In Vivo Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Trigger Vγ9Vδ2 T-cell Antitumor Cytotoxicity through ICAM-1 Engagement

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Abstract

Purpose: Nitrogen-containing bisphosphonates (N-BP) such as zoledronate and risedronate exhibit antitumor effects. They block the activity of farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway, leading to intracellular accumulation of mevalonate metabolites (IPP/ApppI), which are recognized as tumor phosphoantigens by Vγ9Vδ2 T cells. However, mechanisms responsible for Vγ9Vδ2 T-cell recognition of N-BP–treated tumors producing IPP/ApppI remain unclear.

Experimental Design: The effects of N-BPs on Vγ9Vδ2 T-cell expansion and anticancer activity were evaluated in vitro and in animal models of human breast cancers. The modalities of recognition of breast tumors by Vγ9Vδ2 T cells in N-BP–treated animals were also examined.

Results: We found a strong correlation between Vγ9Vδ2 T-cell anticancer activity and intracellular accumulation of IPP/ApppI in risedronate-treated breast cancer cells in vitro. In addition, following risedronate treatment of immunodeficient mice bearing human breast tumors, human Vγ9Vδ2 T cells infiltrated and inhibited growth of tumors that produced high IPP/ApppI levels but not those expressing low IPP/ApppI levels. The combination of doxorubicin with a N-BP improved, however, Vγ9Vδ2 T-cell cytototoxicity against breast tumors expressing low IPP/ApppI levels. Moreover, Vγ9Vδ2 T-cell cytotoxicity in mice treated with risedronate or zoledronate did not only depend on IPP/ApppI accumulation in tumors but also on expression of tumor cell surface receptor intercellular adhesion molecule-1 (ICAM-1), which triggered the recognition of N-BP–treated breast cancer cells by Vγ9Vδ2 T cells in vivo.

Conclusion: These findings suggest that N-BPs can have an adjuvant role in cancer therapy by activating Vγ9Vδ2 T-cell cytotoxicity in patients with breast cancer that produces high IPP/ApppI levels after N-BP treatment. Clin Cancer Res; 18(22); 6249–59. ©2012 AACR.

Introduction

Bisphosphonates, especially nitrogen-containing bisphosphonates (N-BP), are widely used to treat bone loss disorders, such as postmenopausal osteoporosis and skeletal morbidity associated with malignant bone diseases, because they bind avidly to bone mineral and inhibit osteoclast-mediated bone resorption (1). N-BPs also inhibit angiogenesis (2). N-BPs also inhibit angiogenesis (3). Moreover, the prenylation of small G proteins essential for cancer cell adhesion, migration, and invasion is impaired (2). N-BPs also inhibit angiogenesis in vivo (3). Moreover,

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Translational Relevance

Human γδ T cells straddle the border between innate and adaptive immunity and have potent antitumor activity. We recently provided in vivo evidence that bisphosphonate zoledronate induces accumulation of IPP/ApppI phosphoantigens in human breast tumors implanted in animals, and that human Vγ9Vδ2 T cells infiltrate and inhibit growth of these tumors producing high IPP/ApppI levels but not those expressing low IPP/ApppI levels. We found here similar findings using the bisphosphonate risedronate, and we showed that estrogen receptor (ER)-positive breast tumors are more likely to produce IPP/ApppI after bisphosphonate treatment compared with ER-negative breast tumors. Moreover, our study shows for the first time that the ability of risedronate and zoledronate to activate Vγ9Vδ2 T-cell anticancer activity not only depends on IPP/ApppI accumulation in ER-positive tumors but also on expression of tumor cell surface receptor intercellular adhesion molecule-1, which triggers the recognition of bisphosphonate-treated breast cancer cells by Vγ9Vδ2 T cells in vivo.

by blocking the release of bone-derived growth factors during osteoclast-mediated bone resorption, N-BPs can indirectly slow tumor cell proliferation and survival (2). Importantly, there is now clinical evidence that adding zoledronate to endocrine therapy improves disease-free survival and overall survival of patients with estrogen-responsive early breast cancer in a low estrogen environment (i.e., following ovarian suppression therapy or in women with established menopause at diagnosis; refs. 4–6). How zoledronate mediates this antitumor activity remains however elusive.

