Host Nuclear Factor-κB Activation Potentiates Lung Cancer Metastasis

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Abstract
Epidemiologic and experimental evidence suggests that a link exists between inflammation and cancer, although this relationship has only recently begun to be elucidated for lung cancer, the most frequently fatal human tumor. Nuclear factor-κB (NF-κB), a transcription factor that controls innate immune responses in the lungs, has been implicated as an important determinant of cancer cell proliferative and metastatic potential; however, its role in lung tumorigenesis is uncertain. Here, we specifically examine the role of NF-κB–induced airway inflammation in lung cancer metastasis using a model of intravenous injection of Lewis lung carcinoma cells into immunocompetent C57Bl/6 mice. Induction of lung inflammation by direct and specific NF-κB activation in airway epithelial cells potentiates lung adenocarcinoma metastasis. Moreover, we identify resident lung macrophages as crucial effectors of lung susceptibility to metastatic cancer growth. We conclude that NF-κB activity in host tissue is a significant factor in the development of lung metastasis. (Mol Cancer Res 2008;6(3):364–71)

Introduction
Although the association between inflammation and cancer was originally postulated long ago, it is only recently that we have begun to understand this relationship (1-3). It is now apparent that carcinogenesis and tumor progression are governed not only by genetic changes in cancer cells, but also by the host environment in which tumors develop and grow (4, 5). Inflammation profoundly affects the host milieu surrounding tumors by recruitment of a variety of inflammatory cells and secretion of a multitude of cytokines, chemokines, angiogenic factors, and matrix-degrading enzymes (4, 6). Although host immune cells are classically believed to mediate antitumor effects (paradigms of immune antitumor surveillance and tumor rejection), immune cells may promote tumor progression and metastasis when aberrantly accumulated at sites of ongoing inflammation (1). In this regard, tumor-associated macrophages have been linked to enhanced tumor progression and metastasis (7, 8).

The nuclear factor-κB (NF-κB) family of transcription factors are ubiquitous heterodimeric or homodimeric proteins that control innate immunity and inflammatory responses (9). Although NF-κB proteins are normally sequestered in the cytoplasm by inhibitory proteins known as IκBs, proteosomal degradation of the IκBs can be triggered by a variety of signals, resulting in translocation of the NF-κB dimer to the nucleus where it can activate transcription of target genes. NF-κB–controlled genes include cytokines, adhesion molecules, angiogenic factors, and enzymes that all have been associated with tumor progression and metastasis (4). Moreover, NF-κB affects neoplastic transformation, cell cycling, apoptosis, invasion, and metastasis in tumor cells (10-15). A recent study revealed that NF-κB activity in metastasizing colon cancer cells is critical in determining the effect of host proinflammatory mediators on the proliferative or apoptotic response of tumor cells (16). Thus, NF-κB is centrally positioned to provide a mechanistic link between inflammation and cancer progression and metastasis.

The lungs harbor the majority of human primary and metastatic neoplasms (17, 18). Although surgery often provides definitive cure and high rates of long-term survival for localized primary disease, the development of metastases precludes surgical treatment and dramatically compromises life expectancy and quality of life (19). In the lungs, conditions that increase the risk of primary lung cancer development, such as cigarette smoking, environmental pollutants, and chronic obstructive pulmonary disease, have been associated with NF-κB activation (20-23). In these studies, we hypothesized that a proinflammatory host lung microenvironment, such as that generated by persistent NF-κB activation, directly increases the host lung permissiveness to cancer cell metastasis. We tested this hypothesis in mice by direct interventions into the canonical NF-κB pathway in the host lungs by infection with NF-κB–modulating recombinant adenoviruses. We studied an adenocarcinoma cell line to emulate the majority of human disease, and we used immunocompetent mice to recapitulate the whole spectrum of host-tumor interactions (24). Here, we show...
that increased NF-κB activity in host lung results in an enhanced metastatic phenotype. Importantly, our results suggest that targeting of host inflammatory cells, particularly macrophages, would confer a protective advantage in premetastatic disease.

