下して飲食の量が減る．
③時間や場所について混乱が見られ，時に知っているはずの人がわからなくなる．
④ときどき不穏状態となり，奇妙な動きをしたり大声をあげたりする．
⑤尿や便の失禁がみられる．
⑥唇が乾燥して粘稠な分泌物が口の中にたまり，呼吸のときにゴロゴロという音がする．
⑦手足が冷たくなり皮膚が蒼白状態になる．
⑧身体の下になった部分が暗赤色になることがある．
⑨尿量が減少して，時にはまったく出なくなる．
⑩呼吸は不規則になり，時には15秒くらい止まることもある．

(2) 実際に死が訪れたときの徴候
①呼吸が完全に止まる．胸や顎の動きがなくなる．
②心臓の動きが止まり，脈拍が触れなくなる．
③揺り動かしても，大声で呼んでもまったく反応がない．
④眼球が固定されて動かない．まぶたは開いていることも閉じていることもある．
⑤尿や便の失禁が見られることもある．
⑥手足は末梢の方から徐々に暗紫色に変わっていく．

出典／小笠原一夫：ホスピス・在宅ケア，3（1），p.12. より改変．

る．事実を受け止めていくまでの期間は，患者の気持ちを聞き続けるといった姿勢が必要である．また医師から受けた説明が，十分に理解できているかについて確認したり，補足の説明をすることもある．必要であれば，再度医師と患者との話し合いの場を設けるといった支援を行っていく．

悪い情報を伝えられた患者は，周囲の人との温かなコミュニケーションを必要とすることが多い．言語的なコミュニケーションだけでなく，非言語的なコミュニケーションも重要であり，「何かをする」ことだけでなく，「そばにいる」という姿勢でかかわる必要がある．

患者は徐々に真実を受け止められるようになると，今後の療養をどのようにしていくかについて意思決定する必要がある．多くの選択肢があるなかで，最もよい選択を自己決定していくことには困難を伴う．自らの意思決定が困難な場合には，何が阻害要因になっているかをアセスメントすることも看護の重要な役割であり，納得のできる自己決定を支援することが重要である．

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ORIGINAL PAPER

The effect of zoledronic acid and osteoprotegerin on growth of human lung cancer in the tibias of nude mice

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Abstract The pathogenesis of bone metastases may require the activation of osteoclasts by tumor-secreted factors, which promote important interactions with the bone microenvironment. We utilized an intratibial model of bone metastasis with bioluminescent imaging (BLI) to measure the effect of osteoclast inhibition on the interaction of human lung cancer cells with bone, and on tumor growth. Mice were injected with luciferase-transduced tumor cells (HARA, human pulmonary squamous carcinoma) and divided into three groups: (1) untreated, (2) twice weekly treatment with the bisphosphonate zoledronic acid (ZOL), or (3) osteoprotegerin (OPG). Histomorphometry and imaging were used to evaluate tumor burden, and parameters of osteoclast activity. Mice in the treated groups had increased bone density and decreased osteoclast numbers in nontumor-bearing tibiae. There was greater than 60% reduction in mean tumor volume in both treatment groups when evaluated by histomorphometry ($P = 0.06$ [OPG], $P = 0.07$ [ZOL]). However, bioluminescent imaging failed to show a reduction in tumor burden due to wide variability in the data. Osteoclast numbers along tumor-associated bone were significantly increased compared to tumor-free bone, and were not reduced by either treatment. Plasma calcium concentration was increased in all groups. Plasma tartrate-resistant acid phosphatase 5b was reduced in both treatment groups. Plasma PTHrP was significantly increased in the untreated tumor-bearing group, but was not significantly different in the two treatment groups compared to normal mice. OPG or ZOL did not change tumor cell proliferation, but ZOL increased HARA cell apoptosis. OPG and ZOL reduced tumor growth in the tibiae of treated mice, however, PTHrP production by HARA cells may have resulted in a high concentration in the bone microenvironment, partially overriding the antiosteoclast effects of both OPG and ZOL.

Keywords Bioluminescent imaging · Bisphosphonate · Bone metastasis · Histomorphometry · Hypercalcemia · Mouse models · Osteoclast · Osteoprotegerin · Parathyroid hormone-related protein · Zoledronic acid

Abbreviations

BLI	bioluminescent imaging
IP	intraperitoneal
Luc	luciferase
OPG	osteoprotegerin
PTHrP	parathyroid hormone-related protein
SCC	squamous cell carcinoma
SQ	subcutaneous
TAB	tumor-associated bone
TRAcP	tartrate-resistant acid phosphatase
YFP	yellow-fluorescent protein
ZOL	zoledronic acid

Introduction

Metastases to bone cause significant morbidity and contribute to mortality in cancer patients. Death in association

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with cancer is more commonly due to complications from metastases rather than from the primary tumor [1]. Deleterious effects of bone metastases include bone pain, pathologic fracture, spinal cord compression, and the development of hypercalcemia [2]. Tumor-associated hypercalcemia can result in nausea, vomiting, dehydration, alteration of mental status, and renal failure [3].

Certain types of cancers more commonly metastasize to bone. Tumors originating in the breast, prostate or lung result in 80% of all bone metastases. Other types of cancers, such as gastrointestinal cancer, very rarely spread to bone [4]. The propensity of a certain type of cancer to spread to bone strongly supports the "seed and soil" hypothesis of Paget, namely that certain types of malignant cells (the 'seeds') are attracted to, and grow preferentially in, specific 'soils' (such as the bone microenvironment) [5]. Once the cells have implanted themselves in the bone microenvironment, their continued survival and growth is supported by the so-called 'vicious cycle of bone destruction'. This cycle is characterized by the production of various osteoclast-activating factors by the tumor cells (parathyroid hormone related protein [PTHrP], interleukins-1 and 6, tumor necrosis factor, and others). The activation of osteoclasts results in destruction of bone, releasing bone-associated growth factors (such as transforming growth factor-β, bone morphogenetic proteins, platelet-derived growth factors, fibroblast growth factors, and others), which in turn stimulate the tumor cells to proliferate and secrete more of the osteoclast-stimulating factors [3]. The cycle results in both tumor growth and increased destruction of bone, which contributes to hypercalcemia. The osteoclast plays a central role in the vicious cycle and makes an attractive therapeutic target.

There are several different mechanisms for cancer-associated hypercalcemia, including ectopic secretion of parathyroid hormone by tumor cells, tumor cell secretion of the active form of vitamin D, local osteolytic hypercalcemia, and humoral hypercalcemia of malignancy (HHM) [6]. In humoral hypercalcemia of malignancy (HHM) there is production of humoral factors [e.g., PTHrP] by tumor cells (in the primary tumor and its metastases) and secretion of the factors into the systemic circulation. In the case of PTHrP, hypercalcemia results from the stimulation of PTH1 receptors in both the kidney (increasing calcium reabsorption), and in bone (increasing osteoclastic bone resorption). In local osteolytic hypercalcemia, a large tumor burden in bone is associated with localized osteoclast activation and bone resorption, leading to the release of calcium into the systemic circulation [7].

