Failure to Detect Measles Virus Ribonucleic Acid in Bone Cells from Patients with Paget's Disease

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Background: Paget's disease is a condition of focal accelerated bone turnover. Electron-microscopy investigations of osteoclasts from pagetic lesions have identified nuclear inclusion bodies that have a similar appearance to viral nucleocapsid particles. Subsequently, RNA from several paramyxoviruses has been detected in pagetic tissue, and it was suggested that these viruses, in particular measles, might play a role in the etiology of Paget's disease. We have tested for measles virus sequences in osteoblasts and bone marrow cells collected from pagetic lesions and healthy bone.

Methods: Bone and bone marrow samples were taken from Paget's patients and control subjects, and cells were cultured from each of these tissues. RNA was extracted from 13 osteoblast cultures and 13 cultures of bone marrow cells derived from pagetic lesions, and from 26 and 23 control osteoblast and bone marrow cultures, respectively. These samples were sourced from 22 patients with Paget's disease and 31 controls. RT-PCR-nested PCR amplification was used for the detection of the genes for the measles nucleocapsid and matrix proteins.

Results: Measles virus sequences were not detected in any of the pagetic or control samples. However, measles virus sequences were identified in samples of a measles virus culture isolate included as a positive control, and in a brain sample from a patient with subacute sclerosing panencephalitis, a condition associated with chronic measles infection.

Conclusion: The results of the study do not support the hypothesis that measles virus plays a role in the pathogenesis of Paget's disease. **(*J Clin Endocrinol Metab* 93: 1398–1401, 2008)**

Paget's disease of bone is characterized by focal regions of accelerated bone turnover. In the pagetic lesion, there is increased resorption by large, highly nucleated osteoclasts along with disordered bone formation by osteoblasts. Although Paget's disease affects up to 5% of the elderly population in some western countries, the etiology is not well understood. Genetic factors are certainly important, with mutations in SQSTM1 found in some familial and sporadic cases, but environmental factors may also play a role.

Studies suggest that viruses could be one of the nongenetic factors involved in the condition. Nuclear inclusions resembling paramyxoviral particles are often observed in osteoclasts from pagetic patients, and a number of publications have reported detection of measles virus mRNA or protein in samples from Paget's patients (1–5). However, others have repeatedly failed to detect viral RNA or antigens (6–8). There is also evidence that infection of bone marrow with measles virus or measles virus nucleocapsid (N) protein can cause the development of osteoclasts with a pagetic phenotype (9, 10). However, live measles virus has never been isolated from pagetic cells or tissue.

We have previously collected and cultured bone tissue and bone marrow from pagetic and control patients to characterize gene expression in this condition (11). The RNA collected from these cultures provides an opportunity to reexamine the involvement of measles virus in Paget's disease using a highly sensitive nested PCR technique.

0021-972X/08/$15.00/0

Printed in U.S.A.

doi: 10.1210/jc.2007-1978 Received September 6, 2007. Accepted January 22, 2008.

First Published Online January 29, 2008

Abbreviations: M, Matrix; N, nucleocapsid; RANK, receptor activator of nuclear factor-κB; SSPE, subacute sclerosing panencephalitis; TRAP, tartrate-resistant acid phosphatase.

Subjects and Methods

Tissue collection

Bone samples were collected from consenting subjects undergoing hip or knee replacements. Bone marrow was also collected from some of these subjects during surgery or, in seven of the patients with Paget's disease, by aspiration from an affected iliac crest. The details of the patients and samples are given in Table 1. The study had the approval of the local institutional review board. Samples collected from affected areas of the skeleton are described as "pagetic." "Non-pagetic" samples were collected from subjects without Paget's disease as well as from unaffected bone of patients with the condition. Most of the patients with Paget's disease had been treated with bisphosphonates.

Cell culture and RNA extraction

Primary osteoblast and bone marrow cultures were prepared as described previously (11). Briefly, osteoblasts and bone marrow were cultured in DMEM 10% fetal calf serum containing 5 μg/ml ascorbate-2-phosphate for 2–3 wk until confluent, then RNA was extracted.

