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Osteoprogenitor Cells and Osteoblasts Are Targets for Hepatitis C Virus

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The goal of this study was to determine whether human osteoblasts might harbor the hepatitis C virus. We tested for positive-strand and negative-strand (replicative) hepatitis C virus RNA by reverse transcriptase-polymerase chain reaction, by in situ reverse transcriptase-polymerase chain reaction for intracellular localization of the hepatitis C virus, and by amplicon sequencing in in vitro differentiated mature osteoblasts from STRO-1+ osteoprogenitor cells from patients with chronic hepatitis C and from healthy individuals. We only detected the hepatitis C virus genome in STRO-1+ cells and mature osteoblasts from carriers with chronic hepatitis C, and we found hepatitis C virus negative strands expressed sporadically in these patients. Using in situ hepatitis C virus reverse transcriptase-polymerase chain reaction, we determined that the percentage of infected carrier osteoblasts ranged from 8.0–15.3%. These data provide evidence of hepatitis C virus presence and replication in human osteoprogenitors and osteoblasts, which may have important implications for bone allograft processing.

Hepatitis C virus (HCV) is a single-stranded RNA virus of the flaviviridae family that causes chronic infection in approximately 170 million people worldwide.[19] A serious health concern related to HCV infection is the potential for hidden reservoirs of replication in extrahepatic cells,[4,12,18,21,29,30] which may lead to clinical relapse after treatment and inadvertent infection of recipients after tissue and organ transplantation. For instance, there are reports of HCV infection in recipients of bone transplantations receiving unprocessed[9] and minimally processed[7] cadaveric bone allografts from donors who are HCV-antibody negative. Contaminated serum trapped in the porosity of bone probably is responsible for inadvertent HCV infection from bone allograft transplants, but bone cells also might be reservoirs for additional virus.

Potential hepatitis C infection of bone cells is supported by the detection of intracellular HCV genomic sequences in CD34+ pluripotent stem cells of the bone marrow,[32] which contain approximately 0.5% osteoprogenitor cells[8] and are capable of differentiating into osteoblasts.[8,13] Additionally, evidence for an association between HCV and bone cells derives from a rare syndrome termed hepatitis C-associated osteosclerosis, which is characterized by a marked increase in skeletal mass in patients who have chronic HCV.[33] Osteoblasts represent a large proportion of cells in bone.[15,22,38] Therefore, a potential association of HCV with osteoblasts would change the current concept of serum and blood as the known contributors to the viral load of bone.[6]

To determine whether HCV infects bone cells, we examined HCV presence and virus replication in osteoprogenitors and in vitro in differentiated mature osteoblasts from patients with chronic hepatitis C. We also were interested in intracellular HCV signal detection and estimation of the infection rate of osteoblasts.

MATERIALS AND METHODS

Samples of trabecular bone were obtained aseptically from the endosteal surfaces of the subtrochanteric region of the femur from 12 patients during hip replacement. Six patients tested posi-

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tive for HCV antibodies, as detected by third-generation enzyme-linked immunosorbant assay (ELISA). Viral infection was confirmed in these patients by reverse transcriptase-polymerase chain reaction (RT-PCR) detection of HCV RNA in the patients' sera. The mode of infection, when known, was related to blood transfusion or parenteral drug abuse. No evidence of HIV or hepatitis B virus (HBV) infection was documented in these patients. Samples from six patients who were HCV-negative served as control subjects. Immediately after surgery, 10 cm^3 of bone samples were placed in 30 mL phosphate-buffered saline (PBS) supplemented with 7500 IU heparin. The samples were minced finely with scissors and then were filtered to remove bone spicules. A single-cell suspension was prepared and mononuclear cells were isolated by density-gradient centrifugation using Ficoll Hypaque (density, 1.077 g/mL; Biochrom AG Seromed, Berlin, Germany).