The human lung squamous carcinoma cell line (HARA) used in this study represents a subtype of non-small cell lung cancer (NSLC) which has a propensity to metastasize to bone and is commonly associated with the production of PTHrP [7]. The normal keratinocyte both produces and secretes PTHrP, thus it is not surprising that squamous cell carcinomas are commonly associated with production of this polyhormone [8]. PTHrP is a major contributor to osteolytic (bone destructive) metastases due to its ability to indirectly activate osteoclasts through the PTH1 receptor on osteoblasts and stromal cells. This pathway involves the increased expression of RANKL (or 'osteoclast differentiation factor') on the osteoblast or stromal cells, which binds RANK on osteoclast precursors, resulting in osteoclast differentiation/maturation and activation. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL, and binding of RANKL to OPG results in the failure of RANK activation, and inhibition of osteoclast differentiation and activation [9]. The resorption of bone by mature osteoclasts results in the release and activation of bone-associated growth factors such as transforming growth factor-beta (TGF-β), which can act in a paracrine action on nearby tumor cells to upregulate the production of PTHrP by increasing mRNA stability [10].

The importance of PTHrP to the development and progression of bone metastases has been demonstrated in several rodent models of bone metastasis, including the MDA-MB-231 breast cancer mouse model. Treatment of mice with a monoclonal anti-PTHrP antibody after intracardiac injection of MDA-MB-231 cells resulted in a reduction in the formation of bone metastases and decreased bone destruction [11]. Rabbani et al. [12] demonstrated that overexpression of PTHrP in rat MatLyLu prostate cancer cells resulted in increased osteoclast activity in skeletal metastases in an intracardiac injection model.

Several treatments for cancer-associated hypercalcemia and metastases are currently available, including bisphosphonates [13, 14]. Bisphosphonates are inorganic pyrophosphate analogues which, upon administration, rapidly accumulate in bone and result in high levels in the bone microenvironment. Bisphosphonates are internalized by osteoclasts, and result in decreased bone resorption due to apoptosis of mature osteoclasts, suppression of osteoclastogenesis, and decreased function of mature osteoclasts [15]. More recently it has been demonstrated that nitrogen-containing bisphosphonates can have direct effects on neoplastic cells, including the induction of apoptosis, inhibition of migration (by decreasing matrix metalloproteases), and reduction in proliferation [16, 17]. Zoledronic acid is a nitrogen-containing third generation bisphosphonate which has demonstrated clinical efficacy in treating bone metastases and hypercalcemia in several types of solid cancer (including breast and non-small cell lung carcinoma) [18, 19].

The evaluation of osteoprotegerin for treatment of bone metastases is less well developed. Administration of OPG is expected to result in a local reduction in the RANKL/OPG ratio and inhibition of osteoclastic bone resorption.

Studies in mouse models of metastases have demonstrated decreased tumor burden and less bone destruction with OPG treatment using both breast and prostate cancer cells lines [20, 21].

We investigated whether treatment with zoledronic acid or OPG would decrease tumor burden and select plasma parameters of tumor burden and bone resorption by inhibiting osteoclastic bone resorption and tumor growth in an intratibial injection model of bone metastasis of a human pulmonary squamous cell carcinoma (HARA).

Materials and methods

Cell line

HARA, a cell line derived from a human pulmonary squamous cell carcinoma, was obtained from Dr. Haruo Iguchi, Shikoku Cancer Center, Matsuyama, Japan. The original human patient had increased serum parathyroid hormone-related protein (PTHrP) concentration and hypercalcemia, but no apparent bone metastases. The cell line expresses and secretes PTHrP, and mice given intracardiac injections of HARA cells develop bone metastases [22]. Cells were maintained in RPMI 1640 (Invitrogen Co., Carlsbad, CA) supplemented with 10% fetal bovine serum, 250 units/ml penicillin, and 250 μg/ml streptomycin (Invitrogen Co.) in a humidified 37°C incubator with 5% CO_2.

Generation of lentiviral vectors and HARA transduction

A 2,183 bp Luciferase/Yellow Fluorescent Protein fusion construct flanked by *Sfu*I and *Xho*I was PCR amplified from pCDNA3.1(+).yLuc-YFP (obtained from Dr. Christopher Contag, Stanford University, Stanford, CA) and subcloned into pCR4TOPO (Invitrogen Co., Carlsbad, CA), excised, and gel purified. The Luc/YFP construct was cloned into a retroviral vector, pHIVSIN-Luc (obtained from Dr. Kathleen Boris-Lawrie, The Ohio State University, Columbus, OH), replacing a 1,491 bp Luc fragment in the plasmid. The new plasmid was named pHIVSIN-Luc/YFP. Lentiviral vectors were produced by transient triple transfection of 293T cells (obtained from Dr. Michael Lairmore, The Ohio State University, Columbus, OH) in DMEM (Invitrogen Co., Carlsbad, CA) with 10% FBS using calcium phosphate (Sigma–Aldrich Co., St. Louis, MO) and 10 μg of packaging plasmid pCMVΔR8.2, 2 μg of envelope plasmid pMD.G (both obtained from Dr. Kathleen Boris-Lawrie) and pHIVSIN-Luc/YFP. The vector particles in the supernatant were filtered through a 0.2 μm filter and sedimented by ultracentrifugation at 6,500×*g* for 16 h at 4°C. The pellets were redissolved in DMEM resulting in a 1,000-fold concentration. HARA cells (in RPMI with 10% FBS) were transduced with polybrene (8 μg/ml) (Sigma–Aldrich Co., St. Louis, MO) and the virus by spin-inoculation at 2,700 rpm for 1 h at 32°C (HARA-YFP/Luc).

Mice, tumor inoculation

Male nu/nu mice were purchased from the Charles River Laboratories (Wilmington, MA). Mice were housed in microisolator cages, and were provided food pellets and water ad libitum. Mice were anesthetized with 3.5 mg ketamine/0.15 mg xylazine intraperitoneally (IP), and 50,000 HARA-YFP/Luc cells were suspended in 10 μl sterile Dulbeccos's PBS and injected through the skin into the proximal left tibia using the tibial crest as a landmark. Injections were performed using a 25-gauge needle and a 100 μl Hamilton syringe (Hamilton Co., Reno, NV). Mice were imaged on day one post-injection, then weekly for 4 weeks.

