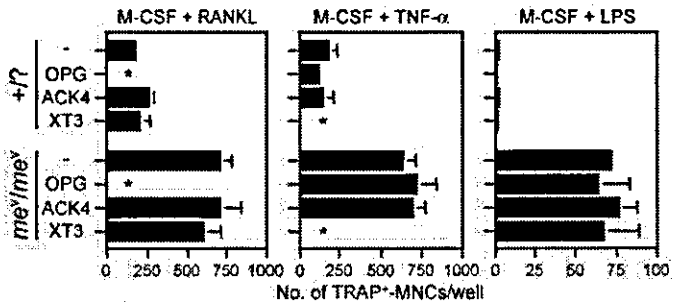

FIG. 4. LPS-induced osteoclastogenesis is not inhibited by OPG or anti-TNFα antibody. BM cells (2×10^4) from +/? littermates (*top*) or me^v/me^v (*bottom*) were cultured with M-CSF and 50 ng/ml RANKL (*left*), 50 ng/ml TNFα (*center*), or 20 ng/ml LPS (*right*) for 6 d in the presence or absence of OPG, XT3 (anti-TNFα antagonistic antibody), or control antibody (ACK4) for 6 d. The mean number and SD of $TRAP^+$ MNCs per well from the simultaneous experiments are shown. Significant differences compared with the responses of untreated cultures are indicated by an *asterisk* ($P < 0.05$). In all experiments, no $TRAP^+$ cells were observed without M-CSF.

LPS-responsive OCPs in me^v/me^v BM cells are enriched in the Kit-positive population

It has been reported that OCPs in the BM are enriched in the Kit^+ cell fraction when osteoclastogenesis was induced with stromal cells (40) or with M-CSF and RANKL (41). To assess the phenotypic characterization of LPS-responsive OCPs in BM cells, we enriched the Kit^+ cells using magnetic cell sorting (Fig. 5A) and induced osteoclastogenesis by addition of M-CSF plus RANKL or M-CSF plus LPS. In +/? BM cells, OCPs were enriched in the bound fraction of anti-Kit antibody and magnetic beads. Although a few $TRAP^+$ MNCs were induced from unfractionated +/? BM cells, the Kit^+ cell-enriched fraction gave rise to significant numbers of $TRAP^+$ MNCs in cultures. A few $TRAP^+$ mononuclear cells (data not shown), but few $TRAP^+$ MNCs, were observed in the Kit^- cell fraction (Fig. 5B).

Kit^+ cell-enriched populations from me^v/me^v BM cells contained more cells that expressed high levels of Kit than those from +/? BM cells (Fig. 5A). When the same numbers (2×10^4/well) of Kit^+ cells enriched from me^v/me^v BM cells as +/? BM cells were cultured with M-CSF and RANKL or M-CSF and LPS, the numbers of $TRAP^+$ MNCs in unfractionated and Kit^+ cell-enriched BM cells were comparable, although those from the cell fraction passed through the magnetic bead column were dramatically reduced (Fig. 5B). It is possible that macrophage-like cells overgrew in the wells of the Kit^+ cell-enriched fraction, resulting in saturation of osteoclastogenesis. Because Kit^+ cells proliferated at high levels in the presence of M-CSF, and when 25% of me^v/me^v BM cells were cultured, M-CSF plus RANKL-responding and M-CSF plus LPS-responding OCPs were significantly enriched in the column-bound fraction (Fig. 5B). Therefore, the majority of OCPs in me^v/me^v BM cells might also be present in the Kit^+ cell-enriched fraction.

Moreover, to confirm whether LPS-responsive OCPs express Kit, we precultured BM cells with M-CSF for 3 d and dish-adherent cells were harvested. A majority (+/?; 87.4%, and me^v/me^v; 88.6%) of the precultured cells expressed Mac-1, and half (+/?; 52.2%, and me^v/me^v; 50.2%) of the cells