The monoclonal antibody STRO-1, a murine anti-human partly purified immunoglobulin M (ppIgM) that recognizes all clonogenic stromal precursors in human bone marrow, including all assayable osteoprogenitors,[10,14,26,34] was chosen for positive selection of osteoprogenitor cells. The STRO-1 selected cells have been shown to give rise to functional osteoblasts.[13] For the isolation of STRO-1+ cells, the technique described by Encina et al[10] was modified, and two different sources of mouse monoclonal anti-STRO-1 antibody (University of Iowa, Developmental Studies Hybridoma Bank, Department of Biological Sciences, Iowa City, IA) were used: a cell-free supernatant (derived from a myeloma cell line producing STRO-1 antibody) containing STRO-1-IgM and a partly purified STRO-1-IgM (ppIgM). Briefly, 4.0×10^7 mononuclear cells [suspended in 1 mL PBS and 1% bovine serum albumin (BSA); pH, 7.4] were incubated with 4 μg ppIgM or with supernatant containing mouse monoclonal anti-STRO-1 antibody for 1 hour at room temperature on a rotary shaker. The cell suspension was washed twice with PBS and 1% BSA, and incubated for 2 hours at room temperature on a rotary shaker with 1.0×10^8 super paramagnetic goat anti-mouse IgG-coated polystyrene beads (Dynabeads M-450, Dynal Biotech Smestad, Oslo, Norway) that cross-react with IgM. The cell-bead mixture was placed on a magnet for 5 minutes to recover STRO-1+ cells. The supernatant was discarded and the cell-bead mixture was washed three times with PBS and 1% BSA. Approximately 8.0×10^4 STRO-1+ osteoprogenitors were isolated per patient, which is within the estimated range described for cell isolation using anti-STRO-1 antibody.[10] Freshly isolated STRO-1+ cells were round and small and only began to proliferate after 7 to 10 days of culture.

All reagents for the following cell cultures were of analytical grade and obtained from Sigma (Sigma–Aldrich, Vienna, Austria), unless stated otherwise. The cell-bead mixture was resuspended in α-modified Eagle's medium (α-MEM) (Biochrom AG Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 100 mmol/L nonessential amino acids) and cultured in 25 cm^2 flasks. The STRO-1+ cells were incubated in a humidified incubator at 37° C and 5% CO_2 for 4 weeks. Half the medium was replaced with fresh medium once a week. After cells had established cell to cell contacts (usually toward the end of the third week of culture and the beginning of the fourth week), the expression of osteopontin (OP) and osteocalcin (OC) was determined by RT-PCR analysis in STRO-1-selected cells to look for osteoprogenitor cell characteristics (RNA isolation). At 4 weeks of culture, cells had formed semiconfluent layers, and OC transcripts became detectable by RT-PCR analysis, but OP transcripts could not be detected (Fig 1). Repeated HCV RT-PCR assays were done for each patient sample during the first 4 weeks of culture. At the end of the fourth week of culture, confluent adherent monolayers of STRO-1+ cells were detached with 0.25% trypsin-EDTA for 15 minutes at room temperature. Detached cells were resuspended with FCS, washed with PBS, and used for additional cultivation.

Osteoprogenitors and the human osteoblast cell line MG63 (ATCC, Nr. CRL-1427) were cultured in α-MEM with 10% FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 mmol/L nonessential amino acids supplemented with 1.0×10^{-7} mol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 50 μg/mL ascorbic acid at 37° C and 5% CO_2 for 1–5 weeks. The MG63 cells were used as a positive control for osteoblasts. Osteocalcin mRNA and OP mRNA evaluations were repeated after treatment with osteogenic factors for 1 week to confirm maturation of osteoblasts. Osteocalcin mRNA and OP mRNA were clearly expressed, providing evidence of osteoblast differentiation and maturation (Fig 1).[3,26,40]

For observation of intracellular expression of STRO-1, OC, OP, and alkaline phosphatase (ALP), osteoblasts were cultured