Treatments

Mice were divided into three groups of 20 mice each. One group was injected with HARA cells and did not receive osteoprotegerin or zoledronic acid. The other two groups of mice were treated twice weekly for 4 weeks with recombinant human Fc-osteoprotegerin, 50 μg/mouse, IP (Amgen, Thousand Oaks, CA) or zoledronic acid, 0.625 μg/mouse, subcutaneously (SQ) (Novartis, Basel, Switzerland). The first dose was administered the day before tumor cell injection. Twice weekly injections of vehicle in untreated mice were not performed. Weekly bioluminescent imaging (involving intraperitoneal injections of luciferin and anesthesia with box induction; details below) was performed on all mice and equalized the levels of stress experienced by each group.

Imaging

In vivo bioluminescent imaging was performed on a cooled CCD IVIS™ system equipped with a 50 mm lens (Xenogen Corp., Alameda, CA). Results were analyzed using LivingImage® software, version 2.2 (Xenogen Corp.). Mice received IP injections of D-luciferin (Xenogen Corp.), 4.3 mg/mouse, dissolved in sterile PBS. The exact time of luciferin injection was recorded. Mice were anesthetized with 3% isoflurane/induction in a box system and 1.5% isoflurane/maintenance via a nose cone delivery system. Oxygen was delivered at 1L/min. Body temperature was maintained throughout imaging using a 37°C platform in the chamber. Lateral images of five mice were acquired simultaneously at several set time points extending to a maximum of 20 min after luciferin injection (Fig. 1). Integration times and binning varied depending on the saturation level of the group of mice being imaged. Times ranged from 10 s to 1 min, binning was small (4) or medium (8), and the f/stop used was 1. Field of view (FOV) was maintained at position C (20 cm). Pseudo-color images of photon emissions were overlaid on grayscale images of animals to aid in fixing spatial distribution of signals. Photon quantitations were calculated within regions of interest (ROIs) overlying the left tibias, as well as a background determination from a signal-free region of the mouse. Tumor burden at day 30 was determined by dividing photons/s emission at day 30 by that from day 1. This allowed normalization of data for the actual numbers of cells initially injected.

Histopathology

Mice were sacrificed at 30 days post-injection, blood was collected and plasma was separated using heparinized Microtainer® tubes (Becton Dickinson, Franklin Lakes, NJ) and stored at −80°C. Legs were separated at the coxofemoral joint, defleshed and fixed in 10% neutral-buffered formalin for 24 h at 4°C. Legs were decalcified in 10% EDTA, pH 7.4 at 4°C for 2–3 weeks. Decalcified tibias and lungs were embedded in paraffin, cut into 5 μm sections, mounted on slides, and stained with hematoxylin and eosin. Freshly cut sections of the tibias were also stained for osteoclasts utilizing tartrate-resistant acid phosphatase enzyme histochemistry as described by van der Pluijm et al. [23]. Briefly, 5 μm paraffin sections were rehydrated and incubated with substrate solution for 30 min containing naphthol AS-BI phosphate as substrate dissolved in Michelis veronal acetate buffer, hexazonium pararosaniline as a coupler, and 10 mM of L(+)tartaric acid as an inhibitor (Sigma–Aldrich Co., St. Louis, MO). Slides were counterstained with hematoxylin. Histomorphometry was performed using the Bioquant Nova image analysis system (Bioquant Image Analysis Corp., Nashville, TN). Trabecular area of metaphyseal bone was measured in one 200 mm^2 window in each tibia. Osteoclast parameters (perimeter and number) were measured in two windows in each tibia (400 mm^2 total). Tumor area was measured in four sections from each tibia. Osteoclast numbers and perimeter were measured along tumor-associated bone by evaluating one section from each tibia.

Plasma calcium concentration

Calcium concentration was measured using 10 μl of plasma and a colorimetric assay performed on a dry chemistry analyzer (VITROS DT60; Ortho-Clinical Diagnostics, Rochester, NY). Ten nontumor-bearing age- and sex-matched nude mice were used as controls for normal plasma calcium concentrations.

Plasma PTHrP concentration

Plasma PTHrP (1–86) concentration was measured using a two-site immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX). Radioactivity was measured using a gamma scintillation counter for 1 min. Results were calculated using a log–log curve fit, then converted to pmol/L by multiplying pg/ml by 0.101. Four nontumor-bearing age- and sex-matched nude mice were used as controls for normal plasma PTHrP concentrations.

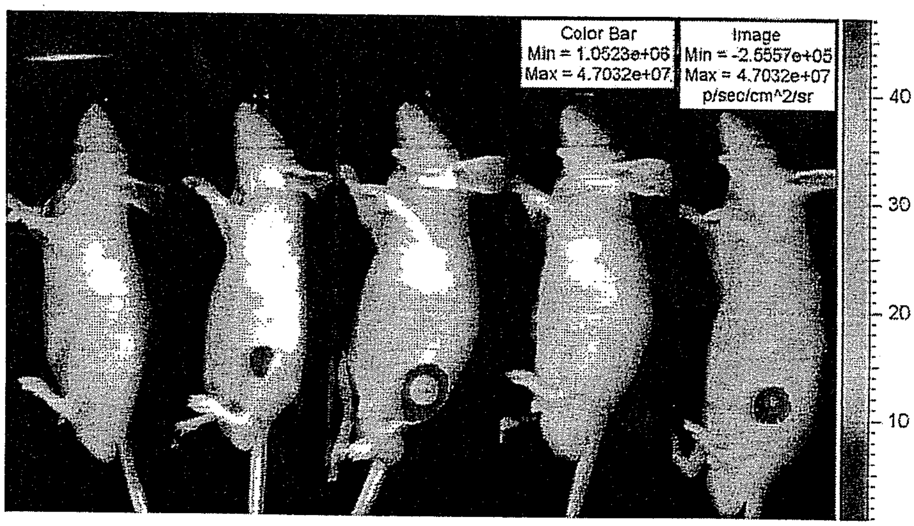


Fig. 1 Bioluminescent imaging of mice from the osteoprotegerin-treated group. Thirty days post-HARA injection, after intraperitoneal injection of luciferin. A region of interest was used to analyze the photons emitted/second from a specified area. The highest level of photon emission corresponded to the orange/red end of the color spectrum. The fourth mouse was injected with cells; photon emission was present but very low. Note the wide variability in tumor growth in the mice

Plasma TRAcP 5b activity

Tartrate-resistant acid phosphatase 5b activity specific to mouse osteoclasts was measured using a solid phase immunofixed-enzyme activity assay (MouseTRAP Assay) (Immunodiagnostic Systems, Inc., Fountain Hills, AZ). Absorbencies were measured at 405 nm wavelength on an UVmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

Ki67 Immunohistochemistry

Ki-67 immunohistochemistry of tumors was performed at The Ohio State University Hospitals, Department of Surgical Pathology. Three 400× fields were evaluated for each tumor. Proliferation rate was estimated by dividing the number of positive tumor cells by the number of total tumor cells counted in the three fields. The average number of cancer cells counted per mouse was 1,100.