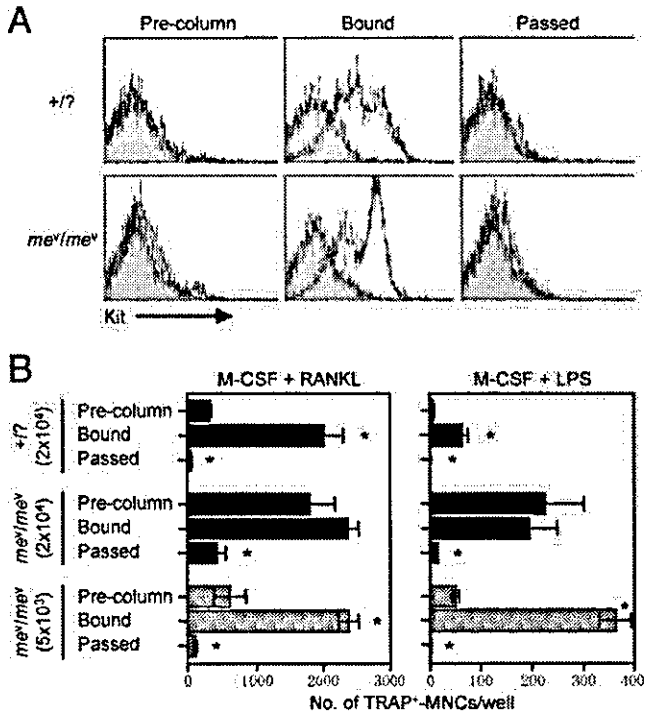

FIG. 5. Osteoclastogenesis from the Kit^+ BM cell-enriched population. The me^v/me^v and +/? littermate BM cells were incubated with PE-labeled anti-Kit antibody (ACK2) and subsequently with anti-PE antibody-conjugated beads (pre-column cells). The cells were applied to the magnetic bead columns, and nonbound cells (passed) and cells bound to the columns (bound) were recovered. A, One aliquot of cells was analyzed by flow cytometry. Cells stained with PE-streptavidin were used as a negative control (*shadowed*). B, Other aliquots of cells (■, 2×10^4/well; □, 5×10^3/well) were cultured with 50 ng/ml M-CSF and 50 ng/ml RANKL or with M-CSF and 20 ng/ml LPS. On d 6 of culture, the number of $TRAP^+$ MNCs was counted. Addition of M-CSF alone induced no $TRAP^+$ MNCs or fewer than seven $TRAP^+$ mononuclear cells from either mouse strain.

were also Fms^+; however, few (+/?; 0.32%, and me^v/me^v; 0.12%) of the precultured cells expressed Kit. The harvested cells were further cultured with M-CSF and RANKL, LPS, or RANKL plus LPS for 6 d. In the presence of M-CSF and RANKL, $TRAP^+$ MNCs were generated from both me^v/me^v and +/? precultured BM cells, and me^v/me^v cells gave rise to significantly higher numbers of $TRAP^+$ MNCs than +/? cells (Fig. 6B). In contrast, few of precultured BM cells gave rise to $TRAP^+$ MNCs in the presence of M-CSF and LPS, and addition of LPS inhibited osteoclastogenesis induced by M-CSF and RANKL (Fig. 6, B and C) (29). These results indicate that BM cells precultured with M-CSF lose Kit-expression, and these cells from me^v/me^v mice lose the potential of LPS-responsive differentiation into osteoclasts.

Reduction of osteoclastogenesis from me^v/me^v PECs

Recently, we reported that OCPs in BM and extramedullary organs, such as peritoneal cavity expressed distinct characteristics (29). To assess the influence of SHP-1 deficiency on OCPs in peritoneal cavities, we cultured PECs from me^v/me^v mice and their +/? littermates with M-CSF plus RANKL for 6 d. In the presence of M-CSF and RANKL, the number of $TRAP^+$ MNCs generated from me^v/me^v PECs was signifi-

cantly reduced compared with that from +/? littermates (Fig. 7A). The *me*^{v}/*me*^{v} BM cells cultured in corresponding experiments (experiments 1–3 in Fig. 1A) generated higher numbers of TRAP^{+} MNCs than the wild-type controls. The addition of LPS, TNFα, or IL-1α did not induce osteoclastogenesis in the presence of M-CSF (Fig. 7B). LPS and TNFα inhibited osteoclastogenesis induced by M-CSF and RANKL in control PECs as reported previously (29). IL-1α increased slightly the number of TRAP^{+} MNCs from wild-type PECs by M-CSF and RANKL. In contrast, osteoclast development from *me*^{v}/*me*^{v} PECs was absent or extremely low in all conditions tested (Fig. 7B).