Emerging data suggest that N-BPs have immunomodulatory properties by stimulating the expansion of Vγ9Vδ2 T cells, a subset of human T cells that straddles the border between innate and adaptive immunity and exhibits anticancer activity (7). Evidence for the stimulation of Vγ9Vδ2 T cells by N-BPs was first found when increased numbers of γδ T cells were observed in patients who had flu-like acute-phase reactions after their first intravenous infusion of pamidronate (8). N-BPs are indeed internalized by peripheral blood mononuclear cells (PBMC), such as monocytes and dendritic cells, in which they inhibit the mevalonate pathway, leading to the intracellular accumulation of IPP, which in turn, activates Vγ9Vδ2 T cells and the release of inflammatory cytokines (TNF-α and IFN-γ), thereby contributing to the acute-phase reaction (7, 9, 10). In addition, ApppI could represent an inactive storage form of phosphoantigen, which would require conversion to IPP to activate Vγ9Vδ2 T cells from PBMCs (11).

N-BPs also induce intracellular accumulation of IPP/ApppI in a wide variety of human tumor cell lines in vitro, and these mevalonate metabolites can be sensed by Vγ9Vδ2 T cells as tumor phosphoantigens (2, 7). We recently provided in vivo evidence that zoledronate induces IPP/ApppI accumulation in human breast tumors implanted subcutaneously in animals and that human Vγ9Vδ2 T cells infiltrate and inhibit growth of these tumors producing high IPP/ApppI levels but not those expressing low IPP/ApppI levels (12). However, mechanisms responsible for Vγ9Vδ2 T-cell recognition of zoledronate-treated tumors producing IPP/ApppI remain unclear.

The modalities of recognition of N-BP–treated tumor cells by Vγ9Vδ2 T cells are likely γδ T-cell receptor (TCR)-dependent for IPP (7), but may also involve additional cell–cell contacts through unrelated TCR cell surface receptors expressed by Vγ9Vδ2 T cells, such as lymphocyte function-associated antigen-1 (LFA1), CD6, and natural killer group 2D (NKG2D; ref. 13). LFA1, CD6, and NKG2D interact with intercellular adhesion molecule-1 (ICAM-1), CD166, and MHC class I–related chains A/B (MICA/MICB), respectively, which are expressed by tumor cells (13). These cell surface receptors have been involved in mediating Vγ9Vδ2 T-cell cytotoxicity against zoledronate-treated tumor cells in vitro (13). However, there is no evidence of their engagement for recognition of zoledronate-treated tumors by Vγ9Vδ2 T cells in vivo. Another consideration is whether other N-BPs might exert in vitro immunomodulating effects similar to those we previously reported for zoledronate (12). This question is of great importance as different N-BPs (ibandronate, risedronate, alendronate, etc.) are used to prevent cancer treatment–induced bone loss in breast cancer, and clinical trials investigating their potential role in the adjuvant setting to prevent metastasis are ongoing (1, 14). In this study, we address whether risedronate can activate human Vγ9Vδ2 T cells in vitro and in animal models of breast cancer. In addition, we examine cell surface receptors through which Vγ9Vδ2 T cells could recognize human breast tumors from mice treated with risedronate or zoledronate.

Materials and Methods

Drugs

N-BPs risedronate, NE-58025, NE-58051, and NE-10790, and fluorescent risedronate were obtained from Procter & Gamble. Zoledronate was provided by Novartis Pharma AG. Recombinant interleukin-2 (IL-2) was provided by Novartis Pharmaceuticals Ltd. Sterile stock bisphosphonate solutions were prepared in PBS (pH 7.4; Invitrogen).

Human cells and cell culture

Blood donated from healthy volunteers or obtained from the Blood Transfusion Center (Etablissement Français du Sang, Beynost, France) was collected in heparin. Human PBMCs were isolated after Ficoll-Paque (Amersham Biosciences) density gradient centrifugation, as previously described (12). The Vγ9Vδ2 T cells were expanded by exposing PBMCs to 10-μmol/L risedronate plus 100-U IL-2 for 14 days. Vγ9Vδ2 T cells were purified by positive selection of TCR γδ cells using immunomagnetic cell sorting (12).
Human cell lines from breast cancer (T47D, MCF-7, BT-474, and ZR-75-1) and cervical carcinoma (Hela) were obtained from the American Type Culture Collection (ATCC)-LGC Promochem and used within 6 months. The human B02 breast cancer cell line, a subpopulation of MDA-MB-231, was prepared as previously described (15). All cell lines were authenticated using short tandem repeat analysis. ZR-75-1 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and penicillin/strepptomycin. BT-474 cells were cultured in Hybri-Care medium (ATCC) supplemented with 1.5 g/L NaHCO3, 10% (v/v) FBS, and penicillin/streptomycin. MCF-7 cells were cultured in Eagle’s minimal essential medium (EMEM) (ATCC) supplemented with 0.01 mg/mL bovine insulin (Invitrogen), 10% (v/v) FBS, and penicillin/streptomycin. Other cancer cell lines were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin.