TUNEL Staining

Apoptosis was detected utilizing the TdT-FragEL™ DNA fragmentation detection kit (Calbiochem, San Diego, CA). The staining procedures were performed based on the manufacturers' recommendations. Briefly, after deparaffinization, rehydration and washing in 1× TBS, tissues were digested with proteinase K (20 μg/ml) for 20 min at room temperature and washed. Endogenous peroxidases were blocked with 3% H_2O_2 and slides were incubated with equilibration buffer. Sections were treated with terminal deoxynucleotidyltransferase (TdT) enzyme for 90 min in a humidified chamber at 37°C. After histochemical staining with peroxidase streptavidin and diaminobenzidine, slides were counterstained with methyl green, dehydrated and cover slipped. Control slides were included with the kit and consisted of HL-60 cells incubated with 0.5 μg/ml actinomycin D for 19 h to induce apoptosis and uninduced HL-60 cells. A negative control was generated by omitting TdT. For each section evaluated, the total area of tumor (μm^2) was calculated using BioQuant Nova image analysis system (Bioquant Image Analysis Corp., Nashville, TN). The total number of apoptotic neoplastic cells was counted for each section and results were expressed as the number of apoptotic cells per area of tumor.

Exclusion of data

Mice were removed from all analyses if it was determined by histology that the initial injection of cells was not directly into the tibia (most commonly into surrounding muscle). This resulted in the removal of one mouse from the untreated group, two mice from the OPG-treated group, and three mice from the zoledronic acid-treated group.

Statistical analysis

Statistical analysis of data was performed using Sigmastat 3.2 (Systat Software, Inc., Richmond, CA). A one-way ANOVA was used to compare normally distributed data. A Kruskal–Wallis one-way analysis of variance on ranks was used to compare data that was not normally distributed. Secondary tests included Dunn's and Holm-Sidak Methods. Incidence of lung metastases were analyzed by Chi Square Independent testing and Fisher's Exact Test.

Results

Trabecular area and osteoclast measurements of non-injected (right) tibia (Fig. 2)

Evaluation of the non-injected (right) leg was used to confirm the biological effect of drug administration. Trabecular area of metaphyseal bone was significantly increased in both treatment groups when compared to untreated mice (Fig. 3a). Osteoclast perimeter (the percentage of metaphyseal trabecular perimeter lined by osteoclasts) and osteoclast number (per mm of trabecular bone) were significantly decreased in both treatment groups when compared to untreated mice (Fig. 3b, c). The significant increase in trabecular area (due to continued bone growth but decreased osteoclastic bone resorption), and decrease in osteoclast measurements in metaphyseal bone were expected after treatment with antiosteoclast agents. These results indicated that the dose and route of administration of zoledronic acid and OPG were sufficient to result in the appropriate physiologic effect in normal metaphyseal bone.

Tumor burden

The mean tumor areas in the OPG and zoledronic acid-treated groups were reduced by 62% and 67%, respectively ($P = 0.06$ for OPG, $P = 0.07$ for zoledronic acid), when evaluated by histomorphometry (Fig. 4a). The data did not fit a normal distribution. The mean tumor volumes between groups as evaluated by bioluminescent IVIS imaging were reduced by 14% (OPG) and 43.4% (zoledronic acid) (Fig. 4b). The data did not fit a normal distribution and there was no significant difference from controls ($P = 0.60$).

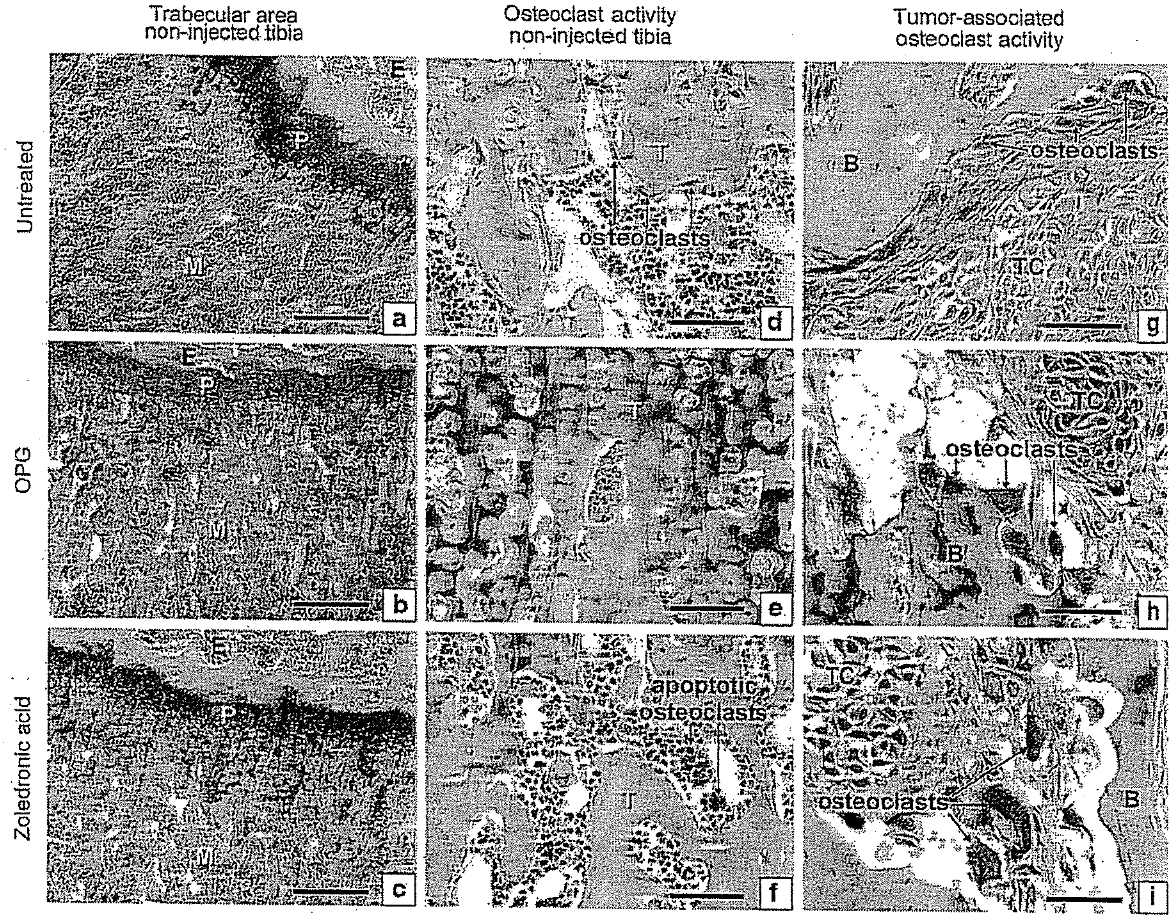


Fig. 2 Representative tibias from non-injected and injected mice. Panels **a–c** (non-injected leg) illustrate the marked increase in trabecular area in the osteoprotegerin (OPG) (**b**) and zoledronic acid (**c**)-treated mice when compared to the untreated group. Panels **d–f** (non-injected leg) illustrate the marked decrease in osteoclast perimeter and number in the OPG (e) and zoledronic acid (**f**)-treated mice when compared to the untreated group. Panels **g–i** (injected leg) illustrate the increased number of osteoclasts along tumor-associated bone in all three groups. Panels **a–c**: magnification = 100×, Bar = 300 μm. Panels **d–i**: magnification = 400×, Bar = 75 μm. All sections stained for tartrate-resistant acid phosphatase with hematoxylin counterstain. (P = physis; E = epiphysis; M = metaphysis; T = trabeculae; B = bone; TC = tumor cells)

