Onset and Dynamics of Osteosclerosis in Mice Induced by Reilly-Finkel-Biskis (RFB) Murine Leukemia Virus

Increase in Bone Mass Precedes Lymphomagenesis

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Newborn NMRI strain mice were infected with Reilly-Finkel-Biskis (RFB) murine leukemia virus (MuLV), a murine leukemia virus that has been shown to induce lymphomas, osteosclerosis, and osteomas in susceptible strains of mice. Bone histomorphometry of the distal femoral metaphyses at 3-month intervals showed osteosclerosis 3 (100%), 6 (100%), and 9 (93%) months after infection. This was represented by significantly augmented cancellous bone mass and accompanied by distinct changes in bone architecture. High numbers of provirus copies were detected at 2–4 weeks in femora, humeri, and calvaria, and viral protein was highly expressed in trabecular and cortical bone cells, particularly in osteocytes. Infected mice showed enhanced bone formation and smaller numbers of osteoclasts relative to sex- and age-matched controls. Osteoclastic differentiation was significantly reduced in cocultures of spleen or bone marrow cells with RFB MuLV-infected osteoclastogenic, osteoblast-like cells. However, RFB MuLV did not impair the activity of mature osteoclasts. In infected mice lymphomas were only observed at 6 (22%) and 9 months (40%) of age. At 3 months, IgG gene and TCR- β gene rearrangements were not detectable, and new proviruses showed a heterogeneous integration pattern, indicating the absence of lymphoma in early osteosclerotic mice. In contrast, lymphomas, which developed in 8- to 9-month-old in-

fectected mice, showed IgG rearrangements indicating development of B-cell lymphomas, together with mono- or oligoclonal expansion of distinct patterns of proviral integrations. These results indicate that RFB MuLV-induced osteosclerosis develops within 3 months after infection and precedes lymphomagenesis. It may therefore be considered an independent skeletal lesion in MuLV-infected mice. (*Am J Pathol* 1999, 155:557–570)

Murine leukemia viruses (MuLVs) are nonacute transforming retroviruses, which induce lymphomas and leukemias after several weeks to months after infection of newborn mice of susceptible strains. These effects are mediated by diverse mechanisms^{1,2} depending on the various virus isolates. Well-characterized, exogenous, highly tumorigenic murine leukemia viruses, such as Moloney MuLV (MoMuLV), SL3–3 MuLV, or Friend MuLV, are potent inducers of thymic lymphomas or erythroleukemias,^{3–5} whereas germline-derived or activated endogenous murine leukemia viruses are considered weakly tumorigenic and induce lymphomas and leukemias after long latency periods.^{6–10} Accumulating evidence suggests that activated endogenous retroviruses also induce pathogenic effects in the skeleton in experimentally infected mice.^{10–15}

A recently characterized, highly bone-pathogenic murine leukemia virus, RFB MuLV,¹¹ induces lymphomas, increased bone mass, and osteomas^{16,17} in NMRI mice, indicating a pathogenic potential in both hematopoietic and skeletal tissues. Recombinant studies between RFB MuLV and the T-lymphomagenic SL3-3 further revealed that a major osteomagenic determinant of RFB MuLV maps to non-long terminal repeat (LTR) regions of the viral genome.¹⁴ An increase in bone mass was also ob-

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served with a high incidence in mice infected with MuLVs isolated from the germline of AKR¹⁷ or BALB/c mice,¹⁸ from normal bone tissue of C57BL/6 and BALB/c mice^{10,18} as well as from benign¹⁹ and malignant bone tumors,^{16–18} pointing to a common pathogenic potential of these closely related viruses in skeletal cells. Among these, RFB MuLV has been shown to be the most potent bone-pathogenic MuLV known to date.^{11,14} In contrast to avian leukosis virus-induced osteopetrosis in birds,² MuLV-induced skeletal lesions appear radiologically as a thickening of the cortex along the endosteal surface. They show a progressive increase in trabecular bone mass, but maintain the overall shape of the affected skeleton. In severe cases the bone marrow cavity is completely filled with excessively accumulated bone.¹⁷

It is unclear at present whether MuLV-induced increase in bone mass is caused by increased bone formation or by decreased osteoclastic bone resorption, or a combination of both. Whereas an increase in cancellous bone mass due to defective osteoclastic bone resorption is now generally considered to be osteopetrosis, an increase in bone mass due to other causes is generally termed *osteosclerosis*.²⁰ Mouse models for osteopetrosis, which are not associated with endogenous or exogenous retroviruses, include M-CSF deficient *op/op* mutant mice^{21,22} and *mil/mi* mutant mice.²³ Recent approaches using *c-src*,^{24–26} *c-fos*,^{27,28} Acp 5²⁹ or PU.1³⁰ knock-out mice, or NF- κ B1/NF- κ B2 double knock-out mice,^{31,32} which show various defects in osteoclast differentiation and/or bone resorption and remodeling, have contributed substantially to the understanding of osteoclast development and function. Common to all of these models are different genetically determined loss-of-function phenotypes in which osteoclast function or osteoclast differentiation and maturation are affected at various stages, and the mice present with variable degrees of osteopetrosis.

In MuLV-infected NMRI mice, lymphomas are the life-limiting lesion, and skeletal lesions in the lymphoma-moribund mice were only detected in post mortem X-ray analyses. Therefore, the onset and the dynamics of the development of skeletal effects are as yet unresolved. Moreover, it is unclear to date whether the MuLV-induced increase in bone mass develops independently of lymphoma, or whether lymphomagenesis is a precondition for the induction of skeletal lesions. In the present study we present a detailed histomorphometric analysis of osteosclerosis development and show that this disease starts to develop already within 3 months after infection, at a time when none of the infected mice show signs of lymphoma, indicating that the skeletal and hematological lesions evolve independently. Infected mice showed rapid dissemination and expression of RFB MuLV in osteoblasts and osteocytes together with enhanced bone formation. These findings, together with the absence of morphological evidence for a functional defect in the osteoclasts in osteosclerotic bone, indicate that retrovirus-induced osteosclerosis represents a gain-of-function model in which expression of viral proteins in cells of the osteoblastic lineage may be a critical factor.

Materials and Methods

Cell Cultures

NIH3T3 cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS). MB1.8 osteoblast-like cells, which support osteoclastogenesis³³ (generously provided by Dr. G. A. Rodan, Merck, West Point, PA) were grown in α -MEM (Biochrom, Berlin) supplemented with 10% FCS and 2% L-glutamine. MB 1.8-RFB cells were generated by infection of MB1.8 cells with RFB MuLV.¹¹ Infection was controlled by immunohistochemistry, using an anti-virus CA Capsid (p30) protein antibody. One hundred percent infected MB1.8-RFB cells were used.

Osteoclast Formation Assay

Osteoclast-like cell formation was studied in a coculture assay by a method essentially described by Takahashi et al.³⁴ To this end, MB1.8 cells were cocultured with spleen or bone marrow cells of 11- to 12-day-old mice. The mice were killed by cervical dislocation, and the spleens and bone marrow from both femora were removed aseptically. Spleens were minced, suspended in phosphate-buffered saline (PBS), and filtered through a cell strainer with a 100- μ m nylon mesh (Becton-Dickinson, Heidelberg, Germany). The cells were harvested by centrifugation and resuspended in a buffer containing 10 mM Tris, 0.83% NH₄Cl (pH 7.4) to lyse the erythrocytes. Suspensions of bone marrow cells were prepared by gentle pipetting of bone marrow plugs, which were flushed from the femora with a 23 gauge needle. MB1.8 or MB1.8-RFB cells (4.5×10^3 cells/cm²) were seeded into cavities of 48- or 96-well tissue culture plates (Costar, Bodenheim, Germany) and cultured in α -MEM containing 10% FCS (Biological Industries, Tel Aviv, Israel), 4% glutamine, 100 U/ml penicillin/100 μ g/ml streptomycin, and 10^{-8} M 1,25(OH)₂ vitamin D₃ (generously provided by Dr. R. Goralczyk, Hoffmann-LaRoche, Grenzach, Germany). After three to four hours, 2.5×10^5 freshly isolated spleen cells or 7.5×10^4 bone marrow cells were added per cm² and cocultivated for 7 days, and the medium was changed twice at 2-day intervals. At the end of the culture period, the cells were fixed with Ca-saturated formalin for 10 minutes at room temperature and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells containing three or more nuclei were evaluated microscopically. Because spleen and bone marrow cells showed similar osteoclastogenic potential in this coculture assay, spleen cells were further used in these assays.