**Cellular uptake assay for risedronate**

T47D, MCF-7, and B02 cells were seeded overnight to 10-cm Petri dishes at 3 × 10^6 cells per dish, then treated with fluorescent-labeled FAM-RIS for 1 hour. Cells were then rinsed with PBS, scraped, and extracted with acetonitrile and water. Extracts were separated by centrifugation (14,000 × g, 2 minutes). Precipitates were analyzed for total protein content by a modified Bradford procedure. The soluble acetonitrile/water extracts were measured at 445 nm and given free access to food and water. All procedures involving animals, including the method by which they were culled and experimental protocols were conducted in accordance with a code of practice established by the ethical committee of the University of Lyon (Lyon, France).

**Animals**

Five-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) immunocompromised mice were purchased from Charles River Laboratories. Animals were maintained in a 12-hour light–dark cycle and given free access to food and water. All procedures involving animals, including the method by which they were culled and experimental protocols were conducted in accordance with a code of practice established by the ethical committee of the University of Lyon (Lyon, France).

**In vitro and in vivo Vγ9Vδ2 T-cell expansion**

PBMCs were seeded in 24-well plates (1 × 10^6 cells/well), then treated with vehicle (PBS), or with bisphosphonates zoledronate, risedronate, NE-58025, NE-58051, and NE-10790 (10 μmol/L) ± IL-2 100 U/mL for 14 days. IL-2 was renewed at day 4 and then every 3 days. In vivo expansion of Vγ9Vδ2 T cells was conducted in NOD/SCID mice. Human PBMCs (3.5 × 10^7) were inoculated intraperitoneally and mice were treated with vehicle or risedronate (10, 30, 150, 300, or 600 μg/kg), intraperitoneally and 10,000-U IL-2. Mice then received IL-2 plus vehicle or risedronate every other days for 14 days. On day 14, mice were sacrificed and peritoneal cells collected for analysis of Vγ9Vδ2 T cells. Percentages of Vγ9Vδ2 T cells in PBMCs and peritoneal cells were determined by flow cytometry, as previously described (12). Human IFN-γ in the serum from mice was measured using a commercial ELISA kit (Biosource).

**Vγ9Vδ2 T-cell–mediated cytotoxicity assay**

Human T47D, MCF-7, BT-474, ZR75-1 (2 × 10^4 cells/well), and B02 (7,000 cells/well) breast cancer cells were incubated overnight, then treated for 1 hour with vehicle (PBS) or risedronate (1–25 μmol/L). Cell monolayers were then washed; 18 hours later, human cancer cells were cocultured with or without purified Vγ9Vδ2 T cells (cancer cell: Vγ9Vδ2 T-cell ratio was 1:12.5) for 4 or 24 hours. Viability was assessed by MTT assay (12).

**Subcutaneous growth of human breast tumors in animals and treatments**

Five-week-old female NOD/SCID mice were injected subcutaneously in the flank with 5 × 10^6 B02 or T47D cells in 100-μL PBS. For the estrogen receptor (ER)–positive T47D cells, host mice were implanted with subcutaneous 60-day release pellets containing 1.7-mg 17β-estradiol (Innovative Research of America) 4 days before tumor cell inoculation. Four weeks later, when B02 and T47D tumors had reached a volume of 50 mm³, mice were randomly assigned to 4 treatment groups (n = 6–7 mice/group): placebo (PBS); risedronate at 150 μg/kg body weight; human PBMCs (3.5 × 10^7) injected intraperitoneally plus 10,000-U IL-2 administered alone or with 150-μg/kg risedronate. In the relevant groups, IL-2 and risedronate in 0.5-μL PBS were administered intraperitoneally every other days for 14 days. Tumor size was calculated by external measurement of the width (m_1) and length (m_2) of subcutaneous tumor xenografts using a Vernier caliper.

**IPP/Apppl analysis**

Bisphosphonate-induced IPP/Apppl production was measured in human cancer cell lines in vitro and in subcutaneous tumors in vivo, as previously described (17). Briefly, following bisphosphonate treatments, tumor cells were
scraped, washed in PBS, and extracted using ice-cold acetonitrile (300 µL) and water (200 µL) containing 0.25-mmol/L NaF and Na3VO4 to prevent degradation of IPP and AppI. Tumor xenographs were collected from mice after sacrifice and snap-frozen in liquid nitrogen, pulverized, and extracted using ice-cold acetonitrile. IPP and AppI in cell and tumor extracts were then quantified by high-performance liquid chromatography negative ion electrospray ionization mass spectrometry.