Osteoclast measurements along tumor-associated bone (Fig. 2)

Osteoclast activity was significantly increased along tumor-associated bone (TAB) when compared to trabecular bone in non-injected legs in all groups. There was no significant difference in osteoclast perimeter (Fig. 5a) or numbers (Fig. 5b) along TAB between the untreated group and either of the treatment groups.

Incidence of tumor growth in lung

One mouse of nineteen in the untreated group, one mouse of seventeen in the zoledronic acid-treated group, and four mice of eighteen in the OPG-treated group developed small foci of tumor growth in the lung. The incidence of tumor growth in the lung did not differ significantly between groups (Chi Square Independent testing ($P = 0.17$) and Fisher's Exact Test ($P = 0.30$)).

Plasma calcium concentration

Plasma calcium concentrations were significantly increased in all three groups ($P < 0.05$) when compared to normal nontumor-bearing mice, however there was no significant difference in plasma calcium between the three tumor-bearing groups (Fig. 6a).

Plasma PTHrP concentration

Plasma PTHrP concentration was significantly increased in the tumor-bearing untreated mice compared to normal

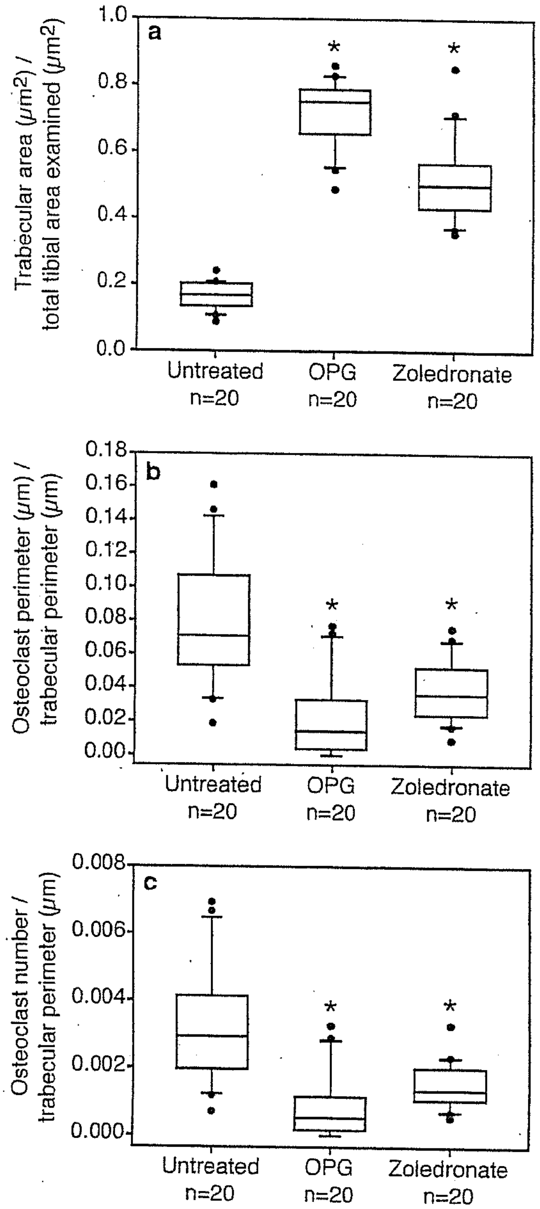


Fig. 3 Trabecular area and osteoclast measurements, non-injected tibias. Trabecular area (**a**) was significantly increased in both treatment groups when compared to untreated mice (*P < 0.001). The area was calculated from a 200 μm^2 field in the metaphysis. Osteoclast perimeter (**b**) and number (**c**) were significantly decreased in both treatment groups when compared to untreated mice (*P < 0.001 for both). Osteoclast measurements were calculated from two 200 μm^2 fields in the metaphysis. (**a–c**) Boxes represent quartiles and the whiskers represent the 10th and 90th percentiles. Outliers are represented as separate points. OPG = osteoprotegerin. Zoledronate = zoledronic acid

nontumor-bearing mice (Fig. 6b). This is likely due to production and secretion of PTHrP by the HARA cells in vivo. Plasma PTHrP in the zoledronic acid-treated group was significantly decreased compared to the tumor-bearing untreated mice. Plasma PTHrP levels in the OPG-treated mice were not significantly different from tumor-bearing untreated mice. Zoledronic acid appeared to have a greater effect on reduction in plasma PTHrP levels than OPG (Fig. 6b).

Plasma TRAcP 5b

Plasma TRAcP 5b is present in high levels in osteoclasts, is secreted into the systemic circulation, and is a specific, sensitive marker of bone resorption [24]. Inhibition of osteoclasts is expected to result in decreased plasma TRAcP 5b concentration. Plasma TRAcP 5b was significantly decreased in both treatment groups when compared to the untreated group (Fig. 6c). This was interpreted to reflect an overall net decrease in osteoclast activity throughout the mouse in response to both treatments. The increased osteoclast numbers along tumor-associated bone were felt to represent a local phenomenon, which did not contribute appreciably to increasing circulating TRAcP 5b levels.

Ki67 immunohistochemistry

The tumor cells in all three groups had a high level of immunostaining for Ki67, a marker of cell proliferation. Positive staining of HARA cells in the untreated group was 69 ± 2% (mean ± SEM) (n = 11), in the OPG group was 72 ± 4% (mean ± SEM) (n = 7), and in the zoledronic acid group was 62 ± 4% (mean ± SEM) (n = 8). Treatment with OPG (P = 0.233) or zoledronic acid (P = 0.097) did not statistically significantly alter Ki67 immunostaining as compared to the untreated group.

TUNEL assay

The rate of apoptosis of the HARA cells in the untreated group was 1.4×10^{-2} cells/mm^2 (n = 10), in the OPG group was 1.0×10^{-2} cells/mm^2 (n = 6) and in the zoledronic acid group was 3.5×10^{-2} cells/mm^2 (n = 5). Although OPG did not induce apoptosis, mice treated with ZOL had a modest increase in tumor cell apoptosis (P = 0.068).