Isolation and Resorption Activity of Osteoclasts

Osteoclasts were isolated from femurs of 1-day-old rats (Wistar) as described.³⁵ Briefly, femurs were dissected and freed of adherent soft tissue. The bones were cut across the epiphysis, and the marrow was removed by using a size 10 dental needle (100 μ m in diameter). The femurs were placed in a dish containing 1 ml Medium199 (Gibco) supplemented with 0.5% gentamicin. Osteoclasts were gently released from the femurs, using, in

succession, calibrated dental needles of size 20 and 30, suspended in Medium 199 and added to ivory slices kept individually in plastic wells (2.0×1.0 cm). Elephant ivory (BEF/WA/BNatSchG 27-62.02) was cut into $4 \times 4 \times 0.1$ mm slices (Isomet low-speed saw; Buehler Instrument, Evanston, IL), cleaned by ultrasound for 30 seconds in deionized water, air dried, gas sterilized, degassed under vacuum for 24 hours, and used as a mineral substrate to assess osteoclast resorption activity. After 25 minutes' incubation at 37°C and 5% CO_2 /air, nonadherent cells were removed by lateral agitation, and slices (eight for control and eight for each treated group) were individually transferred into single wells of a 24-well plastic tissue culture plate. The cultures were carried out in control minimum essential medium (MEM) in the presence or absence of 10% FCS, or in conditioned media from fibroblasts or RFB MuLV-infected osteoblasts ($500 \mu\text{l}$) for 24 h at 37°C and 5% CO_2 /air. Thereafter, the ivory slices were freed of adherent cells by ultrasound (70% propanol), washed, air dried, and subsequently sputter-coated with gold (SCD 004 Coater; Bal-Tec, Balzers, Lichtenstein). The number of resorption pits on each ivory slice was counted by using a light microscope equipped with a tangential light, at $\times 200$ magnification. A pit was defined as a depression in the ivory surface with a continuous rim and an area of at least $250 \mu\text{m}^2$. Osteoclast resorption activity was expressed as the number of excavations per slice. Pit areas were calculated from pit images, which were captured by a camera attached to the reflected light microscope with the aid of image analysis software (NIH Image).

Animal Experiments

Newborn NMRI mice, which are free of ecotropic proviruses,¹² were infected intraperitoneally (i.p.) (within 36 hours after birth) with $100 \mu\text{l}$ of RFB MuLV¹¹ containing 2×10^5 to 2×10^6 infectious virus particles. Mock-infected mice were injected with $100 \mu\text{l}$ of cell culture supernatant of NIH3T3 cells. Viraemia was investigated 10 and 30 days after infection by cocultivation of whole blood with NIH3T3 cells and immunohistochemical analysis of viral p30 (CA) expression in the target cells. To quantify bone formation and mineralization rates in infected and mock-infected control mice, calcein (Sigma, Deisenhofen, Germany) (20 mg/kg) was injected twice i.p. on days 9 and 4 before death at 3, 6, and 9 months. To monitor tumor development, the mice were checked 5 days a week. They were killed at the time indicated or when they showed illness or tumor development. A complete necropsy including X-ray analysis was performed. The tumors were diagnosed as described previously.¹⁹ According to these criteria, enlargement of one of the following organs to the stated minimum dimensions had to be fulfilled for a diagnosis of malignant lymphoma: peripheral lymph nodes, 10 mm; thymus, 10 mm; mesenteric lymph node, 20×5 mm; spleen, 30 mm; left lobe of the liver, 30 mm, or abundant effusions in the thoracic or abdominal cavity. In all cases the diagnosis was confirmed histologically according to the criteria described

by Pattengale.³⁶ Bone changes were detected by X-ray analysis and histologically.

Histology

At necropsy, the distal femora and humeri were freed from adherent tissues and fixed immediately in 40% ethanol for 48 hours at 4°C , or in 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (pH 7.4) for 24 hours at 4°C . The bone samples fixed in PFA were washed overnight at 4°C in 0.1 mol/L phosphate buffer (pH 7.4) containing 10% sucrose. Subsequently, the bones were dehydrated and embedded undecalcified in methylmethacrylate, as described.³⁷ Three-micron-thick sections were prepared in the midsagittal plane of the distal femora and humeri with a HM 360 microtome (Microm, Walldorf, Germany) and stained with von Kossa/toluidine blue³⁸ and with toluidine blue at acid pH.³⁹

Histochemistry and Immunohistochemistry

For TRAP histochemistry, deplasticized sections were placed in 0.1 mol/L acetate buffer at pH 5.0 for 5 minutes. TRAP reaction was subsequently performed as described,³⁹ using hexazotized pararosaniline (Merck, Darmstadt, Germany) as an azo dye and naphthol AS-TR phosphate (Sigma) as the substrate. Tartaric acid (Sigma) was added to the incubation medium at a concentration of 100 mmol/L. Control sections, incubated in incubation solution without substrate, showed no staining. Counterstaining was performed using Mayer's hematoxylin (2 minutes). The sections were subsequently mounted with an aqueous mounting medium (Kaiser's glycerol gelatin; Merck). For the demonstration of virus CA (p30) antigen, immunohistochemistry was performed by the ABC (avidin-biotin complex) method on specimens fixed with 40% ethanol. Deplasticized sections were placed in 0.1 mol/L Tris-HCl buffer at pH 8.2 for 5 minutes. After the sections were blocked with 10% goat serum (Vector, Burlingame, CA) for 20 minutes, they were incubated for 2 hours at room temperature with a polyclonal rabbit anti-CA (p30) antibody at 1:50 and 1:100 dilution in Tris-buffered saline (TBS) (0.1 mol/L Tris, 0.15 mol/L NaCl, pH 8.2) with 1% goat serum. Bone sections of noninfected mice were used as controls. After washing in TBS containing 0.1% Tween 20 (3×5 minutes), the sections were incubated for 30 minutes with biotinylated goat anti-rabbit antibody (Vector) at 1:200 dilution in TBS with 1% goat serum. After washing (3×5 minutes, TBS with 0.1% Tween 20), sections were incubated for 30 minutes with avidin/biotinylated alkaline phosphatase complex (Vector). Thereafter the sections were rinsed (3×5 minutes, TBS with 0.1% Tween 20) and stained with the Vector Red kit according to the supplier's instructions. Levamisole (Vector) was added to the incubation medium at twice the concentration recommended by the supplier. Finally, the sections were counterstained with methyl green (Vector) and mounted with DePeX (Serva, Heidelberg, Germany).

Histomorphometry

All histomorphometric parameters are presented as two-dimensional terms and were calculated and expressed according to the suggestions made by the American Society for Bone and Mineral Research ASBMR nomenclature committee.⁴⁰ Structural parameters in femoral cancellous bone were determined with an automatic image analysis system (VIDAS; C. Zeiss, Oberkochen, Germany) connected to a Zeiss stereomicroscope via a TV camera (Bosch, Stuttgart, Germany), from sections stained with von Kossa/toluidine blue. The area within 0.25 mm of the growth plate was excluded from the measurements. The average measuring area was about 5 mm² in each section. The image analysis system automatically determined the measuring area (tissue area, T.Ar), bone area (B.Ar), bone perimeter, and the number of trabeculae (N.Tb). From these data, the structural parameters were calculated: bone area (B.Ar/T.Ar), trabecular width, trabecular area (Tb.Ar), trabecular number per tissue area, trabecular number per bone area, trabecular number (Tb.N), and trabecular separation (Tb.Sp). Trabecular area was given by dividing B.Ar by N.Tb and represents the mean area (in mm²) of individual trabeculae. Trabecular number per bone area represents the number of individual trabecular profiles found per mm² of cancellous bone, with N.Tb/B.Ar = 1/Tb.Ar. For the calculation of Tb.N and Tb.Sp the parallel plate model was used.⁴⁰

Cellular and dynamic histomorphometric measurements were made using a semiautomatic system (Video-plan; C. Zeiss) and a Zeiss Axioskop microscope with a drawing attachment. In the centrally located cancellous bone of the distal femoral metaphysis, about 1.25 mm² of tissue area was evaluated in each section. The area within 0.25 mm from the growth plate was excluded from the measurements. Osteoclast numbers were measured in sections stained for TRAP, and dynamic, fluorochrome-based parameters were measured in unstained sections. Osteoclasts were defined as TRAP positive cells in contact with bone surfaces, showing one or more nuclei in the section. The numbers of TRAP positive osteoclasts were expressed, using the bone perimeter as referent. The mineralizing perimeter was defined as the percentage of fluorochrome double-labeled bone perimeter. The mineral apposition rate (MAR) between calcein labels was measured at $\times 400$, sampling each double label every 50 μ m. Values for MAR were not corrected for the obliquity of the plane of section. The bone formation rate was calculated by multiplying the mineralizing perimeter with the MAR.