**Immunohistochemistry**

Tumors were embedded in Tissue-Tek (Sakura), snap-frozen in liquid nitrogen-cooled isopentane, and stored at −70°C. Frozen 7-µm sections were cut in a cryostat, air-dried, and fixed in cold acetone. Vy9V62 T cells and proliferating tumor cells were immunodetected using a fluorescein isothiocyanate (FITC)–conjugated anti-human TCR δ2 monoclonal antibody (Beckman Coulter) and a rabbit polyclonal anti–Ki-67 antibody (Abcam), respectively, as previously described (12).

**Real-time PCR**

Total RNA was extracted from T47D and B02 tumors and infiltrating Vy9V62 T cells. Tumors were homogenized with a Polytron device (Kinematica). RNA was extracted using the nucleospin RNA II Kit (Macherey-Nagel) followed by DNase digestion (Macherey-Nagel). Samples of total RNA (1.5–5 µg) were reverse-transcribed using Superscript II (Invitrogen). Real-time PCR was conducted (IQ SYBR Green, Bio-Rad) with primers specific for the human housekeeping gene L32 (100 bp), a ribosomal protein used as an internal standard: 5′-cagaggagctggaagtgctgt; 3′. T cells, monocytes, and B cells are responsive to N-BPs (13). The effect of risedronate on proliferation and IPP/AppI formation in PBMCs was quantified in triplicate samples in the pool of 3 independent tumors for each gene and normalized to corresponding L32 values.

**Flow-cytfluorimetric analysis**

T47D, B02, and MCF-7 breast cancer cell lines were seeded at a density of 1.106 cells per well in 6-well plates. After overnight incubation, the cells were incubated at 37°C for 24 hours with PBS, 10 ng/mL TNF-α, 0.5 ng/mL INF-γ (Biosource), or conditioned medium from risedronate-treated PBMCs. After this induction time, cells were stained with a phycoerythrin-conjugated human anti-ICAM-1 (CD54; Beckman Coulter) monoclonal antibody. All cells were resuspended in 500 µL PBS supplemented with 1% bovine serum albumin and analyzed immediately by cytometry (Canto II, Becton Dickinson). Similar experimental conditions were carried out with untreated T47D, B02, and MCF-7 cancer cells using phycoerythrin-conjugated monoclonal antibodies directed against antiadhesion molecule receptors Alcam (CD166; Beckman Coulter), and MICA/B (eBioscience).

**Statistical analysis**

All data were analyzed using StatView software (version 5.0; SAS Institute Inc.). Pairwise comparisons were carried out by conducting a nonparametric Mann–Whitney U test. P values less than 0.05 were considered statistically significant. All statistical tests were 2-sided.

**Results and Discussion**

**N-BPs induced human Vy9V62 T-cell expansion in vitro and in vivo**

The PBMCs were treated with different N-BPs to evaluate the relation between Vy9V62 T-cell proliferation and inhibition of recombinant human FPPS in vitro. Only potent FPPS inhibitors, such zolendronate (IC50 of FPPS activity: 4.1 nmol/L), risedronate (IC50: 5.7 nmol/L), and to a lower extent, the structural risedronate analog NE-58025 (IC50: 42 nmol/L) significantly stimulated γδ T-cell proliferation in vitro (Fig. 1A). In contrast, structural risedronate analogs NE-58051 (IC50: 337 nmol/L) and NE-10790 (IC50: 250 × 103 nmol/L) had no effect (Fig. 1A). Thus, there was a strong correlation between FPPS inhibition and Vy9V62 T-cell expansion induced by N-BPs in vitro.

The treatment of human PBMCs with increasing concentrations of risedronate stimulated in a time- and dose-dependent manner the expansion of Vy9V62 T cells (Supplementary Fig. S1A). Concomitant to Vy9V62 T-cell expansion, mass spectrometry revealed rapid IPP/AppI accumulation in PBMCs after risedronate treatment (Supplementary Fig. S1B and S1C). These results extend previous findings showing that zolendronate stimulates Vy9V62 T-cell proliferation and IPP/AppI formation in PBMCs in vitro (12). This effect was specific to Vy9V62 T cells. Neither human γδ T cells expressing the Vy9V61 TCR, nor human αβ T cells, monocytes, and B cells are responsive to N-BPs (13).

Murine PBMCs treated with N-BPs do not activate murine or human γδ T cells (7, 13). The effect of risedronate on Vy9V62 T-cell expansion was therefore studied in vivo after intraperitoneal injection of human PBMCs into NOD/SCID immunodeficient mice and RIS-IL-2 treatment for 14 days. Risedronate (30, 150, 300, and 600 µg/kg), in the presence of IL-2, dose-dependently stimulated the expansion of Vy9V62 T cells, such that they comprised up to 50% of human T lymphocytes versus less than 10% for IL-2 alone or IL-2 plus risedronate at a dose of 150 µg/kg (Fig. 1B). We previously observed similar results with the effect of zolendronate treatment on Vy9V62 T-cell expansion from human PBMCs in vivo (12). This is explained by the fact that both zolendronate and risedronate are internalized by antigen-presenting cells from human PBMCs, leading to intracellular IPP/AppI accumulation and subsequent activation of Vy9V62 T cells in a TCR-dependent manner.