Results

Intratracheal Adenoviral Instillation Is a Suitable Method for Increasing NF-κB Activity in the Lung

To generate NF-κB–induced inflammation in the lungs, we instilled adenoviral vectors encoding the active NF-κB molecule, RelA, intratracheally into mice. We have previously shown that intratracheal instillation of adenoviruses expressing NF-κB–activating transgenes results in infection of bronchial epithelial cells, NF-κB activation, and airway inflammation (25). To distinguish specific RelA-induced effects from nonspecific adenoviral-mediated events, we used control viruses (Ad-ctl) encoding either β-galactosidase, green fluorescent protein (GFP), or luciferase. We first used only the luciferase virus in C57Bl/6 mice to establish the time course of expression from intratracheal viral delivery. Efficient transgene delivery was confirmed using 10^9 plaque-forming units (pfu) of Ad-luciferase (Fig. 1A). Following luciferin injection, increased photon emission due to expression of the viral-encoded protein was detectable starting at day 3 (data not shown). Detectable luciferase signal was still present 14 days after virus infection, demonstrating that adenoviral transgene delivery to the lungs produces continued expression over a time period suitable for experimental metastasis assay (Fig. 1B). To show that treatment with Ad-RelA virus results in significant NF-κB activity in the lungs of exposed mice, we took advantage of a transgenic NF-κB reporter mouse model (26). We confirmed that Ad-RelA caused a significant increase in NF-κB–dependent luciferase activity in the lungs at 72 hours postinjection by measurement of chest bioluminescence, whereas control GFP adenovirus had no significant effect [Fig. 1C; luminescence emission (photons/s), mean ± SD: baseline = 5,557,183 ± 3,965,979; control virus = 8,261,200 ± 3,864,549, P = 0.36 compared with baseline; Ad-RelA = 42,761,333 ± 35,490,104, P = 0.029 compared with baseline; n = 3 mice per group].

Airway Inflammation Resulting from Enhanced Host NF-κB Activity Increases Tumor Take

Experimental metastasis (tail vein) experiments allow testing of the later stages of the metastatic cascade, that is, survival in the circulation, extravasation and survival, and growth at a secondary site. Lewis lung carcinoma cells were originally established from a spontaneous lung tumor occurring in a C57Bl/6 mouse (27). In these experiments, our goal was to assess the effect of airway inflammation, as induced by RelA expression, on the metastatic ability of these cells using the experimental metastasis assay. Because this cell line is syngeneic with C57Bl/6 mice, we were able to examine the inflammatory effects on the background of full immune competence. Based on initial time course studies indicating substantial airway inflammation at 3 days (72 hours) after intratracheal Ad-RelA administration, we instilled 10^9 pfu Ad-RelA or control adenovirus at 72 hours before intravenous injection of LLC cells. Following tumor cell inoculation, the mice were left for 14 days and then euthanized. At this 2-week time point, Ad-RelA–infected mice exhibited >2-fold higher surface tumor numbers than control-infected animals (Fig. 2A). Assessment of tumor burden and size in histologic sections confirmed the increase in tumor number in Ad-RelA–infected mice (Fig. 2B). Furthermore, the size of the tumors was larger in the Ad-RelA mice (median sizes: PBS, 0.05 mm^2; Ad-ctl, 0.04 mm^2; Ad-RelA, 0.10 mm^2; Fig. 2C). Surprisingly, when we analyzed bromodeoxyuridine incorporation as a marker of proliferation or immunostaining for cleaved caspase-3 as a marker of apoptosis in tumor sections from this 2-week time point, no differences were observed between the tumors from the control or Ad-RelA–infected mice (data not shown). These results raised the possibility that the difference in tumor number seen in the Ad-RelA–infected lungs was influenced by increased survival or tumor “take.” We therefore assessed whether there was a difference in surviving tumor number in the lungs of virus-infected mice 24 or 48 hours after

**FIGURE 1.** Intratracheal adenoviral instillation is a suitable method for increasing NF-κB activity in the lungs. A. Prolonged expression of adenoviral-encoded luciferase in the mouse lungs. Wild-type C57Bl/6 mice received 10^9 pfu Ad-luciferase and were serially imaged for bioluminescence after intravenous injection of 1 mg luciferin. Left, image taken before virus instillation; right, image taken after 14 d. B. Pooled data from three mice per group treated as in A (*, P = 0.017 compared with baseline). C. Intratracheal Ad-RelA increases NF-κB activity in the lungs. NF-κB reporter mice (HLL) on the C57Bl/6 background received 10^9 pfu control adenovirus or Ad-RelA intratracheally. Increased luciferase expression was seen in Ad-RelA–infected (right), but not control Ad-GFP–infected (middle) HLL mice, 72 h after viral delivery.
tumor cell injection. The LLC cells were loaded with a red lipophilic dye in vitro and then injected as usual into mice that had been pretreated with Ad-RelA or control virus. There was no apparent difference in the number of surviving cells at these time points (Fig. 2D and E and data not shown).