Southern Blot Analysis

DNA from liver, muscle, brain, spleen, thymus, femur, humerus, and calvaria was isolated using a QuiAmp Tissue Kit for DNA purification (Quiagen); digested with *Hind*III, which generates virus/cell junction fragments, with *Eco*RI, which cuts in the host DNA, or with *Pst*I, which cuts in the viral LTRs; separated on 0.7% agarose gels; electrophoresed; and blotted on Zeta-Probe nylon membranes (Bio-Rad). Hybridization was performed with ³²P

random priming-labeled probes (activity: $1\text{--}2 \times 10^6$ cpm/ml) at 65°C overnight (rediprime DNA labeling system; Amersham). Membranes were washed in 2 $\times$ sodium citrate and sodium chloride (SSC) and 0.1% sodium dodecyl sulfate (SDS) (20 minutes, room temperature), 1 $\times$ SSC and 0.1% SDS (20 minutes, 65°C), and 0.2 $\times$ SSC and 0.1% SDS (45 minutes, 65°C). Membranes were exposed either to Fuji phosphor imager screens or Kodak MRX Roentgen films for various time periods.

The probes used for hybridization included an ecotropic MuLV env-specific probe,⁴¹ an immunoglobulin heavy chain probe pJ11,⁴² and a light chain, JK1-5 probe.⁴³ The RBL-5-17 probe was a 730-bp *Pst*I fragment of the murine *C β 1* coding and 3' untranslated region. To generate a 687-bp polymerase chain reaction (PCR) fragment as a TCR β -J1 probe, upstream primer: 5'-GGGTCCCATCAGC TCTTTGGGAG-3', positions 1261–1283, and downstream primer: 5'-GGGTCCAGATGGGAAGGGACGACTCTGT-3', positions 1970–1942, were used. The J2 probe was a 713-bp PCR fragment (upstream primer: 5'-GCTTCTTGG CAACTGCAGCGGGGAGT-3', position: 1295–1320, downstream primer: 5'-CTGGGTCTCCAACACTGCTTCAAGTG-3', position: 2058–2033). PCR was performed with a PE thermal cycler in 50 μ l with Boehringer's PCR Master kit (containing 2.5 U Taq polymerase in Brij 35; 0.005% v/v dATP, dCTP, dGTP, dTTP (each 0.2 mmol/L); 10 mmol/L Tris-HCl; 50 mmol/L KCl; 1.5 mmol/L MgCl₂). Twenty-five picomoles of each primer and 250 ng DNA were used. Procedures were as follows: amplification: 40 cycles; denaturation: 94°C, 1.2 minutes (first cycle 94°C, 5 minutes); annealing: 60°C, 1.2 minutes; extension: 73°C, 3 minutes (last cycle 73°C, 8 minutes). PCR fragments were electrophoresed in a 1.5% agarose gel, cut, and purified with a Qiagen gel extraction kit. Southern hybridizations have been described in detail elsewhere.⁴⁴

Statistical Analysis

Statistics were computed using SPSS for Windows 6.1 (SPSS, Chicago, IL). Because of the small number of animals and high variances for some variables, all data were log-transformed before statistical analyses. Statistical differences between infected and control groups in male and female mice were evaluated using Student's *t*-test. Differences in osteosclerosis and lymphoma incidence were determined by Fisher's exact test. *P* values less than 0.05 were considered significant. The data are presented as means $\pm$ SEM.

Results

Skeletal Tissues Are Primary Target of RFB MuLV

To determine virus dissemination in infected mice, several tissues were analyzed at different time points after infection. At 10, 21, and 30 days after infection, Southern analysis of *Pst*I-cleaved DNA showed faint signals of ecotropic provirus sequences in muscle and brain and weak but distinct signals in spleen, thymus, and liver of infected mice. Strong signals were detected in femur, humerus, and calvaria, indicating a considerably higher

Figure 1. Southern blotting of DNA from liver, muscle, spleen, brain, femur, humerus, and calvaria of two 3-week-old mice (A, B) infected at birth with RFB MuLV. High-molecular-weight DNAs were digested with *Pst*I (top) and *Hind*III (bottom), and the restriction fragments were electrophoresed, blotted, and probed with an ecotropic retrovirus-specific probe. Equal amounts of DNA were loaded to detect variable numbers of proviral copies in the different tissues. The data show high numbers of proviral copies in the skeletal tissues and a lower number in spleen (top). The absence of distinct bands in *Hind*III-digested DNAs indicates heterogeneous integration sites in the infected tissues. X, not investigated.

number of proviral sequences in the skeletal tissues. Whereas *Pst*I restriction cuts in the viral LTR and the signal intensity points to the total number of provirus copies, *Hind*III restriction, cutting once in the viral genome, gave faint signals in the skeletal tissues and spleens, suggesting common integration of proviral sequences in up to 10% of cells in the tissues investigated (Figure 1). At 10 and 30 days after infection, all infected mice showed mean infectious titers of 1.7×10^4 and 3.4×10^4 virus particles, respectively, per ml blood, indicating viraemia for extended periods of time and new integrations of proviruses in the skeletal tissues. Control NMRI mice did not harbor ecotropic proviruses, and infectious virus particles were not detected.

Immunohistochemistry with a polyclonal anti-CA (p30) antibody in plastic sections taken from humeri and femora of 2- and 4-week-old RFB MuLV-infected mice showed viral CA protein expression in the majority of bone marrow cells. Intense staining was detected in the cytoplasm of megakaryocytes (Figure 2A). In agreement with the results obtained from Southern analysis, high expression of CA was detected in periosteal cells (Figure 2C) and endosteal osteoblasts in the primary spongiosa (Figure 2D). The strongest staining was found in osteocytes (Figure 2C). Four weeks after infection, the staining

pattern for CA in bone and bone marrow was very similar; the number of stained osteocytes, however, was higher at 4 weeks relative to 2 weeks after infection (not shown). Labeling of osteoclasts was weak or was not detectable at 2 and 4 weeks (Figure 2, E and F).

RFB MuLV Induces an Increase in Cancellous Bone Mass

X-ray analysis of whole skeletons of RFB MuLV-infected mice showed beginning osteosclerosis in some animals as early as 3 months after infection (Figure 3). In these mice, increased bone mass was most prominent at the distal femora, the proximal tibiae, the cranial part of the os ilium, and the lumbar and tail vertebrae (Figure 3A), indicating that bones mainly composed of trabecular bone represent preferential sites of early osteosclerosis development. In mock-infected control mice there were no radiological signs of osteosclerosis (Figure 3B), even in 18-month-old mice (data not shown).

Histological analysis revealed that RFB MuLV infection induced a striking increase in cancellous bone mass, as shown in the distal femur (Figure 4), which was already accompanied by marked trabecular thickening 3 months after infection in all animals. The increase in cancellous bone was more pronounced in infected males than in females. Moreover, male and female infected mice showed increased cortical bone thickness.