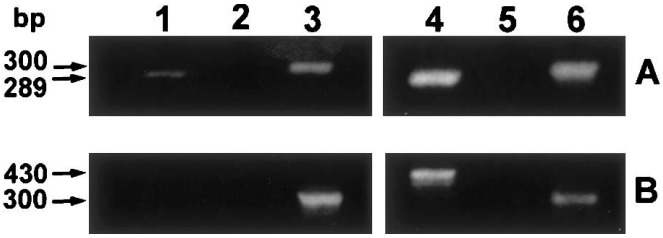

Fig 1A–B. Reverse transcriptase-polymerase chain reaction analyses of osteoprogenitor cell cultures for expression of OC and OP transcripts are shown. Amplifications were done with specific primer sets, and the resulting products were separated by 2% agarose gel electrophoresis and seen with ethidium bromide staining. (A) Reverse transcriptase-polymerase chain reaction analysis for the expression of OC transcripts (289-base pair fragments) shows that when STRO-1–selected cells had established cell to cell contacts, expression of OC transcripts became detectable (Lane 1). After treatment with osteogenic factors, OC mRNA was clearly expressed (Lane 4). Lanes 2 and 5 are negative controls; Lanes 3 and 6 are cDNA controls and β-actin (300-base pair fragments). (B) Reverse transcriptase-polymerase chain reaction analysis for expression of OP transcripts (430-base pair fragments) shows no OP transcripts were detected before treatment with osteogenic factors (Lane 1). After treatment with osteogenic factors, OP mRNA was expressed (Lane 4). Lanes 2 and 5 are negative controls; Lanes 3 and 6 are cDNA controls and β-actin (300-base pair fragments).

for 1 week on slides. Cells were air dried, fixed in acetone for 10 minutes, and stored at –20° C before immunophenotyping. Cells were rehydrated in 0.05 mol/L Tris HCl buffer. The following antibodies were used: anti-STRO–1 antibody (1:10 dilution of the ppIgM); anti-OC antibodies carrying the epitopes oc4-30 (IgG_{2a}; ZYM33-5400) and ocg-3(IgG_3; ZYM33-5700, 1:20 dilution; Zymed Laboratories, South San Francisco, CA); and anti-mouse-OP antibody (1:50 dilution; R&D Systems, Minneapolis, MN).

Antibody incubation was done for 20 minutes at room temperature. After washing in Tris HCl buffer, cells were incubated with an ALP-labeled polymer conjugated rabbit anti-mouse–IgG (secondary antibody; DAKO EnVision System Alkaline Phosphatase, DAKO Corp, Carpinteria, CA) for 10 minutes at room temperature. Slides were rinsed with Tris HCl buffer and incubated with Fast Red (DAKO Corp) for 10 minutes at room temperature and finally counterstained with hematoxylin for 5–20 minutes at room temperature. To detect ALP, staining was done according to the manufacturer's instructions (Fast Red Substrate System, LAB VISION Corp., Fremont, CA). Cells were incubated with naphtol-AS-MX phosphate for 20 minutes at room temperature. Finally, cells were counterstained with hematoxylin for 5–20 minutes at room temperature. Positive staining for ALP, OC, and OP, and negative staining for STRO-1, confirmed the differentiation of osteoblasts (Fig 2).[3,14,26,40]

Total cellular RNA was prepared from one tissue culture flask (approximately 2.0×10^4 differentiated osteoblasts) using the High-Pure Viral RNA isolation kit (ROCHE, Roche Diagnostics Inc., Mannheim, Germany) according to the manufacturer's recommendations. Ribonucleic acid was used as a template for cDNA synthesis, which was prepared using a First-Strand cDNA synthesis kit for RT-PCR (ROCHE, Roche Diagnostics Inc.).