We characterized the inflammatory environment of the lungs both at an early time point (24 hours post-LLC cells) and at the end of study (2 weeks post-LLC cells). This was accomplished by performing bronchoalveolar lavage and assessing cellularity and cytokine levels in the collected fluid. As we have seen previously (25), at early time points following adenoviral delivery of RelA to the lungs, there was a marked increase in neutrophil infiltration (Fig. 3A). Our previous studies have shown that this is a transient early inflammatory response (25). The bronchoalveolar lavage fluid was analyzed by ELISA to measure expression of a number of factors that are indicative of an inflammatory response or that could contribute to tumor growth. Levels of both vascular endothelial growth factor and matrix metalloproteinase 9, two proteins that have been suggested to enhance vascular endothelial growth factor and MCP-5 levels were not different between mice infected with Ad-RelA or control (data not shown). However, at the 2-week time point, levels of the chemokine MCP-1 were increased in bronchoalveolar lavage fluid specifically from Ad-RelA–treated mice (Fig. 3F). As MCP-1 can be expressed by epithelial cells (Fig. 3C; ref. 28), the increased levels are likely a direct result of the Ad-RelA infection of epithelial lining cells and could be responsible for
the observed macrophage influx. The inflammatory response induced by Ad-RelA was confined to the lungs as WBC counts and serum cytokine levels were normal and identical between groups (data not shown).

Taken together, our data show that increasing lung NF-κB activity by adenoviral infection of epithelial lining cells results in a strong up-regulation of chemokine production and concomitant leukocyte influx into the airspaces, composed principally of macrophages at 2 weeks post-LLC cell injection. This inflammatory phenotype correlates with a more permissive environment for outgrowth of metastatic tumors as evidenced by increased lung tumor number and size following intravenous injection of LLC cells.

Alveolar Macrophages Mediate the Effect of Increased Host NF-κB Activity on LLC Metastasis

Because analysis of tumors and bronchoalveolar lavage from multiple experiments revealed a significant correlation between increased tumor number and increased infiltration of macrophages following enhancement of host NF-κB activity, we sought to determine whether this leukocyte population contributed to enhanced tumor cell metastasis. We chose to reduce the numbers of lung macrophages by intratracheal injection of liposomal encapsulated clodronate, which is a bisphosphonate that results in selective apoptosis when phagocytosed by macrophages. When delivered intratracheally, liposomal clodronate targets resident alveolar macrophages and the effect lasts ~5 to 7 days, after which macrophages start to repopulate the lung (29, 30). For these studies, mice received 10^9 pfu Ad-RelA or control adenovirus immediately followed by 75 μL liposomal clodronate or empty liposomes in any combination (n = 6 per group). Four animals received PBS followed by PBS (internal control). Three days later, mice received 2.5 × 10^5 LLC cells via the tail vein and were sacrificed after 14 days for bronchoalveolar lavage and analysis of lung tumors. Despite the relatively short-lived effect of clodronate treatment, we observed partial

**FIGURE 3.** Host lungs respond to RelA overexpression with expression of MCP-1. **A.** Bronchoalveolar lavage was done 24 h following LLC cell injection in mice that had been pretreated with intratracheal PBS, control adenovirus, or Ad-RelA (n = 5 mice per group). A distinct increase in polymorphonuclear leukocytes was seen in the Ad-RelA–treated group that was not present in the other groups (*, *P < 0.0001 compared with Ad-Ctl or PBS). **B.** Increased levels of MCP-1 in bronchoalveolar lavage from the mice described in **A** (*, *P < 0.01 compared with PBS or Ad-Ctl). **C.** Lung tissue harvested from mice 24 h following LLC injection was used for immunohistochemical analysis. MCP-1 was detectable in airway lining epithelial cells (arrows, left) in mice exposed to Ad-RelA but not those exposed to a control adenovirus (right). Bar, 44 μm. **D.** IL-6, a marker of NF-κB pathway activation, was also present in airway lining cells in Ad-RelA–treated mice (arrows, left) but not in control adenovirus–treated mice (right). Bar, 68 μm. **E.** Bronchoalveolar lavage was done 2 wk following LLC cell injection in mice pretreated with intratracheal PBS, control adenovirus, or Ad-RelA (n = 6 mice per group). Ad-RelA, but not Ad-Ctl or sham PBS injection, caused a mononuclear inflammatory cell influx into the lungs (*, *P < 0.001 compared with Ad-Ctl or PBS). **F.** Increased levels of MCP-1 in bronchoalveolar lavage from mice treated as described in **E** (*, *P = 0.005 and *P = 0.007 compared with PBS and Ad-Ctl, respectively). **E** and **F** were recapitulated without tumor cell injection, with identical results.
abrogation of the inflammatory cell influx induced by Ad-RelA at the 14-day time point (Fig. 4A). Significantly, this reduction in inflammatory cell influx was sufficient to completely negate the Ad-RelA–enhanced metastasis effect (Fig. 4B). There was no effect of clodronate administration on the number of metastatic foci evident in the control virus–treated animals, indicating that the enhanced metastasis seen after Ad-RelA treatment is related to newly recruited or activated macrophages. These data show that short-term ablation of macrophages is sufficient to block enhanced tumor formation resulting from NF-κB–induced airway inflammation.