To further characterize the skeletal effects of RFB MuLV quantitatively, bone histomorphometry was performed and the data were plotted as a function of age. In both female and male control mice we observed an age-dependent decline in cancellous bone mass between 3 and 9 months of age (Figure 5). RFB MuLV induced a significant increase in cancellous bone mass in both female and male mice at 3 months of age and at the later time points. Furthermore, the increase in cancellous bone mass in infected mice was accompanied by distinct structural changes in trabecular bone architecture (Table 1). At 3 months, RFB MuLV infection increased trabecular width significantly in both female and male mice. Mean trabecular area increased in RFB MuLV-infected mice by about fourfold in females and 24-fold in males. These structural changes persisted in infected mice throughout the experiment (data not shown). In both female and male mice, the alterations in trabecular bone architecture, especially the increase in trabecular width, appeared to be the most sensitive indicator of the early skeletal effects of RFB MuLV in this study.

Bone formation as assessed by dynamic, fluorochrome-based histomorphometry was augmented in RFB MuLV-infected mice (Table 1, Figure 6). This effect was more pronounced in male mice. At 3 months, infected male mice had an almost fourfold higher bone formation rate, whereas infected female mice showed a 39% increase in bone formation rate, relative to sex-matched controls. It is interesting to note that female control mice had substantially higher dynamic indices of cancellous bone formation compared with age-matched male control mice.

Figure 3. Osteosclerosis development after RFB MuLV infection of mice. **A:** The mouse was infected as a newborn and X-rayed at 3 months. **B:** Representative X-ray photograph of a mock-infected control mouse, showing increased bone density in the os ilium, lumbar and sacral vertebrae, distal femora, and proximal tibia, as well as the normal appearance of the cortical and spongy bone structures.

Retrovirus Infection Reduces the Number of Osteoclasts

To test for the involvement of the cellular compartment responsible for bone resorption in retrovirus-induced osteosclerosis, we investigated whether retrovirus infection affects the number of TRAP-positive osteoclasts. Infected male and female mice showed reduced numbers of TRAP-positive osteoclasts in cancellous bone throughout the observation period (Figure 7). This decrease in osteoclast numbers was more pronounced in male mice than in females, and the differences were greater at later time points.

Cocultivation of osteoblast-like, osteoclastogenic MB1.8 cells with either bone marrow cells or spleen cells from 11- to 12-day-old mice resulted in the formation of similar numbers of TRAP-positive, multinucleated cells (data not shown). After infection of MB1.8 cells with RFB

MuLV, the mean number of TRAP-positive cells per 96-well cavity dropped from 30.9 ± 6.9 to 3.8 ± 0.7 . In individual cultures of MB1.8 cells, spleen cells, or RFB MuLV-infected spleen cells, cultivated in the presence of 10^{-8} mol/L $1,25(\text{OH})_2$ vitamin D_3 , TRAP-positive, multinucleated cells were not detected (Table 2).

Osteoclasts in RFB MuLV-Infected Mice Show Normal Morphology and Function

TRAP staining (Figure 2, F and G) showed the presence of intensely TRAP-positive, mono- and multinucleated cells in the primary and secondary spongiosa of the distal femoral metaphysis and in cortical bone resorption sites in RFB MuLV-infected mice. Furthermore, bone-resorbing osteoclasts with normal morphology were detected in Howship's lacunae (Figure 2H), revealing no morphological evidence for a functional defect in osteoclasts of RFB MuLV-infected mice.

RFB MuLV-Treated Osteoclasts Show Unaltered Bone Resorption in Vitro

In vitro analyses with isolated osteoclasts showed stimulation of osteoclastic bone resorption by conditioned medium of osteoblasts, but not of fibroblasts (Table 3). The addition of 10% fetal bovine serum, known to support osteoclast-mediated bone resorption, increased the number of pits in an additive manner. Pit formation was observed in an equal manner when the osteoclasts were treated with RFB MuLV, present in the conditioned medium of infected osteoblasts (Table 3). Furthermore, the pit areas were not changed in cultures of osteoclasts, which were exposed to RFB MuLV or were in control cultures. These findings further suggest that RFB MuLV did not affect the resorption activity of mature osteoclasts.

Osteosclerosis Development Precedes Lymphomagenesis

Previous studies showed that RFB MuLV-induced lymphomas develop after a latency period of 5–11 months, and at the time of necropsy, the majority of lymphoma-diseased mice presented with osteosclerosis.¹¹ To investigate the onset of osteosclerosis and a possible coupling of the skeletal lesion to lymphomagenesis, cohorts of infected female and male mice were investigated at sev-

Figure 2. Virus expression, and osteoclast morphology and function in RFB MuLV-infected and control mice. **A–F:** Ethanol-fixed humeri from 2- and 4-week-old mice. The sections **A–E** were labeled with a polyclonal rabbit antibody against MuLV capsid protein CA (p30) by the ABC method and counterstained with methyl green. **F:** Section stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with Mayer's hematoxylin. Three-micron-thick undecalcified sections, $\times 360$ (**A, B, D–F**) and $\times 125$ (**C**). **A:** Most cells in bone marrow stain positive with anti-CA antibody 2 weeks after RFB MuLV infection. Virus CA protein expression is most prominent in the cytoplasm of megakaryocytes. **B:** Bone marrow section of a control mouse showing no retrovirus expression. **C:** Longitudinal section of cortical bone, 4 weeks after RFB MuLV infection. The p30 protein is demonstrable in periosteal (open arrow) and endosteal (solid arrow) osteoblasts and is highly enriched in most but not all osteocytes. **D:** Osteoblasts in the primary spongiosa, showing intense labeling with anti-viral CA protein. **E** and **F:** Serial sections of a periosteal bone resorption site showing low or absent viral CA protein in osteoclasts (**E**, arrowheads). The same cells express TRAP activity (arrowheads in **F**). Osteoblasts and osteocytes in these sections show viral CA expression (**E**, arrows). **G** and **H:** Growth plate cartilage-metaphyseal junction in the distal femur (PFA-fixed) of 3-month-old mice neonatally infected with RFB MuLV. Three-micron-thick undecalcified sections, $\times 360$. **G:** Section stained for TRAP and counterstained with Mayer's hematoxylin. It shows numerous TRAP-positive osteoclasts located beneath the growth plate and in the proximal metaphysis. **H:** Toluidine blue-stained section showing osteoclasts with normal morphology located within Howship's lacunae (arrows).

Figure 4. Undecalcified sections (3 μ m thick) of the distal femora of female (A) and male (C) control NMRI mice and female (B) and male (D) NMRI mice neonatally infected with RFB MuLV, 3 months postinfection. In both female and male mice, RFB MuLV infection induced a striking increase in cancellous bone mass, which was accompanied by trabecular thickening. It is evident that the increase in cancellous bone in response to virus infection was more pronounced in male mice. Moreover, infected male and female mice showed increased cortical bone thickness. Note the sexual dimorphism of cancellous bone structure in female (A) and male (C) control NMRI mice. Although the amount of cancellous bone in the femoral metaphysis was similar in male and female control mice, females had thicker trabeculae located predominantly in the metaphyseal area beneath the growth plate, whereas males exhibited a more evenly distributed network of thinner bone spicules. Von Kossa/toluidine blue stain, $\times 20$.

eral time points. The histomorphometric analyses had shown that trabecular width was one of the most sensitive indicators of the early skeletal effects of RFB MuLV infection. Therefore, we used this parameter to assess skeletal changes indicative of osteosclerosis. They were defined as an increase in trabecular width of more than two standard deviations above control levels. Using this method, osteosclerosis was detected in all infected mice at 3 (6/6) months and at 6 (9/9) months, and in 93% (14/15) at 9 months (Table 4). At 3 months the size of the spleen, thymus, and lymph nodes of infected mice was similar to that of controls. At 6 months lymphomas were detected in 22% (2/9) of infected mice, and at 9 months in 40% (6/15). Some of the lymphoma-negative mice showed enlarged spleens; however, gross pathological examination and comparison of the lymphatic organs of infected mice with controls excluded lymphoma, accord-

Figure 5. Development of the cancellous bone area (B.Ar/T.Ar) in the distal femoral metaphysis of female (A) and male (B) control NMRI mice and NMRI mice neonatally infected with RFB MuLV, plotted as a function of age. Each data point represents the mean $\pm$ SEM of three to six animals. Statistical comparisons between groups were performed using a two-sided *t*-test. * *P* < 0.05 versus female or male control group, respectively.

ing to the criteria established earlier,¹⁹ in thymus, spleen, or lymph nodes. Osteosclerosis incidence was significantly higher than that of lymphoma at 3, 6, and 9 months after infection. These data show that RFB MuLV-induced osteosclerosis develops early in the disease process and precedes lymphomagenesis by several months. There were no significant differences in osteosclerosis or lymphoma incidence at the different time points between female and male mice, and none of these lesions were observed in the 27 mock-infected control mice. The data are summarized in Table 4.