Real-time PCR

cDNA synthesized using Superscript III (Invitrogen Corp., Carlsbad, CA) was used for multiplex real-time PCR on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). 18S rRNA was used as the endogenous control, and the $\Delta\Delta$Ct method was used to analyze results.

Measles virus detection

Aliquots of RNA were supplied to the National Institute for Biological Standards and Control, United Kingdom. Measles virus genome detection was performed blinded using the single-step RT-PCR-nested PCR amplification technique described previously (12). The N protein gene (primers: MV1, 5′-TTAGGGCAAGAGATGGTAAGG-3′, and MV2, 5′-GTTCTTCCGAGATTCCTCCCA-3′; and nested primers: MV3, 5′-AGCATCTGAACTCGGTATCAC-3′, and MV4, 5′-AGCCCTCGCATCACTTGCTCT-3′) and the matrix (M) protein gene (primers: MV13, 5′-GCGACAGGAAGGATGAATGC-3′, and MV14, 5′-GTTTGCGTTGAAGACACTCC-3′; and nested primers: MV15, 5′-TATGTACATGTTTCTGC-3′, and MV16, 5′-GTTGTTAGGACCTTTCTCC-3′) were amplified. The sensitivity limits of these assays were 5.5×10^{-2} to 2.5×10^{-4} plaque-forming units of measles virus per reaction (12, 13).

The positive controls used in this study were derived from a measles virus tissue culture isolate and from a brain sample of a subacute sclerosing panencephalitis (SSPE) patient. These controls have been routinely used at the National Institute for Biological Standards and Control for measles virus detection studies.

TABLE 1. Clinical characteristics of patients and sample details

	Paget's disease	Controls
No. of patients	22	31
No. of males/females	16/6	9/22
Median age (range)	78.5 yr (60–89)	67 yr (30–84)
Median serum ALP (range)[a]	98 IU/liter (57–667)	
Median no. of bones involved (range)	2 (1–15)	
Samples		
No. of non-pagetic OB	8	18
No. of pagetic OB	13	
No. of non-pagetic BM	2	21
No. of pagetic BM	13	

ALP, Alkaline phosphatase; BM, bone marrow; OB, osteoblast.

[a] Normal range for alkaline phosphatase 40–110 IU/liter.

Results

The phenotypes of the primary osteoblasts and bone marrow cells were characterized by studying the expression of bone-related genes. Real-time PCR analysis showed that although the bone marrow cells were not cultured under conditions that specifically stimulate osteoclast formation, the monocyte marker CD14 and the osteoclast-specific genes, receptor activator of nuclear factor-κB (RANK) and tartrate-resistant acid phosphatase (TRAP), were expressed in these samples (Fig. 1). These results indicate the presence of cells of the osteoclast lineage in this mixed-cell population from the bone marrow. As previously described, analysis of other bone-specific genes demonstrated the expression of alkaline phosphatase, osteocalcin, and bone sialoprotein in both osteoblasts and bone marrow cultures (11).

The results of RT-PCR-nested PCR amplifications for the N and M genes of the measles virus were negative for all 75 samples from the patients in Table 1. Measles virus specific cDNA fragments corresponding to the N and M gene regions were detected in the positive controls of a measles virus culture isolate and an SSPE brain sample. Testing for the N gene was performed using 75 to 150 ng total cellular RNA from patient samples, and 125 to 500 ng RNA was used for M gene amplification. This assay has previously been shown to detect viral RNA in preparations corresponding to as few as 18 SSPE cells (12), or samples with 16 copies of the measles virus N gene transcript (13). The quantity of RNA from cultured osteoblasts and bone marrow cells used in this study corresponds to at least 7,500 cells in the N gene assay and 12,500 cells for the M gene assay. This suggests that the expression levels of these transcripts, if present, are at least 415- and 690-fold lower, respectively, than in SSPE in all of the samples tested.