Detection of OC and OP expression were assessed by RT-PCR. The reaction mixture contained 10 mmol Tris HCl, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.1 units/μL Taq DNA polymerase, and 1 pmol/μL OC or OP sense and antisense primers, respectively, as described previously by Gronthos et al,[14] in a final volume of 25 μL with 2 μL cDNA. An initial denaturation step at 96°C for 5 minutes was followed by 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension step at 72°C for 10 minutes. Ten microliters of each PCR product were analyzed by 2% agarose gel electrophoresis and observed with ethidium bromide staining. Primers were designed to amplify a 289-base pair fragment for OC and a 430-base pair fragment for OP. Expression of β-actin (housekeeping gene; expected product size, 300 base pair) was assessed as an indicator of cDNA integrity.

To detect HCV RNA, nucleic acids were isolated from adherent cultured cells using a High Pure Viral RNA isolation kit

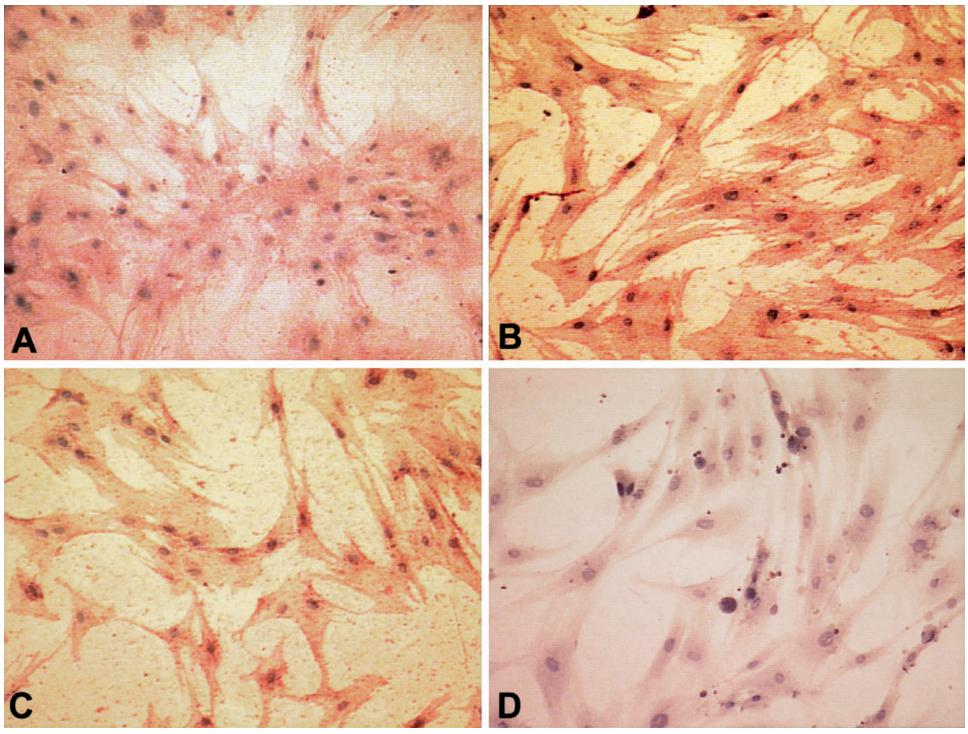

Fig 2A–D. The immunocytochemical analyses of STRO-1 + cells after 6 weeks of culture, cultured in the presence of osteogenic factors for the final 2 weeks are shown. To see the expression of ALP, OC, OP, and STRO-1, cells were cultured on slides, stained with specific antibodies, and counterstained with hematoxylin. (A) Positive staining for ALP (Original magnification, ×100), (B) OC (Original magnification, ×100), and (C) OP (Original magnification, ×100), and (D) negative staining for STRO-1 are shown (Original magnification, ×100).