Young Osteosclerotic Mice Show Absence of Lymphoma and Random Provirus Integration in Lymphatic Tissues

To exclude lymphomas in 3-month-old RFB MuLV-infected mice molecularly by immunoglobulin gene and T-cell receptor- β (TCR- β) rearrangement analysis, and to establish the absence of a clonal evolution of a distinct proviral integration pattern, we investigated spleens and

Table 1. Histomorphometric Data in the Cancellous Bone of the Distal Femoral Metaphysis in 3-Month-Old Male and Female RFB MuLV-Infected and Control NMRI Mice

Variable	Female RFB-infected (n = 3)	Female control (n = 4)	Male RFB-infected (n = 3)	Male control (n = 4)
Tissue area (mm ²)	5.04 ± 0.56	5.41 ± 0.27	6.07 ± 0.75	5.46 ± 0.09
Trabecular number per tissue area (no./mm ²)	3.83 ± 0.90*	7.13 ± 0.09	3.45 ± 1.03*	15.08 ± 0.71
Trabecular number per bone area (no./mm ²)	19.78 ± 2.38*	81.9 ± 22.0	9.66 ± 4.49*	153 ± 14
Trabecular area (mm ² × 10 ⁻²)	5.23 ± 0.72*	1.43 ± 0.27	16.4 ± 7.2*	0.67 ± 0.06
Trabecular width (μm)	90.1 ± 2.1*	48.9 ± 3.4	101 ± 18*	33.6 ± 1.5
Trabecular number (no./mm)	2.72 ± 0.48	2.57 ± 0.33	5.35 ± 0.07*	3.82 ± 0.34
Trabecular separation (μm)	318 ± 58	376 ± 69	107 ± 13*	242 ± 26
Mineralizing perimeter (%)	48.3 ± 3.6	37.5 ± 6.8	39.1 ± 11.3	11.8 ± 5.2
Mineral apposition rate (μm/day)	2.24 ± 0.05	1.92 ± 0.30	1.17 ± 0.12	0.95 ± 0.08

All values are means ± SEM.

*P < 0.05 vs. corresponding male or female control.

thymuses, which are the hematopoietic target tissues of murine leukemia viruses,⁴⁶ in infected and control mice. In the spleens of infected 3-month-old mice (three males and three females) there were no rearrangements detected in either the heavy or light chain Ig gene or the TCR-β J1 region (Figure 8). One female and three male control mice out of eight (four males and four females) showed rearrangements in the heavy or light chain gene or TCR-β J1 region. The thymus of one infected male mouse, although not enlarged, showed rearrangements of both heavy and light chain, with both light chain alleles affected, and loss of the germline band at the TCR-β J2 (Figure 9) and constant region (not shown).

The 6-month-old lymphoma-free mice nos. 336-5 and 336-17, which had an enlarged spleen, showed germline patterns in the Ig and TCR-β J2 regions (not shown), whereas Ig rearrangement and absence of the germline band in the TCR-β J2 region were detected in the thymus of the 9-month-old lymphoma-bearing mouse.

At 3 months after infection, Southern analysis of proviral integrations showed an 8.2-kb band after *Pst*I cleavage of splenic DNA from infected mice. No signals were detected after cutting with *Hind*III, an enzyme that generates virus/cell junction fragments, or with *Eco*RI, which cuts in the cellular DNA, indicating random integration of RFB MuLV in lymphoid tissues at this time point. Some mice showed enlarged spleens at 6 months after infection. DNAs taken from the spleen of these mice also showed an 8.2-kb band after *Pst*I restriction, and no bands after *Hind*III or *Eco*RI restriction. In 8- and 9-month-old mice with follicular center cell lymphoma (FCC) and lymphocytic lymphoma, we detected characteristic *Pst*I bands of 8.2 kb, new *Pst*I bands of 2.3 kb, and distinct new proviral bands of 19, 13, and 6.4 kb after *Eco*RI restriction. These findings indicate clonal expansion of cells harboring newly integrated proviral copies in lymphoma tissues, but not in normal-sized or enlarged spleens of infected mice (data not shown).

Discussion

RFB MuLV is an ecotropic C-type retrovirus isolated from CF-1 strain mice. Previous investigations have shown

that, depending on the mouse's genetic background, RFB MuLV-infected mice develop lymphomas, osteosclerotic lesions, and/or osteomas.^{11,14,15} As leukemias and lymphomas represent the prevalent disease in MuLV-infected mice,^{1,3-5,8,47} it has only recently been established that RFB MuLV, as well as other murine leukemia viruses,^{10,18,19,48} induce skeletal lesions.^{11,14,15} Because

Figure 6. Bone formation rate in the distal femoral metaphysis of female (A) and male (B) control NMRI mice (solid line) and NMRI mice neonatally infected with RFB MuLV (dotted line), plotted as a function of age. Each data point represents the mean ± SEM of three to six animals. Statistical comparisons between groups were performed using a two-sided *t*-test. * P < 0.05 versus female or male control group, respectively.

Figure 7. Number of TRAP-positive osteoclasts/mm in the distal femoral metaphysis of female (A) and male (B) control NMRI mice (solid line) and NMRI mice neonatally infected with RFB MuLV (dotted line), plotted as a function of age. Each data point represents the mean $\pm$ SEM of three to six animals. Statistical comparisons between groups were performed using a two-sided *t*-test. * *P* < 0.05 versus female or male control group, respectively.

of the absence of overt clinical signs of disease, the osteosclerotic lesions have only been diagnosed by routine X-ray analysis performed on lymphoma-diseased, MuLV-infected mice. Recent experiments showed an osteosclerosis incidence of 60% in RFB MuLV-infected NMRI mice presenting with lymphoma after a mean lymphoma latency of 8 months,¹¹ indicating the limitations of X-ray analysis for diagnosing osteosclerosis in small rodents.

Table 2. RFB MuLV-Induced Abrogation of Osteoclast Differentiation

Cells/coculture	No. of wells assayed	Osteoclast precursors and osteoclasts ($\pm$ SEM)
MB1.8 cells + spleen cells	12	30.9 $\pm$ 6.9
RFB MuLV-infected MB 1.8 cells* + spleen cells	12	3.8 $\pm$ 0.7
MB1.8 cells	12	0
Spleen cells	12	0.3 $\pm$ 0.2
RFB MuLV-infected spleen cells	12	0.5 $\pm$ 0.4

The assays were carried out in microtiter plates in the presence of 10^{-8} mol/L 1,25-(OH)₂ vitamin D₃. The numbers of tartrate-resistant acid phosphatase (TRAP)-positive mononuclear osteoclast precursor cells and multinuclear osteoclasts were determined per well. Spleen cells and bone marrow cells were found to generate similar numbers of TRAP-positive mono- and multinuclear cells after cocultivation with osteoblastic MB1.8 cells.

*Immunohistochemical analysis showed 100% of the MB1.8 cells expressing viral CA (p30) protein (data not shown).