**Risedronate-induced IPP/AppI production in human cancer cell lines in vitro**

We have previously shown that zolendronate induces IPP/AppI accumulation in human breast cancer cell lines (MCF-7, T47D, and BT-474) having a luminal molecular subtype (ER-positive and/or progesterone receptor...
might also explain variation in IPP/ApppI production between breast cancer cell lines, the uptake of fluorescently labeled–risedronate (FAM-RIS) was measured in T47D, MCF-7, and B02 cells. FAM-RIS uptake in T47D and MCF-7 cells was similar but 2-fold lower in B02 cells (Supplementary Fig. S2C). We previously reported similar findings for the cellular uptake of zoledronate (12). In addition, we showed that f3-hydroxy-f3-methylglutaryl Coa reductase (HMG-Coa) reductase expression, a mevalonate pathway enzyme upstream of FPPS, was low in B02 cells as compared with that observed in T47D and MCF-7 cells (12). These results agree with an earlier report (18) that high HMG-Coa reductase expression correlated positively with ER expression in tumor tissue from patients with breast cancer. Taken together (12, 18, and this study), these results show that IPP/ApppI production in breast cancer cells depends on cellular uptake of N-BP and on mevalonate pathway activity associated with the ER status of the cell.

**Risedronate-induced IPP/ApppI accumulation in human breast cancer cell lines correlates with Vγ9Vδ2 T-cell-mediated cancer cell death in vitro**

We next examined whether the anticancer potency of Vγ9Vδ2 T cells might depend on intracellular IPP/ApppI production in risedronate-treated ER-positive and -negative breast cancer cells *in vitro* and *in vivo*. A 1-hour treatment with risedronate (1–25 μmol/L) did not affect survival of T47D, MCF-7, BT-474, ZR 75-1, and B02 cells *in vitro* (Fig. 2A, C, E, G, and I, respectively). However, coculturing risedronate-treated T47D and MCF-7 cells with purified Vγ9Vδ2 T cells led to dose-dependent cancer cell death, which was statistically significant with risedronate at concentrations as low as 3 μmol/L for 1 hour (Fig. 2B and D). Similarly, Vγ9Vδ2 T cells mediated cytotoxicity of risedronate-treated BT-474 cells (Fig. 2F). A minimum concentration of 25 μmol/L risedronate was, however, required to trigger the cytotoxicity of Vγ9Vδ2 T cells against this breast cancer cell line. In sharp contrast, Vγ9Vδ2 T cells did not exhibit cytotoxicity against risedronate-treated ZR 75-1 and B02 cells (Fig. 2H and J). Similarly, coculture of zoledronate-treated T47D and MCF-7 cells with purified Vγ9Vδ2 T cells leads to dose-dependent cancer cell death, whereas Vγ9Vδ2 T cells are not cytotoxic against zoledronate-treated B02 cells (12). Altogether, these results strongly suggested that N-BP–induced IPP/ApppI accumulation in breast cancer cells was responsible for Vγ9Vδ2 T-cell–mediated cancer cell death. Further experiments were therefore conducted *in vivo*, using breast tumors cells with low or high IPP/ApppI levels after N-BP treatment.

**Cytotoxic effect of Vγ9Vδ2 T cells against human breast tumor xenografts in vivo**

We observed that RIS+IL-2 treatment of NOD/SCID mice engrafted with human PBMCs stimulated the expansion of Vγ9Vδ2 T cells *in vitro* (Fig. 1B). Vγ9Vδ2 T cells are known only to exist in primates and humans (2, 13), and they only recognize tumor cells of human origin (13), making our animal model most suitable for studying the
potential role of risedronate (and zoledronate) in cancer immunotherapy. Of note, this effect of risedronate on in vivo Vγ9Vδ2 T-cell expansion was observed at a dosing regimen calculated equivalent to 525 μg/kg body weight, weekly, whereas the approved weekly dose of risedronate for prevention of aromatase inhibitor–induced bone loss in breast cancer is 600 μg/kg body weight (14), further supporting the clinical relevance of our animal model. Therefore, in vivo experiments were carried out with NOD/SCID mice that were bearing subcutaneous T47D or B02 breast tumor xenografts. Treatment with risedronate alone or PBMC+IL-2 did not inhibit T47D and B02 tumor growth (Fig. 3A and B). In contrast, a 46% reduction of the volume of T47D tumors (P < 0.05) was observed in mice receiving PBMC+IL-2+RIS, when compared with placebo (Fig. 3A). A similar treatment of B02 tumor-bearing animals with PBMC+IL-2+RIS did not inhibit tumor growth (Fig. 3B).