(ROCHE, Roche Diagnostics Inc.), according to the manufacturer's instructions. Reverse transcription and first-step PCR were done with a reaction mixture containing 1 x PCR buffer, 4 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 5 mmol/L DTT, 10 units/25 μL RNasin, 10 units/25 μL MmuLV reverse transcriptase (Roche Diagnostics Inc), 2 units/25 μL Taq polymerase (Roche Diagnostics Inc), and 1 pmol/μL of each external primer for the 5′ untranslated region (5′UTR), as described previously.[27,39] The primer locations were: external primers, sense (nt 13-nt 45), anti-sense (nt 251-nt 268); and internal primers, sense (nt 138-nt 167), anti-sense (nt 224-nt248). Reverse transcription for 20 minutes at 42°C was followed by a denaturation step at 95°C for 5 minutes and amplification with 40 cycles at 94° C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, following a final extension step at 72°C for 7 minutes. The second-step amplification was done with a mixture containing 1 × PCR buffer, 2 mmol/L $MgCl_2$, 0.4 mmol/L dNTP, 2.5 units/25 μL Taq polymerase (Roche Diagnostics Inc.), 2 μL first-step product (1:10 dilution of the first- step PCR product) and 1 pmol/μL of each internal primer for the 5′UTR region, with 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, following a final extension step at 72°C for 7 minutes. Primers were designed to amplify a 110-base pair HCV fragment. Detection of the HCV minus strand (replicative intermediate), which is synthesized during the course of viral replication in the cultured cells, was accomplished using the PCR assay, as previously described. To avoid false-positive reactions as a result of false priming of the positive-sense genomic RNA during reverse transcription or self-priming mechanisms,[4] detection of the HCV-negative strand RNA was accomplished using standard RT-PCR assay, with the exception of the cDNA, which was synthesized using positive-sense primer only or a tagged forward primer.[17] Polymerase chain reaction was done on an Perkin Elmer Gene Amp PCR System 9600 (Perkin Elmer Cetus, PE Biosystems, Foster City, CA). Amplified products were analyzed by electrophoresis through 4% MetaPhor high-resolution agarose gel (FMC BioProducts, Rockland, ME). Each experiment was validated by the use of known positive and negative control samples. Hepatitis C virus-negative material served as negative controls. Reference samples (VQC proficiency panel for HCV-RNA, VQC Laboratory of CLB, Alkmaar, The Netherlands) with viral loads varying from less than 3 to greater than 3750 genomic equivalents per mL, were used as HCV-positive controls. The limit of the RT-PCR sensitivity was established to be 375 genomic equivalents per 1.0×10^5 cultured cells.

Polymerase chain reaction products were subjected to cycle sequencing and sequence analyses of both strands by SEQLAB (Sequence Laboratories GmbH, Göttingen, Germany). Electropherograms were evaluated by a DNA sequence analysis program (CHROMAS, Technelysium Pty Ltd, Tewantin Qld, Australia). Detected sequences also were compared with the total HCV sequence resources of the EMBL (European Molecular Biology Laboratory, Heidelberg, Germany) nucleotide sequence database.

In situ HCV RT-PCR was done with adherent osteoblasts cultured on silane-coated slides. Formalin-fixed cells were digested with proteinase K (20 mg/mL) at 37°C for 5 minutes, then treated overnight with 1 unit/μL RNase-free DNase I solution (Roche Diagnostics Inc) at 37°C. In situ PCR was done as described previously.[28] Using this method, simultaneous detection of replicative and nonreplicative HCV RNA strains was possible. Reverse transcription was done with a reaction mixture containing 1 × PCR buffer, 4 mmol/L $MgCl_2$, 0.4 mmol/L dNTP, 100 units/50 μL MMuLV reverse transcriptase (Roche Diagnostics Inc), and 2.5 pmol/μL of each external primer and 2.5 pmol/μL of positive-sense primer (only for the 5′ UTR) for 20 minutes at 42° C. The following amplification was done with a mixture containing 1 × PCR buffer, 3 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.4 x Dig DNA labeling mixture, 5 units/50 μL Taq-polymerase (Roche Diagnostics Inc), and 2.5 pmol/μL of each internal primer for the 5′ UTR region, with 20 cycles at 95°C for 2 minutes, 64°C for 2 minutes, and 72°C for 2 minutes, following a final extension step at 72°C for 10 minutes. Samples with the reverse transcription step omitted, and stromal HCV-negative cell cultures (bone-marrow derived) and the osteoblast cell line MG63, served as negative controls. Samples that did not have the DNase step, and the HCV-positive tissue, were used as positive controls. The slides were covered with Ampli Cover Disks and Ampli Cover clips (Perkin Elmer Cetus) and in situ PCR was done using the Gene Amp in situ PCR system 1000 (PE Biosystems). The amplified signals were detected by ALP-conjugated anti-digoxigenin antibody with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics Inc.) as substrate. Quantification of the number of infected cells per sample was achieved by counting by visual inspection, and was expressed as the number of cells showing a positive staining signal per total number of counted cells.