Discussion

NF-κB is a multifunctional transcription factor that has been shown to affect the pathobiology of a variety of diseases (23, 31, 32). In the present study, we have examined the effects of NF-κB activation in host cells on the development of metastatic tumors in the lung. Increasing the levels of NF-κB activity in the host lung resulted in lung inflammation and a substantial protumorigenic effect. The effector cell population mediating this enhanced tumorigenicity seems to be macrophages, which are recruited to the lungs as a consequence of epithelial cell NF-κB activation. Ablation of lung macrophages using liposomal clodronate is sufficient to block the increase in tumor burden resulting from NF-κB–dependent lung inflammation.

In contrast to previous studies on the role of NF-κB in tumor progression that have primarily used loss-of-function approaches, we chose to increase host NF-κB levels. Our goal was to recapitulate the increased NF-κB levels reported in the lung tissue of smokers and patients with chronic obstructive pulmonary disease (22, 31) and to generate airway inflammation as occurs in these individuals. In addition to increasing the risk for lung cancer development (33), chronic inflammatory disorders of the respiratory tract may increase the risk for cancer progression and metastasis to the lungs. A striking result of delivery of adenovirus encoding RelA, but not control adenoviruses, was an induction of an inflammatory infiltrate that was composed largely of macrophages at 17 days postinfection. The composition of the RelA-expressing inflammatory infiltrate in the lungs changes from primarily neutrophilic at an early time point following activation of NF-κB in airway epithelium to a primarily macrophage-rich infiltrate later. Interestingly, MCP-1 expression was found to be increased at the early time point, potentially directing the macrophage recruitment. Notably, the presence of the inflammatory infiltrate correlated with an increase in the number of tumor foci. Both the number and the average size of tumor foci were increased in the group overexpressing RelA. There was no difference in the number of tumor cells surviving in the lungs at 24 or 48 hours postimplantation. This would suggest that the inflammatory phenotype associated with RelA overexpression is most critical for outgrowth of implanted tumors. Surprisingly, proliferation and apoptosis variables were not significantly altered between tumors in the Ad-RelA and control adenovirus groups when measured at the 2-week time point. Hence, the critical time when the inflammatory environment contributes to development of metastatic lung tumors seems to be between the early postimplantation stage (48 hours) and the time of harvest (2 weeks).

This idea that NF-κB–induced lung inflammation primarily affects early growth of a metastatic focus is supported by our studies involving macrophage depletion. To determine whether the increase in tumor numbers observed after Ad-RelA infection could be attributed to macrophage infiltration, we depleted macrophages with liposomal clodronate. Previously, we have shown that a single intratracheal dose of liposomal clodronate depletes alveolar macrophages by 77% (30, 34). The depletion lasts ~5 to 7 days (29, 30). In these experiments, partial ablation of macrophages was sufficient to overcome the tumor-promoting effect of lung inflammation resulting from Ad-RelA administration. This finding supports the idea that macrophage recruitment and/or activation occurring proximate to tumor cell implantation determines development of metastatic foci. Although the specific macrophage products or activity that determines metastasis...
in this model is not apparent from these studies, our findings support a role for the macrophage in tumor metastasis to the lungs. Literature describing tumor-associated macrophages has suggested various roles for these cells in tumor growth and progression (5, 35, 36). For example, the presence of tumor-associated macrophages has been especially associated with increased angiogenesis, which may be related to vascular endothelial growth factor production by macrophages (3, 5, 7). This does not seem to be a critical factor in our studies as vascular endothelial growth factor levels were similar among the different mouse groups at both early and late time points. A recent article from Condeelis and colleagues has identified a possible paracrine loop that exists between epidermal growth factor receptor–expressing tumor cells and colony-stimulating factor receptor–expressing macrophages that apparently serves as a mechanism to enhance tumor cell migration and hence metastasis (8). As we were using an experimental metastasis assay, migration away from a primary tumor was not required. Further, the number of surviving tumor cells present within the lung parenchyma 24 or 48 hours after tumor cell injection was not different among the various groups, suggesting that emigration from vasculature was not affected. In our experiments, only the enhanced macrophage infiltrate induced by lung inflammation seemed to be related to metastatic focus formation as the clodronate-mediated macrophage depletion did not alter the number of foci in the control animals.