In agreement with in vivo data, the in situ immunodetection of the Ki-67 nuclear antigen in T47D tumors from animals treated with PBMC+IL-2+RIS showed a reduction in the proliferative index, when compared with that observed with tumors from placebo-treated animals (Fig. 3C). In contrast, the proliferative index of B02 tumors from animals treated with PBMC+IL-2+RIS did not differ significantly from that obtained with placebo (Fig. 3D). Of note, Vγ9Vδ2 T-cell infiltrates were detected in T47D but not B02 tumors from mice treated with PBMC+IL-2+RIS, as judged by RT-PCR (Fig. 3E and F).

Vγ9Vδ2 T-cell–mediated cancer cell death in vivo correlates with risedronate-induced Apppl accumulation in breast tumor xenografts

Because Vγ9Vδ2 T cells were infiltrating subcutaneous T47D tumor xenografts in mice treated with PBMC+IL-2+RIS (Fig. 3E), mass spectrometry analysis of tumor extracts was conducted to measure Apppl contents. Apppl was barely detectable in T47D tumors from placebo-treated mice (Fig. 4A), whereas substantial Apppl amounts were detected 48 hours after PBMC+IL-2+RIS treatment (Fig. 4B). Apppl was also detected in T47D tumor extracts from mice treated with risedronate alone (data not shown). In contrast, risedronate-induced Apppl production was not detected in B02 tumor xenografts (data not shown). Low IPP levels were detected at 48 hours after the last risedronate dose in PBMC+IL-2+RIS–treated mice (not shown). We previously reported similar results about IPP/Apppl accumulation in T47D tumor extracts 48 hours after the last treatment of mice with PBMC+IL-2+ZOL (12). Conversely, IPP levels were high in T47D tumor extracts at 24 hours after the last zoledronate dose, whereas Apppl levels were barely detectable (12). We did not measure here IPP/Apppl levels in tumor extracts 24 hours after the last risedronate dose in PBMC+IL-2+RIS–treated animals.

Figure 2. Risedronate-induced IPP/Apppl phosphoantigen accumulation in human breast cancer cells correlates with Vγ9Vδ2 T-cell–mediated cancer cell death in vitro. T47D, MCF-7, BT-474, ZR 75-1, and B02 cells were pretreated with control (ctr; PBS) or 1 to 25 μmol/L risedronate for 1 hour, then cultured without drug for 18 hours. After incubation, cells were cocultured without (A, G, E, and I) or with (B, D, F, H, and J) purified Vγ9Vδ2 T cells for 24 hours. The ratio of cancer cells to Vγ9Vδ2 T cells was 1:12.5. A–J are representative of 3 independent experiments (mean ± SD). *, P < 0.05; **, P < 0.01.
Nevertheless, our findings (12 and this study) are consistent with the fact that ApppI formation results from the intracellular accumulation of IPP after FPPS inhibition (2). Importantly, as shown here (Figs. 3 and 4) and in our previous study (12), N-BP uptake in tumors (as judged by IPP/ApppI production) is not enough for tumor growth reduction in vivo. Indeed, tumor growth reduction coincides with infiltration of Vγ9Vδ2 T cells in tumors that produced IPP/ApppI.

Induction of ICAM-1 expression in breast cancer cells in vitro and in vivo

Vγ9Vδ2 T cells recognize N-BP–treated tumor cells through costimulatory non-TCR receptors, such as CD6, NKG2D, and LFA1 in vitro (13). However, there is no evidence of their engagement for recognition of N-BP–treated tumors by Vγ9Vδ2 T cells in vivo. The presence of ligands for these cell surface receptors was evaluated by flow cytometry using T47D, MCF-7, and B02 breast cancer cell lines (Supplementary Fig. S3). Cell adhesion molecule ALCAM (CD166), the ligand for CD6, was strongly expressed in all the 3 cell lines, whereas MICA/B (NKG2D ligand) was not expressed. ICAM-1 (CD54), the ligand for LFA-1, was poorly expressed by all 3 cell lines. ICAM-1 is a member of the transmembrane immunoglobulin superfamily whose expression is induced by TNF-α or IFN-γ (19). In addition, risedronate and zoledronate both strongly induced IFN-γ production by PBMCs in vitro (data not shown). We therefore carried out experiments with the conditioned medium from risedronate- and zoledronate-treated PBMCs to induce cell adhesion molecule expression in breast cancer cells. ICAM-1 expression was significantly increased by conditioned media from N-BP–stimulated PBMCs in all the 3 cell lines when compared with IFN-γ or TNF-α (Fig. 5A). MICA/B was not expressed under these experimental conditions (data not shown).