RESULTS

The positive-strand HCV RNA (genomic, nonreplicative) was consistently expressed in osteoprogenitors derived from all six patients with chronic HCV and was detected after treatment with osteogenic factors for as much as 12 weeks of culture (Fig 3). Reverse transcriptase-polymerase chain reaction products sequenced from osteoblasts of one representative patient showed 94.6% homology with the HCV consensus sequence, which confirmed the specificity of the amplified product.

The negative strand HCV RNA (replicative intermediate) was detected in osteoprogenitor cells in two of six patients with chronic HCV after a minimum of 10 days of culture. This indicates ongoing HCV replication and synthesis of progeny genomic RNA in the cells. In one of the two samples positive for the replicative HCV RNA strand, the replicative HCV RNA strand was detected after 10 days of culture, not detected after 3 weeks of culture (data not shown), and detected again after 6 weeks of culture (including the addition of osteogenic factors for the final 2 weeks), when osteoprogenitor cells had been differentiated into osteoblasts (Fig 3). Therefore, we could

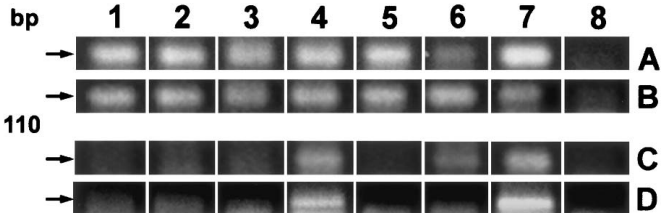

Fig 3A–D. Reverse transcriptase-polymerase chain reaction analyses of HCV RNA isolated from osteoprogenitor cell cultures of six patients chronically infected with HCV (Lanes 1–6) are shown. Primers were designed to amplify a 110-base pair HCV fragment. Polymerase chain reaction products were analyzed by electrophoresis through 4% MetaPhor agarose gel. The (A) positive, nonreplicative-strand HCV RNA was detected in osteoprogenitor cell cultures of all patients after 2 weeks (Lanes 1–6) and in (B) osteoblast cultures of all patients (Lanes 1–6) after 6 weeks (including the addition of osteogenic factors for 2 weeks). The (C) negative, replicative strand HCV RNA was detected in osteoprogenitor cell cultures of two patients after 2 weeks (Lanes 4 and 6) and in the (D) osteoblast culture of one patient (Lane 4) after 6 weeks (including the addition of osteogenic factors for 2 weeks). Lane 7 is HCV-positive liver tissue used as a positive control; Lane 8 is a negative control.

exclude the possibility that our results were a consequence of adherence of replicative HCV RNA because this would be unlikely during the prolonged cultivation with culture medium changes and trypsinization. These results show that during and after the differentiation of osteoprogenitors to osteoblasts, HCV-positive and HCV-negative strands are expressed, but the negative replicative HCV strand is expressed sporadically.

We detected PCR-amplified viral cDNA signals in the cell cytoplasm and in the perinuclear area of osteoblasts of all tested patients with positive results of HCV RNA (Fig 4). Single cells also expressed nuclear signals. We did not detect HCV RNA in control subjects with negative results of HCV RNA (Fig 4). Quantitative evaluation of cells with positive intracellular signals by in situ RT-PCR yielded 104 of 972 cells, 63 of 782 cells, and 92 of 602 cells in the three samples, respectively, resulting in an average 11% (range, 8.0–15.3%) of the cells infected with HCV. These data show that the HCV RNA signals in differentiated osteoblasts were not attributable to cell surface adherence and show the intracellular presence of the HCV genome.