In other studies of NF-κB activation within tumor epithelial cells, the primary result seems to be the generation of a tumor cell intrinsic survival response usually mediated through up-regulation of expression of antiapoptotic Bcl-2 family members (37, 38). In our case, the tumors came from exogenous cells that were inoculated after the infection of the host epithelium with Ad-RelA. By using this system, we have been able to identify another potential function of epithelial NF-κB, that is, the recruitment of tumor-promoting inflammatory cells. Hence, NF-κB in epithelium functions both as an intrinsic tumorigenic promoter by favoring survival and also as an extrinsic promoter by causing the influx of inflammatory cells.

Lung cancer represents an epidemic on the rise (17, 18). At the time of diagnosis, most patients will have local or systemic spread that renders the disease incurable by surgery (39). In addition, metastasis to the lungs from tumors in other organs is a significant clinical problem. Thus, therapies that would halt invasion and metastasis are desperately needed. It is increasingly clear that the biological behavior of lung cancer is influenced not only by progressive changes in cancer cells but also by host factors, including the host immune system. Although an intact immune system may deliver an antitumor effect, an aberrant inflammatory response may actually promote tumor progression/metastasis. Here, we show how increases in host NF-κB activity, a situation known to occur in chronic inflammatory conditions of the respiratory tract as well as in several other organ systems, can enhance the development of tumor lesions in a mouse model of metastatic lung disease. We suggest that the paradigm of treating patients with agents to down-regulate inflammation could be pursued to limit tumor metastasis to the lungs.

Materials and Methods

Cell Line, Plasmid, and Transfection

LLC cells, originally obtained from the National Cancer Institute, were maintained in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen), glutamine (Invitrogen), and 100 mg/L penicillin and streptomycin (Invitrogen). The LLC clone used for all these experiments was one carrying a NF-κB reporter plasmid, as previously described (40).

Reagents and Drugs

d-Luciferin sodium was obtained from Biosynth AG. Liposomal clodronate or empty liposomes were produced as previously described (30). The lipophilic dye CellTracker Red CMTPX was purchased from Molecular Probes/Invitrogen.

Adenoviral Vectors

Replication-deficient adenoviral vectors that express *Photinus pyralis* luciferase (Ad-luc), β-gal (Ad-βgal), GFP (Ad-GFP) as controls, and the activator of NF-κB (Ad-RelA) were constructed and purified as previously described (25, 41). Briefly, an expression cassette containing a cytomegalovirus promoter driving RelA was inserted into the replication-deficient recombinant adenovirus type 5. Adenoviral vectors were propagated, purified, and stored at −70°C.

Experimental Animals

All animal care and experimental procedures were approved by and conducted according to the Institutional Animal Care and Use Committee guidelines. Mice used for experiments were sex matched, weight matched (20-25 g), and age matched (8-12 wk). C57BL6/J mice were purchased from The Jackson Laboratory, crossed, and inbred. Transgenic mice expressing *Photinus pyralis* luciferase cDNA under control of the proximal 5' HIV-long terminal repeat, called HIV-LTR/Luciferase (HLL), have been previously reported (26, 42, 43). HLL mice on the C57BL6/J background (n > 9) were used for this study. For intratracheal injection, mice were anesthetized with isoflurane, anterior cervical skin and soft tissues were dissected, and 105 pfu virus/50 μL PBS or PBS alone followed by 75 μL liposomal clodronate or empty liposomes were injected intratracheally. Three days later, mice received 2.5 × 105 or 5 × 105 LLC cells in PBS or PBS alone via a lateral tail vein. After 24 h or 14 d, mice were euthanized by CO2 asphyxiation, and lungs were lavaged thrice with 1 mL PBS and fixed in Bouin’s solution.