Discussion

We found no evidence for the presence of measles virus in pagetic bone cells of either the osteoclast or osteoblast lineages. Our bone marrow samples contained osteoclast precursors, as demonstrated by the expression of CD14, RANK, and TRAP. These samples are similar to those used by others who have found measles virus transcripts in bone marrow and peripheral blood from many Paget's patients using a less sensitive RT-PCR technique (3–5). Like the samples in our study, some of these positive samples had been cultured for up to 3 wk before RNA extraction (4). Unlike some of these cultures, ours were cultured from total bone marrow mononuclear cells and were not enriched for osteoclast lineage cells. Both the bone marrow and the osteoblast cultures in this study expressed the marker genes alkaline phosphatase, bone sialoprotein, and osteocalcin, indicating the presence of osteoblastic cells. The osteoblastic RNA was also found to be negative for measles virus. Some *in situ* hybridization studies have detected viral RNA in osteoblasts (2, 14), but RT-PCR studies in osteoblastic or stromal cells have been negative (4, 6).

Although some authors have never detected evidence of paramyxoviruses in pagetic tissue (6–8), other groups have

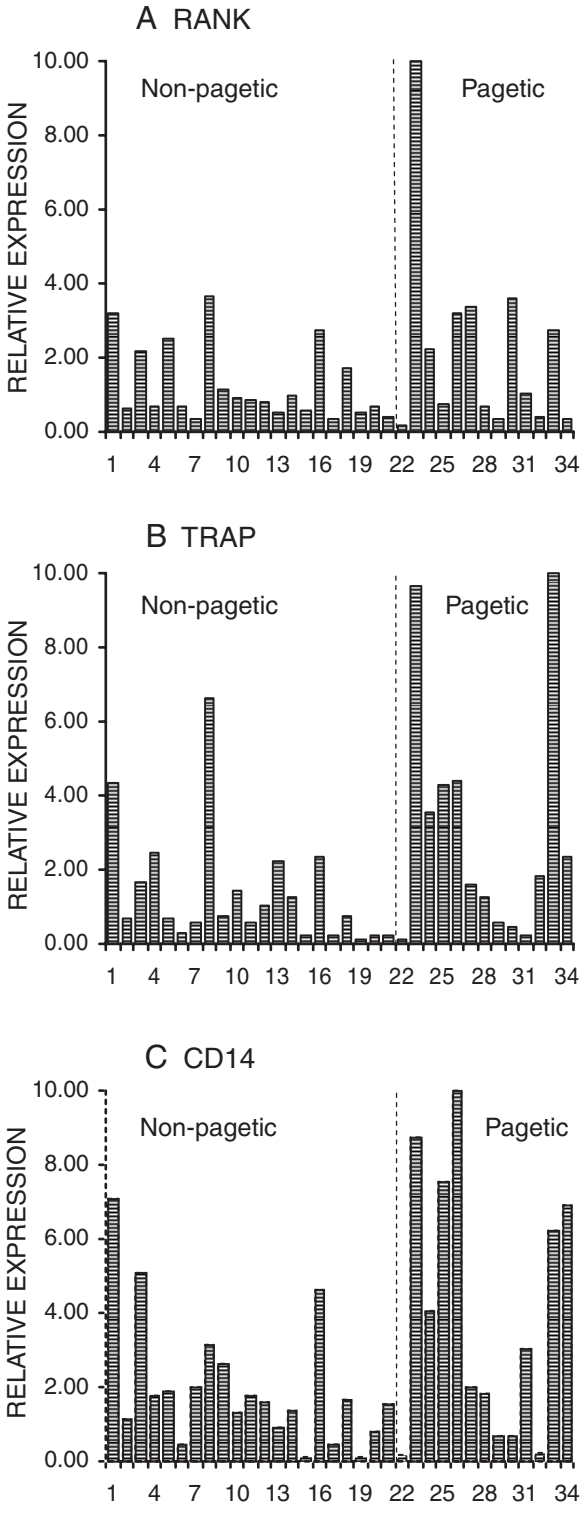

FIG. 1. Relative gene expression in RNA extracted from bone marrow cell cultures. Real-time PCR was used to determine the levels of expression of RANK (A), TRAP (B), and CD14 (C). The expression levels in the different samples were normalized to the expression of 18S rRNA and are presented relative to the sample with the highest expression level, which was normalized to 10.

found evidence for the presence of several different viruses. Apart from measles, positive results have also been reported for canine distemper virus, respiratory syncytial virus, simian virus 5, parainfluenza virus type 3, and mumps (1, 14–16). Studies using *in situ* hybridization have often identified the presence of viral RNA in osteoblasts, osteocytes, and bone marrow cells, in addition to osteoclasts (2, 16).