The current study, investigating time cohorts of RFB MuLV-infected mice at 3-month intervals, has shown for the first time that pronounced skeletal changes are detectable as early as 3 months after infection in all infected mice. At this time point, pathological examination or molecular analysis of lymphatic organs⁴⁶ of infected and control mice did not show any signs of lymphoma in spleen, thymus, or lymph nodes. Surprisingly, Southern analysis showed Ig- or TCR- β rearrangements in the spleen of four control mice. Both Ig- and TCR rearrangements were found in one control mouse in the spleen and in one infected mouse in the thymus. In the NMRI strain mice used in these studies, spontaneous lymphomas develop with low incidence and generally after the first year of life.^{10,11,19} In similar studies with NFS.V mice congenic for ecotropic MuLV, the mean latency of lymphomas was 10–20 months,⁴⁹ and the great majority of lymphomas showed clonal evolution of distinct proviral integration patterns, with similarities even at different sites in one mouse.⁴⁶ The detection of clonal rearrangements of Ig- and TCR- β genes by Southern blot hybridization are usually but not always indicative of B- or T-cell lymphomas.⁵⁰ However, in view of the absence of gross pathological findings in the lymphatic organs, we consider these additional bands in 3-month-old controls to be an indication of focal maturation rather than lympho-

Table 3. Effect of Conditioned Media from RFB MuLV-Infected and Normal Osteoblast-like Cells on Number and Size of Excavation Formed by Isolated Osteoclast Cultured on Ivory Slices

Osteoclast treatment	Pits/ivory slice*($\pm$ SEM)	Pit area (μ m ²)† ($\pm$ SEM)
α MEM (control medium)	1.2 $\pm$ 0.5	1254 $\pm$ 131
α MEM + 10% FCS	4.9 $\pm$ 1.2	1266 $\pm$ 155
CM NIH3T3 + 10% FCS	4.5 $\pm$ 1.4	894 $\pm$ 131
CM OB	8.4 $\pm$ 5.2	1091 $\pm$ 182
CM OB + 10% FCS	14.0 $\pm$ 4.1	967 $\pm$ 97
CM OB RFB MuLV-infected + 10% FCS	11.9 $\pm$ 3.5	937 $\pm$ 128

*Number of pits (means $\pm$ SEM) scored on eight different ivory slices, cultured individually in separate wells of a 24-well tissue culture plate.

†Area per pit (means $\pm$ SEM) of all pits found on eight slices per treatment. FCS, fetal calf serum; CM OB, osteoblast-conditioned medium; CM NIH3T3, fibroblast-conditioned medium.

Table 4. Osteosclerosis and Lymphoma Incidence in RFB MuLV-Infected NMRI Mice

Mice	Months after infection					
	3		6		9	
	Osteopetrosis	Lymphoma	Osteopetrosis	Lymphoma	Osteopetrosis	Lymphoma
RFB MuLV infected	100% (6/6)*	0% (0/6)	100% (9/9) [†]	22% (2/9)	93% (14/15) [‡]	40% (6/15) [§]
Controls	0% (0/9)	0% (0/9)	0% (0/8)	0% (0/8)	0% (0/10)	0% (0/10) [§]

Osteosclerosis was defined as an increase in trabecular width of more than two standard deviations above control levels, as assessed by histomorphometric analyses. Data show osteosclerosis and lymphoma incidence at the different time points: *24 out of 30 mice and [†]15 out of 30 mice were still alive at this time point without signs of lymphoma and were not yet analyzed for osteosclerosis. Osteosclerosis and lymphoma incidence differed significantly at each time point.

[‡]Ten more control mice (four females, six males) were investigated at month 12 without signs of either lymphoma or osteosclerosis.

[§]There were no significant differences of osteosclerosis or lymphoma incidence between infected female and infected male mice.

ma.^{51–53} In the present study, some infected mice showed enlarged spleens without signs of lymphoma. However, these mice did not show proviral *EcoRI* or *HindIII* bands, supporting the absence of clonal evolution of cells with a distinct new proviral integration pattern.⁴⁶ In contrast, clonal patterns were detected in 8- and 9-month-old mice, which showed follicular center cell lymphomas or lymphoblastic lymphoma. The presence of such bands in the tumors indicates that the lymphomas show mono- or oligoclonal expansion of cells with a given provirus integration pattern in infected mice. Taken together, the absence of signs of lymphoma by pathological examination and the lack of clonal evolution of a distinct proviral pattern in the organs of infected mice strongly indicate that lymphoma was not present in 3-month-old RFB MuLV-infected mice. Therefore, our study indicates that the induction of new bone formation and the increase in cancellous bone of RFB MuLV-infected mice precedes lymphomagenesis by several months, suggesting that the skeletal lesions, consistent with the term *osteosclerosis*, and the lymphomas develop independently.

In agreement with the temporal dissociation between the onset of osteosclerosis and the onset of lymphoma,

we have shown here by Southern analysis and immunohistochemistry that skeletal tissues are primary targets for RFB MuLV in the early viraemic state after infection. Immunohistochemical analysis detected intensely labeled osteoblasts and osteocytes with anti-viral CA antibody at 2 and 4 weeks after infection. These data confirm earlier electron microscopic findings showing virus particles budding from osteoblasts and osteocytes in the primary spongiosa of 4-week-old infected mice¹³ and abundant virus particles in osteocyte lacunae in the cortical bone of femurs and lumbar vertebrae in osteosclerotic bone up to 14 months after infection.⁵⁴ In the present study viral protein was either low or was not detectable in osteoclasts of infected mice. However, *in vitro* cultures of RFB MuLV-infected osteoblasts with spleen cells resulted in viral CA expression in all multinucleated, TRAP-positive cells (unpublished data). High susceptibility of osteoclasts or their progenitors to retrovirus infection and virus expression in these cells was recently shown in mice that were infected neonatally with the pBAG retroviral vector⁴⁵. Our *in vitro* data further indicate that treatment of mature osteoclasts with conditioned medium from MB1.8-RFB MuLV-infected osteoblasts, which contains high titers of infectious virus particles, did not affect

Figure 8. Immunoglobulin and T cell receptor gene rearrangements in spleens of RFB MuLV-infected and control mice. Southern blots of DNAs after cleaving with *EcoRI* or *HindIII* and hybridization with probes recognizing the immunoglobulin heavy and light chain and the J1 region of the murine TCR β -chain locus. Ig rearrangements were detected in three control mice and in lymphomas of 8- and 9-month-old mice, but not in infected 3-month-old mice. TCR β -J1 rearrangements were detected in two 3-month-old control mice, but not in lymphomas of 8- and 9-month-old mice, indicating development of B-cell lymphomas after RFB MuLV infection. 336-1 to 336-3, 3-month-old RFB MuLV-infected females; 340-1 to 340-4, control females; 336-5 and 336-17, 6-month-old infected females; 337-1 to 337-3, 3-month-old infected males; 341-1 to 341-4, control males; 337-13 and 337-15, 8- and 9-month-old infected mice with lymphoma. X, not investigated.

Figure 9. Immunoglobulin and T-cell receptor gene rearrangements in thymuses of RFB MuLV-infected and control mice. Southern blots of DNAs, as shown in Figure 6, are of thymic DNA from male mice. Heavy chain and light chain rearrangements and absence of the germ line band in the TCR- β J2 region are shown in one infected 3-month-old mouse and the thymus of a 12-month-old lymphoma-bearing mouse.

their resorption activity *in vitro*. Therefore, although cells of the osteoclastic lineage appear to be susceptible to retroviral infection, viral protein expression in osteoclasts is low *in vivo*, as compared to osteoblasts and osteocytes, and resorption activity is not reduced.

In analogy to a well-characterized bone disorder in chicken,⁵⁵ which is induced by different isolates of avian retroviruses (for a review, see Ref. 56), the increase in bone mass in MuLV-infected mice was originally termed osteopetrosis^{17,19}, although the pathogenesis has been attributed to cells of the osteoblastic lineage in both species^{13,54,57}. The histological appearance of the affected bones in MuLV-infected mice shows considerably increased cancellous bone mass, associated with changes in cancellous bone architecture and characterized by pronounced thickening of individual structural elements. These features are anatomically consistent with the term osteosclerosis. Although the gross pathological pattern observed in infected mice differs from that described for ALV-induced osteopetrosis in chicken (for reviews, see Refs. 2 and 55), the pathogenesis of the two lesions seems to follow similar mechanisms. Osteopetrosis in chicken is due to enhanced osteoblast proliferation and extracellular matrix formation. A reduction of osteoclastic resorption appears to be secondary to the decrease in bone marrow space caused by excessive osteoblast-mediated bone deposition. Thus the term osteopetrosis was considered inappropriate by some authors,⁵⁸ whereas it was continuously used by others.^{55,59} Finally, MAV-2(O) has been shown to stimulate the proliferation and cloning ability of chicken bone cells,⁵⁹ supporting a recent notion that RFB MuLV infection of mouse osteoblasts extends the cells' lifespan *in vitro* and delays senescence⁶⁰. These findings suggest a common underlying mechanism in retrovirus-induced bone growth in both species.