Figure 3. Cytotoxic effect of Vγ9Vδ2 T cells against human T47D and B02 breast tumor xenografts. Mice were inoculated subcutaneously with T47D or B02 cells. Four weeks after tumor cell inoculation, when tumors became palpable, mice were engrafted with PBMCs from healthy human adult blood donors. In addition, mice were treated with IL-2 + RIS (150 μg/kg) every 2 days for 2 weeks. A and B, T47D and B02 tumor growth was determined at day 26, 34, and 42 after tumor cell inoculation, by measurement of the tumor volume with a Vernier caliper. C and D, T47D and B02 Ki-67 proliferative indices were assessed by immunohistochemistry (IHC). E and F, relative expression of Vγ9Vδ2 T cells in T47D and B02 tumors was assessed by RT-PCR. Results are the mean ± SD of 6 to 7 mice per group. ns, not significant; *, P < 0.05 and **, P < 0.01.
We next examined whether ICAM-1 could be induced in experimental human breast tumors in vivo. In our animal models of breast cancer, RT-PCR showed a 3-fold increase ICAM-1 expression in T47D tumors from mice treated with PBMC + IL-2 + RIS versus placebo (Fig. 5B and C). T47D tumors from mice treated with risedronate alone or PBMC + IL-2 showed a modest but significant increased expression of ICAM-1 versus placebo (Fig. 5B and C). In sharp contrast, ICAM-1 was poorly expressed in B02 tumors, irrespective of the presence or absence of PBMCs and/or the use of a N-BP (risedronate or zoledronate; Fig. 5B and C). These results may be explained by the fact that tumor-derived IPP/Apppl phosphoantigens behave like rate-limiting factors. We have previously shown that phosphoantigen production by tumor cells recruits Vγ9Vδ2 T cells to breast tumors in vivo (12). Infiltrating Vγ9Vδ2 T cells then secrete IFN-γ that is growth inhibitory for tumor cells (7, 12, 13). Here, because of the lack of Apppl production by B02 cells, Vγ9Vδ2 T cells did not infiltrate B02 tumor xenografts (Fig. 3F). Consequently, there was no production of IFN-γ by Vγ9Vδ2 T cells and no activation of ICAM-1 expression within B02 tumors. This contention was supported by serum measurements of human IFN-γ. We observed that IFN-γ levels were high-to-moderate in T47D tumor-bearing mice treated with PBMCs + IL-2 + RIS (765 ± 357 pg/mL, n = 6) or
PBMCs barely detected in B02 tumor-bearing animals treated with ZOL and RIS (228 ± 45 pg/mL, n = 5) or PBMCs+IL-2 (12 ± 23 pg/mL, n = 5; P < 0.001).

Thus, our study establishes that IPP/Apppl-producing tumors induce Vγ9Vδ2 T-cell infiltration and secretion of IFN-γ, which in turn, triggers the recognition of tumors by Vγ9Vδ2 T cells through ICAM-1 engagement, leading to tumor growth reduction in vivo.

**A sequential treatment with doxorubicin and zoledronate sensitizes unresponsive B02 breast tumors to Vγ9Vδ2 T-cell cytotoxicity in vivo**

We next determined whether combination of a N-BP treatment with chemotherapy might sensitize low IPP/Apppl-producing B02 tumors to Vγ9Vδ2 T-cell cytotoxicity in vivo. It has been previously shown by Otewell and colleagues (16) that a sequential treatment with doxorubicin followed 24 hours later by zoledronate reduces subcutaneous growth of ER-negative MDA-MB-436 tumors in animals. In addition, Mattarollo and colleagues (20) have shown that chemotherapy and zoledronate sensitize ER-negative MDA-MB-231 breast cancer cells to Vγ9Vδ2 T-cell cytotoxicity in vitro. The human B02 breast cancer cell line used in our study is a subpopulation of MDA-MB-231 (15). Because B02 cells were not susceptible to zoledronate-mediated Vγ9Vδ2 T-cell cytotoxicity, we therefore, carried out combination experiments using a sequential treatment with doxorubicin followed by zoledronate, alone or in combination with PBMC+IL-2 (Fig. 6). Doses of doxorubicin (2 mg/kg body weight) and zoledronate (100 μg/kg body weight) given to animals were calculated clinically equivalent to doses given in patients (16). On the first week of treatment, tumor-bearing animals treated with doxorubicin followed by ZOL+PBMC+IL-2 had statistically significantly smaller tumors (115 ± 43 mm³) than those of animals treated with doxorubicin+ZOL (228 ± 54 mm³; P = 0.0007) or with doxorubicin alone (242 ± 72 mm³; P = 0.0004; Fig. 6). However, on the second week of treatment, tumors...
there was still a modest inhibitory effect compared with doxorubicin were similar to tumor volumes of animals treated with zoledronate, enhanced the antitumor effect of this sequential treatment, which probably explained why doxorubicin followed by zoledronate did not only depend on IPP/ApppI accumulation in tumors but also on expression of tumor cell surface receptor ICAM-1, which triggered the recognition and killing of bisphosphonate-treated breast cancer cells by Vγ9Vδ2 T cells.