The primers used in the present study target the same region of the N gene as those used in the other studies in which measles virus RNA has been detected (bp 1198–1630 for the first amplification in our study, compared with bp 1269–1450) (3, 4). These primers successfully amplify measles virus RNA from SSPE samples, a condition associated with long-term measles infection. In a recently published, blinded study (13), samples of RNA were analyzed for measles virus sequences in five laboratories. The RT-PCR nested-PCR used in the present study proved to be the most sensitive technique, detecting as few as 16 copies of the measles virus N gene. Results from this study showed no evidence for the presence of measles virus in any of the pagetic samples. Given the sensitivity of this assay, and the relatively large number of samples in the present study, it seems unlikely that amplification of both N and M genes in all samples would fail due to the presence of mutations in the primer sites.

Most samples in our study were from patients who had received bisphosphonate treatment. Although bisphosphonates control Paget's disease, they do not cure it, suggesting that if the virus were a causative factor, it should still be present. Measles virus and canine distemper virus have both been detected in bisphosphonate-treated patients by other laboratories (5, 14).

Nuclear inclusions are a feature of pagetic osteoclasts (7) and have been suggested to show features of a paramyxoviral infection. However, similar inclusions have also been found in osteoclasts or macrophages in cases of osteopetrosis, pycnodysostosis, and oxalosis, none of which is attributed to viral infection. Thus, they might represent a non-specific stress response in osteoclasts. Nuclear inclusions are also seen in brain cells from patients with SSPE. These inclusions are of a similar size to those in Paget's disease, but their organization appears different (7). In the past, measles virus has also been implicated in other conditions, such as inflammatory bowel disease, multiple sclerosis, and, autism, however, these links are not currently thought to be etiologically important (12, 17). Such studies of disease association are complicated by the fact that there can be a persistence of measles virus RNA in human tissue long after an acute infection, without evidence of ill effects (17).

Epidemiological evidence suggests a decline in the prevalence and severity of Paget's disease in New Zealand (18). Although this suggests that environmental factors are involved, this decline is significant between cohorts born before 1910 and those born after 1930, whereas vaccination of children against measles did not begin until 1971. Therefore, the decline in the prevalence of Paget's appears to precede the introduction of the vaccine.

A further strand of evidence for a viral etiology of Paget's disease is the demonstration that infection of osteoclasts with paramyxoviruses or measles virus N protein produces Paget-like changes in these cells. Thus, mouse bone marrow cultures infected with measles virus produce increased numbers of large, highly nucleated osteoclasts, and IL-6 levels are increased (9). Similarly, infection of human osteoclast precursors with canine distemper virus stimulates osteoclast formation and resorption, and increases the size and number of nuclei in the cells (19). Mice

expressing the measles N gene under the TRAP promoter develop a pagetic phenotype that worsens with age (10). Although these data suggest that paramyxoviral infection may reproduce some features of Paget's disease, there is no evidence that this response is specific for a particular virus, and it might be mediated by virus-induced increases in cytokines. Large, highly nucleated osteoclasts have also been identified in non-pagetic bone treated with bisphosphonate, again, suggesting a nonspecific response (20).

In conclusion, the present study has not detected evidence of measles virus infection in bone cells from a large cohort of patients with Paget's disease and control subjects. The virus detection method used has been shown to be highly sensitive, specific, and robust. This finding, together with the similar recent report from Ralston *et al.* (13), raises major doubt regarding the role of measles virus infection in the pathogenesis of Paget's disease of bone.

Acknowledgments

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This work was supported by the Health Research Council of New Zealand, Paget's Disease Charitable Trust Inc. (New Zealand), and The Paget Foundation (United States).

Disclosure Statement: The authors have nothing to disclose.

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