A number of gene defects have been reported to cause variable degrees of osteopetrosis in mice.^{21–30,32} These lesions are brought about by partial or complete failure of osteoclast-mediated bone resorption. Although we did not observe any morphological evidence of impaired osteoclast function in RFB MuLV-infected mice or reduced resorption in an *in vitro* assay of RFB MuLV-treated osteoclasts, it cannot be ruled out that a reduced number of osteoclasts, as found in the infected mice, may indirectly contribute to a net increase in bone mass. The significant reduction of osteoclastic differentiation in co-cultures of the RFB MuLV-infected, osteoclastogenic, osteoblast-like MB1.8 cells with bone marrow or spleen cells supports the *in vivo* findings, and indicates that retrovirus infection of cells of the osteoblastic lineage interferes with their capacity to induce osteoclastic differentiation from mononuclear precursor cells and subsequently uncouples bone remodeling in favor of bone formation. Thus the bone structural alterations in infected mice, together with the interference with osteoblast-mediated osteoclastic differentiation, strongly suggest that cells of the osteoblastic lineage mainly mediate the skeletal effects of RFB MuLV. In line with this hypothesis, we have previously shown that infection of murine osteoblasts with osteosclerosis-inducing MuLVs enhances osteoblastic differentiation, extracellular matrix formation,⁶¹ and endochondral ossification⁶² in cell and tissue cultures. Therefore, in contrast to the deletion-^{21–23} and knock-out genotypes,^{24–30,32} resulting in a direct block of osteoclast differentiation or osteoclastic bone resorption, retrovirus-induced osteosclerosis represents a gain-of-function model, which involves cells of the osteoblastic lineage as the primary virus target.

Based on recent viral recombinant studies in which the osteoma-inducing potential of RFB MuLV was mapped to coding regions of the viral genome,¹⁴ it is tempting to speculate that viral proteins expressed in osteoblastic cells induce the pathogenic effects leading to osteosclerosis. As the strong bone anabolic response leading to osteosclerosis appears to be a consequence of infection and insertion of the RFB MuLV genome into the DNA of osteoblasts and its continuous high expression thereafter, this model of profound MuLV-induced up-regulation of cancellous and cortical bone mass may facilitate further studies on the mechanisms underlying bone anabolic actions in general.

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References

1. Kozak CA, Ruscetti S: Retroviruses in rodents. The Retroviridae, vol 1. Edited by JA Levy. New York, Plenum Press, 1992, pp 405–481

2. Teich N, Wyke J, Mak T, Bernstein A, Hardy W: Pathogenesis of retrovirus-induced disease. *RNA Tumor Viruses*. Edited by R Weis, N Teich, H Varmus, J Coffin. New York, Cold Spring Harbor Laboratory, 1982, pp 785–998
3. Ben-David Y, Bernstein A: Friend virus-induced erythroleukemia and the multistage nature of cancer. *Cell* 1991, 66:831–834
4. Ethelberg S, Sørensen AB, Schmidt J, Luz A, Pedersen FS: An SL3–3 murine leukemia virus enhancer variant more pathogenic than wild type obtained by assisted molecular evolution in vivo. *J Virol* 1997, 71:9796–9799
5. Hallberg B, Schmidt J, Luz A, Pedersen FS, Grundström T: SL3–3 enhancer factor 1 transcriptional activators are required for tumor formation by SL3–3 murine leukemia virus. *J Virol* 1991, 65:4177–4181
6. Lovmand J, Sørensen AB, Schmidt J, Østergaard M, Luz A, Pedersen FS: B-cell lymphoma induction by Akv murine leukemia viruses harboring one or both copies of the tandem repeat in the U3 enhancer. *J Virol* 1998, 72:5745–5756
7. Hays EF, Levy JA: Differences in lymphomagenic properties of AKR mouse retroviruses. *Virology* 1984, 138:49–57
8. Speth C, Luz A, Strauss G, Wendel S, Zeidler R, Dorn S, Erfle V, Brem G, Lipp M, Schmidt J: Akv murine leukemia virus enhances lymphomagenesis in myc-kappa transgenic and in wild-type mice. *Virology* 1995, 206:93–99
9. Lenz J, Celander RL, Crowther R, Petarca DW, Perkins DW, Haseltine WA: Determination of the leukaemogenicity of a murine retrovirus by sequences within the long terminal repeats. *Nature* 1984, 308:467–470
10. Schmidt J, Luz A, Erfle V: Endogenous murine leukemia viruses: frequency of radiation-activation and novel pathogenic effects of viral isolates. *Leuk Res* 1988, 12:393–403
11. Gimbel W, Schmidt J, Strauss PG, Luz A, Brack-Werner R, Erfle V, Werner T: Molecular and pathogenic characterization of the RFB osteoma virus: lack of oncogene and induction of osteoma, osteopetrosis and lymphoma. *Virology* 1996, 224:533–538
12. Leib-Mösch C, Schmidt J, Etzerodt M, Pedersen FS, Hehlmann R, Erfle V: Oncogenic retrovirus from spontaneous murine osteomas. II. Molecular cloning and genomic characterization. *Virology* 1986, 150:96–105
13. Murray AB, Schmidt J, Rieke L: Retrovirus-induced osteopetrosis in mice: ultrastructural evidence of early virus production in osteoblasts and osteocytes. *Am J Pathol* 1986, 124:319–323
14. Østergaard M, Pedersen L, Schmidt J, Luz A, Lovmand J, Erfle V, Pedersen FS, Strauss G: Mapping of a major osteomagenic determinant of murine leukemia virus RFB-14 to non-long terminal repeat sequences. *J Virol* 1996, 71:645–649
15. Pedersen L, Behnisch W, Schmidt J, Luz A, Pedersen FS, Erfle V, Strauss PG: Molecular cloning of osteoma-inducing replication-competent murine leukemia viruses from the RFB osteoma virus stock. *J Virol* 1992, 66:6186–6190
16. Luz A, Murray AB, Schmidt J: Osteoma, spontaneous and virus-induced, mouse. *Monograph on Pathology of Laboratory Animals*. Edited by TC Jones, U Mohr, RD Hunt. New York, ILSI, Springer, 1991, pp 182–190
17. Murray AB, Schmidt J, Luz A: Osteopetrosis induced by retrovirus, mouse. *Cardiovascular and Musculoskeletal Systems*. Edited by TC Jones, U Mohr, RD Hunt. Berlin, Heidelberg, New York, Springer Verlag, 1991, 284–291
18. Pedersen L, Strauss PG, Schmidt J, Luz A, Erfle V, Jorgensen P, Kjeldgaard NO, Pedersen FS: Pathogenicity of BALB/c-derived N-tropic murine leukemia viruses. *Virology* 1990, 179:931–935
19. Schmidt J, Erfle V, Pedersen FS, Rohmer H, Schettters H, Marquart KH, Luz A: Oncogenic retrovirus from spontaneous murine osteomas. I. Isolation and biological characterization. *J Gen Virol* 1984, 65:2237–2248
20. Whyte MP: Sclerosing bone dysplasias. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, 3rd ed. Philadelphia, PA, Lippincott-Raven, 1996, pp 363–387
21. Nielsson SK, Bertonecello F: The development and establishment on hemopoiesis in fetal and newborn osteopetrotic (op/op) mice. *Dev Biol* 1994, 164:456–462
22. Philippart C, Theoval E, Moricard Y, Bringuier A-F, Seebold C, Lemoine F-M, Arys A, Dourov N, Labat M-L: Immune cell defects affect bone remodelling in osteopetrotic op/op mice. *Bone Miner* 1993, 23:317–332
23. Steingrimsdottir E, Moore KJ, Lamoreux ML, Ferré-D'Amaré AR, Burley SK, Zimring DC, Skow LC, Hodgkinson CA, Arnheiter H, Copeland NG, Jenkins NA: Molecular basis of mouse microphthalmia (ml) mutations helps explain their developmental and phenotypic consequences. *Nature Genet* 1994, 8:256–263
24. Soriano P, Montgomery C, Geske R, Bradley A: Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 1991, 64:693–702
25. Lowe C, Yoneda T, Boyce BF, Chen H, Mundy GR: Osteopetrosis in Src-deficient mice is due to an autonomous defect of osteoclasts. *Proc Natl Acad Sci USA* 1993, 90:4485–4489
26. Boyce BF, Chen H, Soriano P, Mundy GR: Histomorphologic and immunocytochemical studies of src-related osteopetrosis. *Bone* 1993, 14:335–340
27. Grigoriadis AE, Wang Z, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF: c-Fos a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* 1994, 266:443–448
28. Johnson RS, Spiegelman BM, Papaioannou V: Pleiotropic effects of a null mutation in the c-fos proto-oncogene. *Cell* 1992, 71:577–586
29. Hayman AR, Jones SJ, Boyde A, Foster D, Colledge WH, Carlton MB, Evans MJ, Cox TM: Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. *Development* 1996, 122:3151–3162
30. Tondravi MM, McKercher SR, Anderson K, Erdmann JM, Quiroz M, Maki R, Teitelbaum SL: Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature* 1997, 386:81–84
31. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R: Osteopetrosis in mice lacking NF- κ B1 and NF- κ B2. *Nature Med* 1997, 3:1285–1289
32. Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonardi A, Tran T, Boyce BF, Siebenlist U: Requirement for NF- κ B in osteoclast and B-cell development. *Genes Dev* 1997, 11:3482–3496
33. Wesolowski G, Duong LT, Lakkakorpi PT, Nagy RM, Tezuka K-I, Tanaka H, Rodan GA, Rodan SB: Isolation and characterization of highly enriched, perfusion mouse osteoclastic cells. *Exp Cell Res* 1995, 219:679–686
34. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T: Osteoclast-like formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* 1988, 122:1373–1382
35. Collin P, Guenther HL, Fleisch H: Constitutive expression of osteoclast-stimulating activity by normal clonal osteoblast-like cells: effects of parathyroid hormone and 1,25-dihydroxyvitamin D3. *Endocrinology* 1992, 131:1181–1187
36. Pattengale PK: Neoplastic lesions of the mouse lymphoid system. *Pathology of Neoplasia and Preneoplasia in Rodents*. Edited by P Bannasch, W Goessner. Stuttgart, New York, Schattauer, 1994, pp 168–176
37. Erben R: Embedding of bone samples in methylmethacrylate: an improved method suitable for bone histomorphometry, histochemistry, and immunohistochemistry. *J Histochem Cytochem* 1997, 45:307–313
38. Erben R, Kohn B, Rambeck WA, Zucker H: Histomorphometric analysis of the rat proximal tibial metaphysis by "linear scanning." *Scanning Microsc* 1990, 4:625–638
39. Baron R, Vignery A, Neff L, Silvergate A, Santa Maria A: Processing of undecalcified bone specimens for bone histomorphometry. *Bone Histomorphometry: Techniques and Interpretation*. Edited by RR Recker. Boca Raton, FL, CRC Press, 1983, pp 13–35
40. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR: Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 1987, 2:595–610
41. Chattopadhyay SK, Lander MR, Rands E, Lowy DR: Structure of endogenous murine leukemia virus DNA in mouse genomes. *Proc Natl Acad Sci USA* 1980, 77:5774–5558