Discussion

The marked propensity of some types of cancer to metastasize specifically to bone and the significant contributions to cancer-associated morbidity and mortality that bone metastases make has resulted in an intense search for methods to prevent and treat this manifestation of cancer. The bone pain, pathologic fractures, and hypercalcemia that can be associated with bone metastases have been

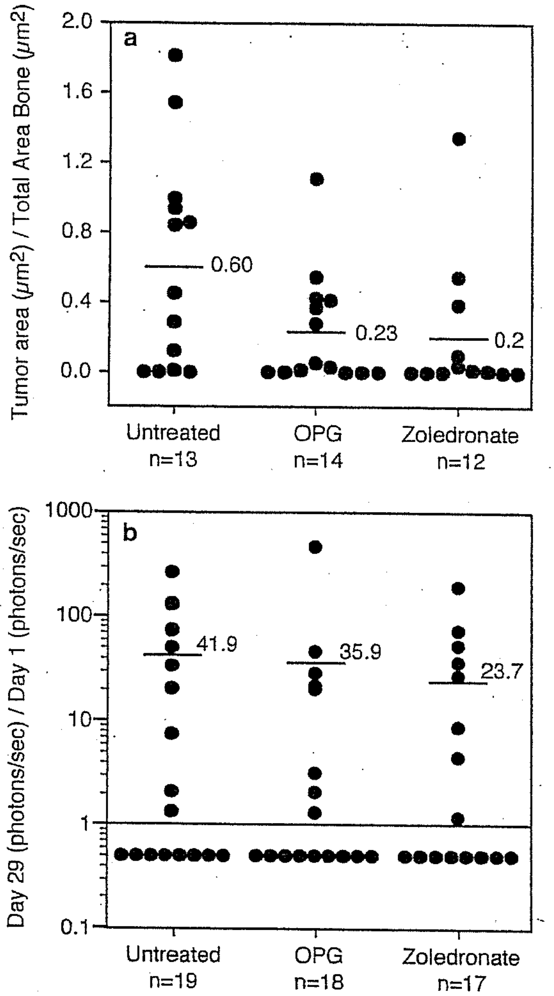


Fig. 4 Tumor burden. (**a**) Histomorphometrical analysis. There was a marked reduction in the mean tumor burden by 62% (OPG) and 67% (zoledronic acid) in treated mice compared to the untreated group; variability in the data precluded attaining statistical significance. The horizontal bars and adjacent numbers represent group means. Values represent the percent of tibial area occupied by tumor. Four sections from each mouse were analyzed. (**b**) In vivo bioluminescent imaging analysis. There was a reduction in the mean tumor burden by 14% (OPG) and 43% (zoledronic acid) compared to the untreated group; variability in the data precluded attaining statistical significance. Variability was greater than the analysis of tumor burden by histomorphometry. Data represents a ratio of photon/second emission on day 29 to that on day 1 to control for the actual number of cells injected into each tibia. Data points < 1 are not represented in calculated values. The horizontal bars and adjacent numbers represent group means. OPG = osteoprotegerin. Zoledronate = zoledronic acid

treated with varying success by using chemotherapy, hormonal therapy, irradiation, surgery, and more recently by agents such as bisphosphonates, which have antiosteoclast and antitumor activity [3].

Bisphosphonates are currently used in the treatment of established bone metastases. Trials with zoledronic acid have demonstrated a delay in skeletal events in patients with breast cancer metastatic to bone [25] and non-small cell lung carcinoma [19], and zoledronic acid has proven benefit in prostate cancer [26].

The use of osteoprotegerin (OPG) for the treatment of bone metastases is less well-established. Animal models have demonstrated that treatment with OPG reduces the growth of both prostate carcinoma and breast cancer in bone [20, 21, 27]. A human trial using OPG has been published (in patients with osteolytic breast carcinoma or multiple myeloma). OPG treatment resulted in suppression of bone resorption as measured by urinary *N*-telopeptide of collagen/creatinine and was well tolerated [28].

Metastasis to bone involves numerous steps starting with the initial detachment of neoplastic cells from the primary tumor mass, invasion through surrounding connective tissue to blood vessels, travel through the blood with evasion of immune detection, and arrest and growth in the secondary site [29]. The intratibial model of bone metastasis represents the final step of the metastatic cascade, namely the survival and proliferation of neoplastic cells in the bone microenvironment. In our intratibial injection model, preventive treatment (treatment started prior to tumor cell injection) with zoledronic acid or osteoprotegerin showed a marked reduction of tumor burden in mice when analyzed by histomorphometry. Analysis of tumor burden using in vivo bioluminescent imaging resulted in a higher degree of variability in the data and was less sensitive than histomorphometry, since the amount of bioluminescence did not always correlate to tumor size as visualized grossly and measured using histopathological sections. Standard histomorphometry, with evaluation of four sections from each tumor-bearing tibia, was interpreted to have resulted in a more accurate quantification of tumor burden.

Others have demonstrated that in vivo bioluminescent imaging (BLI) can be a useful technique to estimate tumor burden [30, 31]. The advantages of such a system include the ability to serially analyze response to treatment and tumor growth without euthanasia of animals, the use of fewer animals in each experiment, and the ability to analyze the volume of viable tumor tissue since light emission from the entire tumor is measured, compared to the traditional two-dimensional area measurements used by standard histomorphometry. However, there are challenges with quantitation of viable tumor tissue by BLI as demonstrated by our study. In this investigation, and using this transduced cell line, histomorphometry was more accurate than BLI at measuring tumor volume and emphasizes the need to confirm BLI data with morphometry. This was likely due to the unexpected wide variability in the BLI data. We controlled for variable injection volumes by dividing day 30 data by that from day 1. BLI is a very sensitive technique that is useful to