Bioluminescent Imaging

For in vivo imaging, mice were anesthetized, received 1 mg of d-luciferin retro-orbitally, and were imaged in an IVIS cooled charged coupled device (Xenogen Corporation) as described previously (25, 43). Data were collected and analyzed using Living Image v.2.50 (Xenogen Corporation) and IgorPro (Wavements) software. Briefly, a photographic image of the animals was first acquired. Then, a bioluminescent image was acquired by integration of photon flux over each group of pixels (bin) in the field of view, and graphically represented using an arbitrary pseudo-color scale. Fifteen seconds of acquisition time were selected in order not to saturate the camera. Standard-sized
circular regions of interest encompassing the murine chest were determined and photon flux was measured over these areas.

**Dye Labeling of Tumor Cells**

Tumor cells were labeled in vitro with the CellTracker Red dye for 30 min at a concentration of 10 μmol/L as per manufacturer’s protocol. After washing with PBS, the cells were counted and used for intravenous injection.

**Lung Surface Tumor Enumeration**

Tumors on the lung surface were enumerated by at least two experienced blinded readers under a dissecting microscope; tumor counts were averaged and statistically analyzed.

**Histology**

The explanted mouse lungs were fixed in Bouin’s fixative solution for 24 h and 70% ethanol for 3 d. Tissues were embedded in paraffin and 5-μm-thick sections were serially cut at a median transverse level of the lungs. The sections were mounted on glass slides and stained with H&E. For assessment of tumor burden, the area of each section that was tumor, as well as the total tissue area, was measured using Metamorph (Universal Imaging Corp.) software. This software was also used to calculate the area of each tumor. Four sections at different depths 300 μm apart were used for these analyses.

Alternatively, lungs were covered in optimum cutting temperature medium and frozen in liquid nitrogen. Sections were rinsed and counterstained with Hoechst 33258 (Sigma), coverslipped using fluorescence-saving mountant (Gel/Mount, Biomeda Corp), and examined using fluorescent microscopy. Four random fields from each section, four sections per animal, were analyzed for the total number of red-labeled tumor cells and the total tissue area using Metamorph software.

**Immunohistochemistry**

Five-micrometer Bouin’s-fixed, paraffin-embedded sections of lungs from either the 24-h or 2-wk time points were assessed for expression of IL-6 and MCP-1 using antibodies purchased from Abcam and following supplier-recommended staining procedures.

**Enzyme-Linked Immunosorbent Assays**

Tumor necrosis factor-α, MCP-1 and MCP-5, vascular endothelial growth factor, and pro–matrix metalloproteinase 9 were assayed in bronchoalveolar lavage using murine ELISA kits (R&D Systems) according to the manufacturer’s instructions (detection limits 3.0, 2.0, 1.58, 5.1, and 8.0 pg/mL, respectively).

**Clodronate Treatment**

Liposomal encapsulation of clodronate (dichloromethylene diphosphonate) was done as previously reported (30). Liposomes containing PBS were used as a control. Briefly, a mixture of 8 mg cholesterol (Sigma) and 86 mg egg phosphatidylcholine (DOPC, Avanti) was dissolved in chloroform and then evaporated under nitrogen. Chloroform was further removed under low vacuum in a speedvac Savant concentrator. The clodronate solution was prepared by dissolving 1.2 g of dichloromethylene diphosphonic acid (Sigma) in 5 mL of sterile 1× PBS. The entire clodronate solution (5 mL) was added to the liposome preparation and mixed thoroughly. The solution was sonicated and ultracentrifuged at 10,000 × g for 1 h at 4°C. The liposome pellet was removed, resuspended in 5 mL PBS, and ultracentrifuged at 10,000 × g for 1 h at 4°C. Liposomes were removed, resuspended in 5 mL PBS, and used within 48 h. The final concentration of the liposomal clodronate solution was 5 mg/mL.

**Statistical Analysis**

All values given represent mean values ± SE. The number of observations (n) is given in parentheses. To compare the two groups, the Student’s t and Mann-Whitney U tests were used (for normally or nonnormally distributed data). ANOVA was used to detect significant differences between multiple groups. All P values are two-tailed; P values ≤0.05 were considered significant. Statistical analyses were done using the Statistical Package for the Social Sciences Software Version 11.0 (SPSS).

**References**


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