42. Marcu KB, Banerji NA, Lang R, Arnheim N. 5' flanking region of immunoglobulin heavy chain region displays length heterogeneity in germ lines of inbred mouse strains. *Cell* 1980, 22:187-196
43. Lewis S, Gifford A, Baltimore D: DNA elements are asymmetrically joined during the site-specific recombination of kappa immunoglobulin genes. *Science* 1985, 228:677-685
44. Amtoft HW, Sørensen AB, Bareil C, Schmidt J, Luz A, Pedersen FS: Stability of AML1 (Core) site enhancer mutations in T lymphomas induced by attenuated SL3-3 murine leukemia virus mutants. *J Virol* 1997, 71:5080-5087
45. Okimoto MA, Fan H: Identification of directly infected cells in the bone marrow of neonatal Moloney murine leukemia virus-infected mice by use of a Moloney murine leukemia virus-based vector. *J Virol* 1999, 73:1617-1623
46. Tang JCO, Ho FCS, Chan ACL, Srivastava G: Clonality of lymphomas at multiple sites in SJL mice. *Lab Invest* 1998, 78:205-212
47. Corcoran LM, Adams JM, Dunn AR, Cory S: Murine T lymphomas in which the cellular myc oncogene has been activated by retroviral insertion. *Cell* 1984, 37:113-122
48. Schmidt J, Krump-Konvalinkova V, Luz A, Goralczyk R, Snell G, Wendel S, Dorn S, Pedersen L, Strauss PG, Erfle V: Akv murine leukemia virus enhances bone tumorigenesis in hMT-c-fos transgenic mice. *Virology* 1995, 206:85-92
49. Hartley JW, Chattopadhyay SK, Morse HC III, Fredrickson TN: Charlotte Friend Memorial Lecture: murine leukemia virus (MuLV) tumorigenesis. *Leukemia* 1997, 11(Suppl 3):147-148
50. Warnke RA, Weiss LM, Chan JKC, Cleary ML, Dorfman RF: Tumors of lymph nodes and spleen. Washington, DC, Armed Forces Institute of Pathology, 1994
51. Torlakovic E, Cherwitz DL, Jessurun J: B-cell gene rearrangement in benign and malignant lymphoid proliferations of mucosa-associated lymphoid tissue and lymph nodes. *Hum Pathol* 1997, 28:166-173
52. Collins RD: Is clonality equivalent to malignancy: specifically, is immunoglobulin gene rearrangement diagnostic of malignant lymphoma? *Hum Pathol* 1997, 28:757-759
53. Fishleder A, Tubbs R, Hesse B, Levine H: Uniform detection of immunoglobulin-gene rearrangement in benign lymphoepithelial lesions. *N Engl J Med* 1987, 316:1118-1121
54. Schmidt J, Casser-Bette M, Rodan GA, Baumgartner-Decker C, Erfle V: Bone Tumor-Inducing Retroviruses and Osteoblastic Cells in Vitro: Target Cell Specificity and Effects of Viral Infection. Edited by S Hurwitz, HJ Sela. Jerusalem, Heiliger, 1987, pp 73-91
55. Smith RE, Ivanyi J: Pathogenesis of virus-induced osteopetrosis in chicken. *J Immunol* 1980, 125:523-530
56. Payne LN: Biology of avian retroviruses. The Retroviridae, vol 1. Edited by JA Levy. New York, Plenum Press, 1992, pp 299-404
57. Foster RG, Lian JB, Stein G, Robinson HL: Replication of an osteopetrosis-inducing avian leukosis virus in fibroblasts, osteoblasts and osteopetrotic bone. *Virology* 1994, 205:179-187
58. Boyde A, Banes AJ, Dillaman RM, Mechanic GL: A morphological study of an avian bone disorder caused by myeloblastosis-associated virus. *Metab Bone Dis Relat Dis* 1978, 1:235-242
59. Schmidt EV, Smith RE: Avian osteopetrosis virus induces proliferation of cultured bone cells. *Virology* 1981, 111:275-282
60. Goralczyk R, Appold A, Luz A, Riemann S, Strauss PG, Erfle V, Schmidt J: Establishment and characterization of osteoblast-like cell lines from retrovirus (RFB MuLV)-induced osteomas in mice. *Differentiation* 1998, 63:253-262
61. Schmidt J, Casser-Bette M, Murray AB, Luz A, Erfle V: Retrovirus-induced osteopetrosis in mice. Effects of viral infection on osteogenic differentiation in skeletoblast cell culture. *Am J Pathol* 1987, 129:503-510
62. Livne E, Schmidt J, Closs EI, Silbermann M, Erfle V: Effects of leukemogenic retroviruses on condylar cartilage in vitro: an ultrastructural study. *Calcif Tissue Int* 1989, 44:25-35