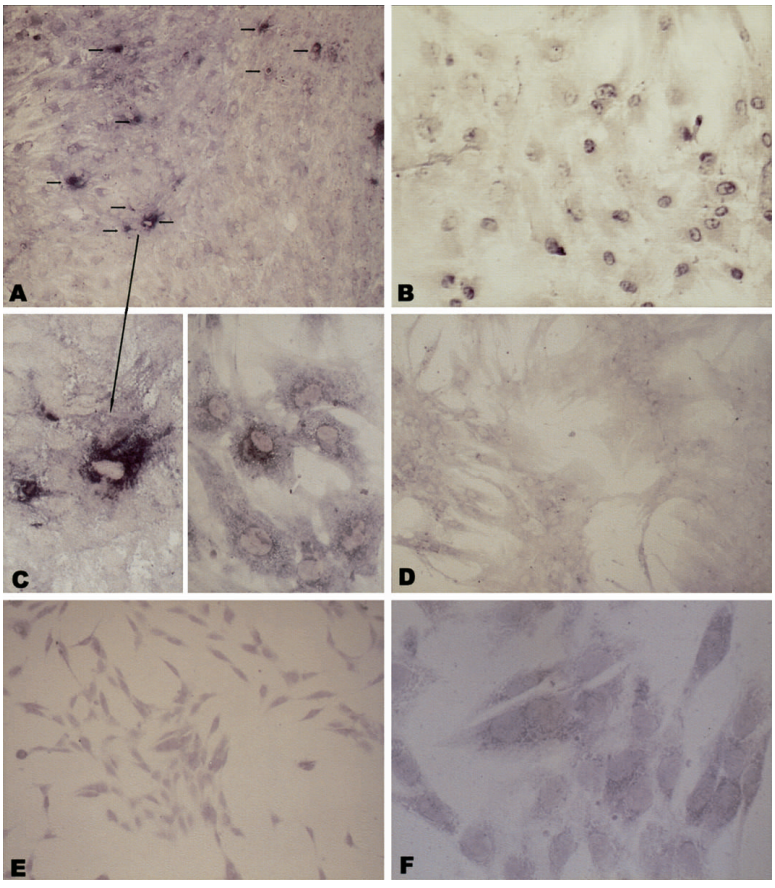

Fig 4A–F. Localization of positive and negative HCV RNA in osteoblasts using in situ HCV RT-PCR is shown. Osteoblasts were cultured on silane-coated slides, and in situ PCR was done using the Gene Amp In Situ PCR System 1000. The amplified signals were detected by ALP-conjugated anti-digoxigenin antibody with NBT-BCIP as substrate. No counterstain was used. (A) Osteoblasts show most intensive HCV cDNA signals at the interphase of the cell cytoplasm and in the perinuclear region (Original magnification, ×100). (B) Single osteoblasts are shown expressing nuclear HCV cDNA signals (Original magnification, ×100). (C) A higher magnification of the marked region in Fig 4 A is shown (Original magnification, ×400). (D) No positive signals were evident in the osteoblast cultures of patients who were HCV-negative (Original magnification, ×100), and (E) no positive signals were evident in osteoblasts of the cell line MG63 (Original magnification, ×100). (F) A higher magnification of the in situ HCV RT-PCR–negative osteoblast cell line MG63 is shown (Original magnification, × 400).

DISCUSSION

This study showed that positive-strand and negative-strand (replicative) HCV RNA are present in bone-marrow derived STRO-1–selected cells and in in vitro differentiated osteoblasts obtained from patients with chronic HCV infection.