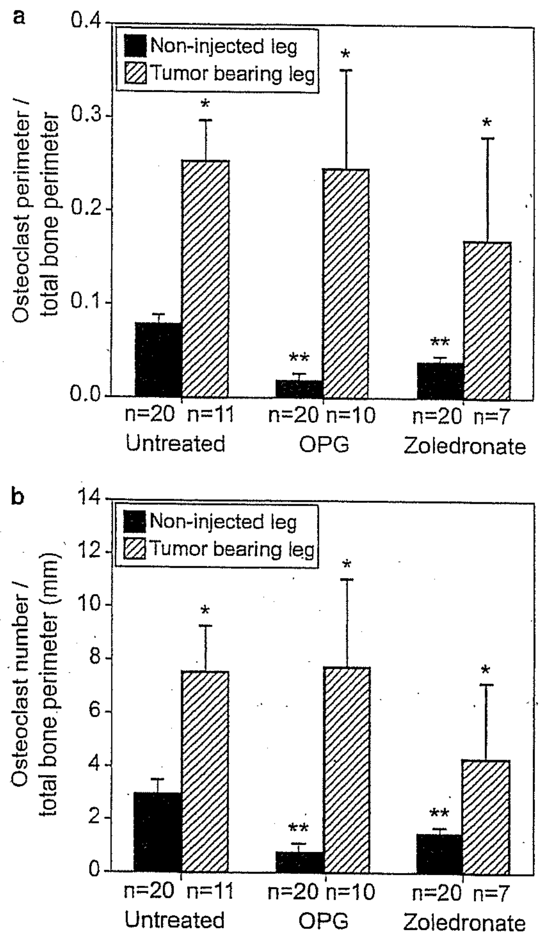


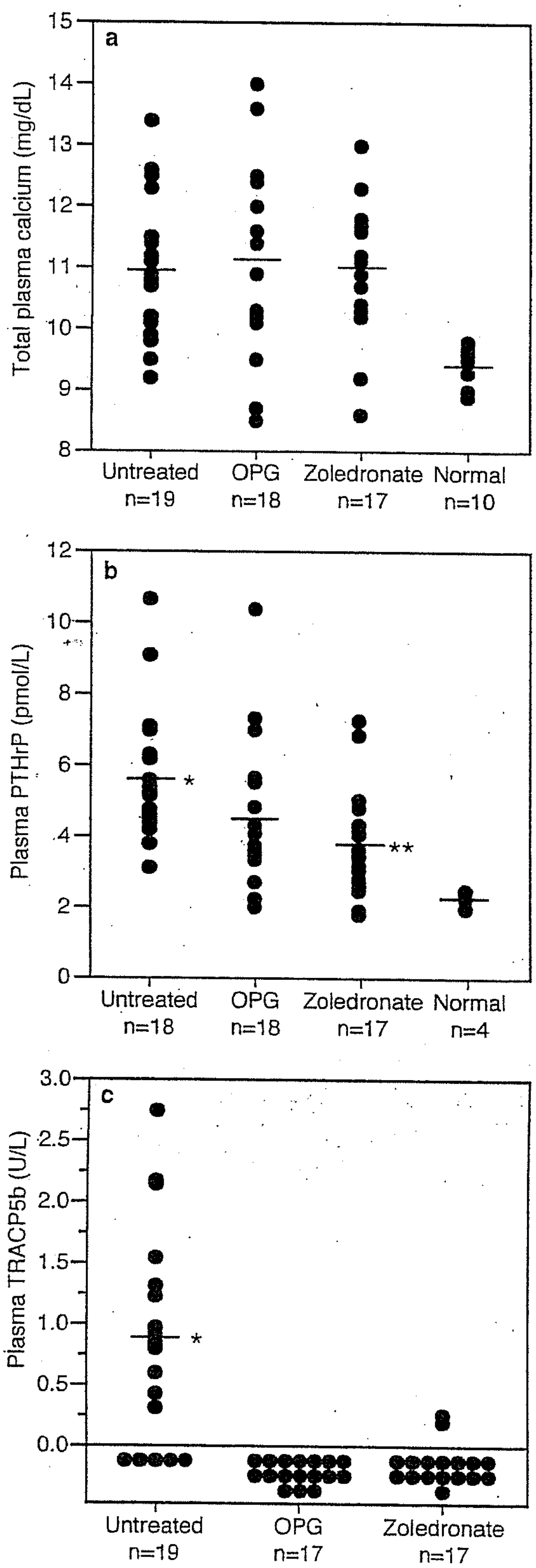
Fig. 5 Osteoclast measurements along tumor-associated bone. Osteoclast perimeter (**a**) and number (**b**) along tumor-associated bone were significantly increased compared to the non-injected (tumor-free) leg (*$P < 0.05$), and treatment with either osteoprotegerin (OPG) or zoledronic acid (zoledronate) did not significantly reduce osteoclast measurements along tumor-associated bone. Treatments significantly decreased osteoclast perimeter (**a**) and number (**b**) in the non-injected (tumor-free) legs (**$P < 0.001$). The bars represent the mean of each group, with the standard error mean indicated by the whisker.

identify small numbers of tumor cells at metastatic sites; however, variability in tumor activity or expression of luciferase can lead to a wide variation in the in vivo data. To obtain an accurate representation of tumor volume using BLI, the neoplastic cells must be actively expressing luciferase at the time of imaging. Loss of expression of the construct (in all or subsets of the tumor cells), areas of necrosis within the tumor, or variable luciferase expression by tumor cells would result in inaccurate representation of tumor volume. While the tumors in our model did not have large areas of necrosis, it is possible that loss or variable expression of the luciferase construct contributed to the variability in tumor burden when measured by this method.

The HARA human pulmonary squamous cell carcinoma line produces and secretes high levels of the potent osteoclast-activating factor, parathyroid hormone-related protein (PTHrP) [22]. We hypothesize that production of PTHrP by the tumor cells resulted in a high localized concentration of PTHrP in the bone microenvironment, at least partially overriding the antiosteoclast effect of either OPG or zoledronic acid. The production of PTHrP would explain not only the increased osteoclasts along tumor-associated bone (TAB) in the untreated animals, but also the lack of reduction in osteoclast perimeter and number along TAB in both treatment groups. We have recently reported that PTHrP mRNA was increased in HARA cells up to 20-fold in bone metastases in mice compared to subcutaneous tumors (Deng et al. (2006) Cancer Res in review). In contrast, the nontumor bearing leg in treated mice showed the expected response to OPG or zoledronic acid treatment (reduction in osteoclast measurements and increase in trabecular area).

In other models of bone metastasis using PTHrP-producing cell lines (PC-3, MDA-231, LNCaP) and treatment with OPG or zoledronic acid, investigators reported significantly reduced tumor burden and osteoclasts along TAB, however doses, routes of administration (subcutaneous versus intravenous), initiation of treatment (preventive versus treatment mode), and cell type (breast versus lung versus prostate) varied, making it difficult to directly compare results from other studies [20, 21, 32]. For OPG, doses ranged from 2 mg/kg to 25 mg/kg and treatment frequency from twice a week to three times a week. Although the dose and frequency of administration of OPG used in this experiment was at the lower end of those used in other experiments, the marked reduction in osteoclast number and perimeter in the non-injected tibiae in our model demonstrated that levels of OPG were high enough to have a physiologic effect in normal tibias. It is possible that the antiosteoclast effect was not attained in the injected tibias as a result of tumor-induced changes, such as localized disruption of blood flow or high local concentrations of osteoclast-stimulating factors, such as PTHrP. The dose of zoledronic acid used in this experiment (0.025 mg/kg) was lower than that used in other animal ▸