There is clinical evidence that zoledronate durably activates γδ T cells in patients with early breast cancer (21). In other small trials, the modulation of γδ T cells with zoledronate has been shown to improve clinical outcome in patients with lung or prostate cancer (22). In large phase III clinical trials, it has been shown that adding zoledronate to standard systemic therapy (endocrine therapy or chemotherapy) improves disease-free survival and overall survival of patients with ER-positive breast cancer in a low estrogen environment (i.e., following ovarian suppression therapy or in women with established menopause at diagnosis; refs. 4–6). The mechanisms responsible for this antitumor effect of zoledronate in early breast cancer are unknown but may involve changes in the bone marrow environment and, therefore, our findings showed that a sequential treatment with doxorubicin followed by ZOL+PBMC+IL-2 increased cytotoxic activity of Vγ9Vδ2 T cells against low IPP/ApppI-producing breast tumors in vivo.

Conclusion

Our study provides new insights into how N-BP (risdronate and zoledronate) could empower the immune system to pinpoint breast tumors and inhibit their outgrowth. We showed that ER-positive breast tumors were more likely to produce phosphoantigens IPP/ApppI after N-BP treatment compared with ER-negative breast tumors, thereby promoting Vγ9Vδ2 T-cell activation in vivo. Moreover, the ability of risdronate and zoledronate to activate Vγ9Vδ2 T cells not only depended on IPP/ApppI accumulation in tumors but also on expression of tumor cell surface receptor ICAM-1, which triggered the recognition and killing of bisphosphonate-treated breast cancer cells by Vγ9Vδ2 T cells.

Disclosure of Potential Conflicts of Interest

P. Clézardin has served as a consultant/advisory board member of Novartis, Amgen, and Transgene; has received lecture fees and a research grant from Amgen; and has a honoraria from Speakers Bureau of Amgen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: I. Benzaid, H. Mönkkönen, J. Mönkkönen, P. Clézardin

48 hours after the last zoledronate dose in mice treated with doxorubicin followed by ZOL+PBMC+IL-2 showed that phosphoantigen amounts did not differ significantly from that measured in tumors from animals treated with doxorubicin followed by zoledronate (120 ± 85 vs. 113 ± 80 nmol/L). Low ApppI amounts were detected in tumor extracts from animals treated with the placebo (45 ± 35 nmol/L) or doxorubicin alone (75 ± 65 nmol/L). Nevertheless, our findings showed that a sequential treatment with doxorubicin followed by ZOL+PBMC+IL-2 from animals treated with doxorubicin alone (480 ± 240 mm3). The second group of animals received doxorubicin followed 24 hours later by 100 μg/kg doxorubicin. Animals from the first group received doxorubicin were engrafted 24 hours later with PBMCs from healthy human adult blood donors and were treated with ZOL+IL-2. In the relevant groups, IL-2 ± ZOL (100 μg/kg) was then administered every other day for 2 weeks. Tumor growth was determined at day 26, 34, and 42 after tumor cell inoculation by measurement of the tumor volume with a Vernier caliper. Results are the mean ± SD of 6 to 7 mice per group. *, P < 0.05 and **, P < 0.01. DOX, doxorubicin.
Development of methodology: I. Benzaid, H. Mönkönen, P. Clézardin

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Benzaid, H. Mönkönen, E. Bonnelye

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Benzaid, H. Mönkönen, E. Bonnelye, P. Clézardin

Writing, review, and/or revision of the manuscript: I. Benzaid, H. Mönkönen, E. Bonnelye, J. Benzaid, P. Clézardin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Benzaid, H. Mönkönen

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In Vivo Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Trigger Vγ9Vδ2 T-cell Antitumor Cytotoxicity through ICAM-1 Engagement

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