The monoclonal antibody STRO-1 is highly specific for human osteoprogenitors.[10,34] Importantly, it does not select any kind of bone marrow cells that have been found to be capable of harboring HCV, such as granulocytes, monocytes, macrophages, and B-lymphocytes.[17,21,34] Although STRO-1–selected cells give rise to functional osteoblasts under appropriate conditions,[10,13,26] screening of cultured STRO-1–selected cells for specific osteoblast gene expression and by immunocytochemic analysis is mandatory.[3,5,26,38] We used OC mRNA expression as one marker for a major bone-related protein, and found detectable OC expression after 4 weeks of culture.[26] Clear expressions of OC and OP mRNAs, found after the addition of differentiation media to osteoprogenitor cell cultures combined with the results obtained by immunocytochemic analysis, are in accordance with published findings on the development of the mature osteoblast phenotype in vitro.[3,5,26,38]

Our study has some limitations. We calculated the 11% HCV osteoblast infection rate from samples from only three patients. Although our findings change the current concept of serum and blood as the known contributors to the viral load of bone,[6] a numerical factor that would allow for calculating the increase of the viral load in bone is not provided by our data. In addition, other factors that would help determine the proposed additional viral load, such as the infection rate of osteoprogenitor cells, the number of virus copies per infected osteoblast, or the potential association of HCV with osteocytes, were not investigated in this study. It is unclear what implications for bone bank-related safety measures, such as bone allograft processing, may arise from the newly found cellular HCV reservoir.

In general, studies of HCV replication have been hampered by the lack of an efficient cell culture system for propagating HCV in vitro. It has been shown that HCV is able to replicate in a limited number of cultured extrahepatic cell types.[2,19,28,35] Also, efficient long-term viral replication has not been described in any system. In our study, the replicative intermediate RNA was detected in osteoprogenitor cells, but also in differentiated osteoblasts after 6 weeks of cultivation. Therefore, an adsorption of replicative HCV RNA on the cellular surface during such prolonged cultivation and intensive cell culture treatment could be excluded. Because the detection of positive-strand HCV RNA in STRO-1+ cells and osteoblasts indicates the presence of virions, but does not constitute a sufficient argument to determine the intracellular process of viral replication, the detection of negative-strand HCV RNA, being the theoretical intermediate of replication, is a stronger indication for putative replication in a cell reservoir.[21] We noted intermittent detection of the negative-strand HCV RNA in osteoblast cultures. Similarly, intermittent detection of HCV genes has been found in hepatocyte cultures, infected in vitro specimens, and in infected humans, indicating that the mechanism of virus persistence in cultured osteoblasts closely mimics the intrinsic cycles of HCV replication in vivo.[31]

In situ HCV RT-PCR allowed us to see HCV in intact cells, even when present as one copy.[25] The in situ HCV RT-PCR method used in our study detected replicative and nonreplicative HCV RNA strands simultaneously. Polymerase chain reaction-amplified HCV signals were observed in the cell cytoplasma and in the perinuclear area of differentiated osteoblasts, but single cells also expressed nuclear signals. So far, it seems hepatocytes, which are the natural HCV reservoir in vivo, that are positive for replicative intermediate RNA usually have one dense signal particle in the cytoplasm. In contrast, the nonreplicative HCV RNA signals are detected in perinuclear areas and in the cytoplasm.[7,28] This pattern of distribution of nonreplicative and replicative intermediate HCV RNA varied widely among different hepatic and nonhepatic cell types.[7] The observation that single osteoblasts also expressed nuclear signals is consistent with the findings of Nuovo et al,[24] who showed that HCV RNA strands can be present in the cell nucleus as part of an RNA-protein complex. In addition, published data on the localization of HCV nucleocapsid-like particles and HCV core protein in hepatocytes of patients who were chronically infected confirmed the presence of the virus in the cell nucleus.[11,22,25,28,36]

The results of our study are the first to provide evidence of HCV presence and replication in osteoprogenitors, and HCV replication and subcellular localization in in vitro differentiated osteoblasts. These data may have important implications for processing of bone allografts because the viral burden of such grafts may be greater than currently is assumed.

Acknowledgments

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