Fig. 6 Plasma parameters. (**a**) Plasma calcium concentration was significantly increased in all three treatment groups compared to normal mice ($P < 0.05$). Treatment with either drug did not significantly decrease plasma calcium concentration. (**b**) Plasma PTHrP was significantly increased above the nontumor-bearing mice ("normal") in the untreated group (*$P < 0.05$). Plasma PTHrP in the zoledronic acid group (but not the OPG group) was significantly decreased below that of the untreated group (**$P < 0.05$). (c) Plasma TRAcP 5b was significantly decreased (*$P < 0.05$) in both treatment groups when compared to the untreated mice. The limit of detection for this assay was 0.1 U/L. All horizontal bars indicate the group means. OPG = osteoprotegerin. Zoledronate = zoledronic acid



models of bone metastasis. For example, in an intratibial prostate carcinoma model, Corey et al. [32] used 0.2 mg/kg. Based on allometric scaling, which takes into account inherent differences in metabolic activity between different species, our dose is more than 10× lower than that typically used clinically in humans [33]. However, in our experiments, zoledronic acid was administered twice weekly, which is more frequent than the current clinical use in humans (where it is typically administered as a 4 mg intravenous infusion once every 3–4 weeks) [34]. Despite the dose used, the decreased numbers of osteoclasts and increased trabecular bone observed in the nontumor-bearing tibial metaphyses indicated a physiologic effect in normal bone. As with OPG, it is possible that disruption of the normal blood flow to the metaphyses in the tumor-bearing legs (due to the presence of the tumor) affected the ability of the drug to reach this region, or that increased local levels of a pro-osteoclast agent, such as PTHrP, antagonized the effects of the zoledronic acid. We would predict that using higher doses of either drug, or inhibiting PTHrP production by the HARA cells, would result in a greater reduction in tumor burden and a reduction in osteoclastic bone resorption along tumor-associated bone.

Bisphosphonates are the treatment of choice for cancer-induced hypercalcemia due to their antiosteoclast activity [35]. OPG has been used to reduce cancer-associated hypercalcemia in several mouse models [36, 37]. In our study, mice in all three groups had significant increases in plasma calcium compared to normal mice, with no reduction in plasma calcium concentration by treatment with zoledronic acid or OPG. We hypothesize that OPG and zoledronic acid were unable to block the effects of locally high levels of PTHrP in the immediate vicinity of the tumor leading to osteoclast activation along TAB and hypercalcemia in the mice. The untreated mice also likely had a humoral contribution from increased plasma concentrations of PTHrP.

Nitrogen-containing bisphosphonates have been reported to decrease the proliferation rate and increase apoptosis of cancer cells [38, 39]. In contrast, PTHrP has been reported to increase the proliferation rate of cancer cells [40], and to protect against apoptosis in breast cancer cells [41]. Osteoprotegerin has been reported to be an anti-apoptotic survival factor through its binding of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [42, 43], as well as to have no effect on apoptosis in vitro [21]. The HARA cells in the zoledronic acid-treated group had a mild increase in apoptosis. Increased PTHrP in the bone microenvironment due to secretion from HARA cells may have interfered with the ability of zoledronic acid and OPG to significantly alter the proliferation rate and level of apoptosis of the HARA cells.

In vitro studies that have demonstrated antiproliferative and pro-apoptotic effects of bisphosphonates used high concentrations of the drugs, ranging from 5 to 2,000 μM [17]. Bisphosphonates concentrate in bone by avid binding to hydroxyapatite, which likely leads to high concentrations of the drugs in the bone microenvironment [34]. However, bisphosphonates are not metabolized and are cleared rapidly from the plasma [15]; peak plasma levels after intravenous infusion of zoledronic acid are reported to be in the 1–2 μM range, far lower than that used in in vitro experiments [J. Green, personal communication]. It is uncertain whether the high concentrations used in cytostatic in vitro studies accurately reflect what occurs in the bone microenvironment in the in vivo setting [34]. Osteoprotegerin has not been shown to have a cytostatic effect on cancer cells in vitro [21]. In our study, the similar reduction in tumor burden using both zoledronic acid and osteoprotegerin supports the concept that the effects on tumor burden were indirect. In other words, the reduction was likely related to the antiosteoclast effect of each drug, rather than a cytostatic effect.

The prevention of bone metastases by bisphosphonates is less well established than their use for the treatment of bone metastases. Human clinical trials and animal models have had mixed results. Two human clinical trials using clodronate for the prevention of skeletal metastases in patients with early stage-breast cancer had contrasting results. One trial had a survival benefit, and one trial had decreased survival. Trials using the more potent zoledronic acid in a preventive manner are planned [34]. Animal models of bone metastasis prevention using bisphosphonates have also yielded contrasting results as well as interesting information on the role of bisphosphonates in the development of soft tissue metastases. In such animal models some studies demonstrated a decrease in bone metastases but an increase in soft tissue metastases [44]. Other studies have demonstrated a decrease in bone metastases and no effect on soft tissue metastases [45]. While others demonstrated a decrease in soft tissue metastases [46]. The variation in results likely reflects, at least partially, the administration of different bisphosphonates by different routes and doses, and the use of different tumor cell types and routes of tumor cell inoculation in these models. The variable results with regard to the effect of bisphosphonates on soft tissue tumor growth and metastasis make the use of such drugs in patients without clinical evidence of bone metastases (in a 'preventive' capacity) potentially riskier [34, 47]. In our intratibial injection model of lung cancer preventive treatment with zoledronic acid demonstrated no difference in the growth of tumor cells in the lungs in spite of a reduction in tibial tumor burden, but lung metastasis was an infrequent finding in this investigation.

The effect of OPG treatment on the development of visceral metastases has not been investigated as thoroughly as it has been for bisphosphonates. Morony et al. [20] demonstrated that administration of OPG at the time of tumor cell injection had no effect on the development of visceral metastases, and Zhang et al. [21] found that OPG had no effect on subcutaneous growth of prostate cancer when administered at the time of tumor cell injection. Despite the lack of an effect on the growth of neoplastic cells in soft tissue, both experiments demonstrated a marked decrease in skeletal growth of neoplastic cells with OPG treatment. Similarly, in our intratibial injection model of lung cancer, the use of OPG in a preventive manner did not result in any difference in the growth of metastatic tumor cells in the lung, despite reducing tumor burden in injected tibias.

In conclusion, we utilized an intratibial model of a luciferase-expressing human pulmonary squamous cell carcinoma line (HARA) to investigate the effects of treatment with the antiosteoclast agents zoledronic acid and osteoprotegerin. There was a marked reduction in tumor burden with both treatments when analyzed by histomorphometry. The lack of a decrease in osteoclasts along tumor-associated bone was likely due to the production of high levels of PTHrP or other cytokines antagonizing the antiosteoclast effects of zoledronic acid or OPG that were evident on bone surfaces in the nontumor-bearing tibia. Further evaluation of this model using HARA cells with reduced PTHrP production could clarify the role of PTHrP in the bone microenvironment.

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