In flow cytometric analysis, Mac-1^{+} cells were 22.6% and 21.9%, and Mac-1^{+} Fms^{+} cells were 12.6% and 9.6% of the cell fraction in +/? and *me*^{v}/*me*^{v} PECs, respectively. Therefore, the contents of the cell lineage in both strains of mice were similar. The *me*^{v}/*me*^{v} PECs may contain cells that inhibited osteoclast maturation, resulting in suppression of osteoclast development from *me*^{v}/*me*^{v} PECs, although cells with the potential to differentiate into mature osteoclasts in the *me*^{v}/*me*^{v} peritoneal cavity may be present. To examine this possibility, we performed mixing experiments with PECs from B6 mice. The number of TRAP^{+} MNCs in mixed cultures was almost additive to that in each PEC from B6 and *me*^{v}/*me*^{v}, and B6 and +/? mice (Fig. 7C). This indicates that *me*^{v}/*me*^{v} PECs may lack OCPs, or the OCPs in *me*^{v}/*me*^{v} PECs may lose differentiative potential, rather than be inhibited by cells in their peritoneal cavity.

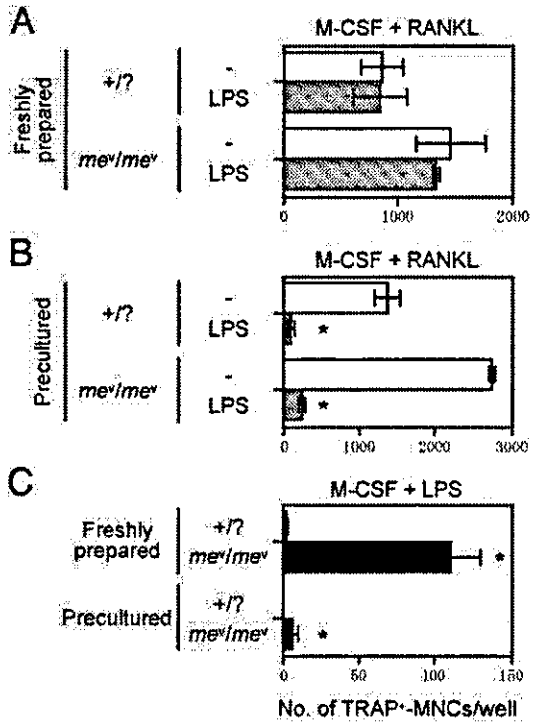

FIG. 6. Few precultured BM cells with M-CSF give rise to osteoclasts in the presence of M-CSF and LPS. A, Freshly prepared BM cells (2 × 10^{4}/well) were cultured with (▨) or without (□) LPS in the presence of M-CSF and RANKL for 6 d. B, BM cells (5 × 10^{6}/dish) were cultured with 50 ng/ml M-CSF. On d 3, the harvested cells (4 × 10^{3}/well) were cultured with (▨) or without (□) LPS in the presence of M-CSF and RANKL for 6 d. C, Freshly prepared (2 × 10^{4}/well) or precultured (4 × 10^{3}/well) BM cells from *me*^{v}/*me*^{v} mice (■) or +/? littermates (□) were cultured with LPS and M-CSF for 6 d. The number of TRAP^{+} MNCs in a well were counted. Significant differences compared with the responses (B) without LPS or those of +/? littermates (C) are indicated by an *asterisk* ($P < 0.05$).

Discussion

In this study we showed that BM cells from *me*^{v}/*me*^{v} mice defective in SHP-1 gave rise to mature osteoclasts in the presence of M-CSF and LPS without exogenous RANKL or TNFα in culture. It has been reported that the multinucleation and bone resorption of *me*^{v}/*me*^{v} osteoclasts induced by M-CSF and RANKL or by coculturing with stromal cells are accelerated (12, 13). Therefore, the total numbers of TRAP^{+}-cells (mononuclear and multinuclear cells) were relatively comparable, but the numbers of TRAP^{+} MNCs in *me*^{v}/*me*^{v} cultures were significantly higher than those in +/? littermates (12, 13) (Hayashi, S.-I., unpublished observation). Wild-type BM cells gave rise to TRAP^{+} mononuclear cells in the presence of M-CSF and LPS; however, none or only a few multinucleated cells were observed in culture. Moreover, the numbers of TRAP^{+} mononuclear cells in +/? control mice were also significantly lower than those in SHP-1-deficient *me*^{v}/*me*^{v} BM cells.

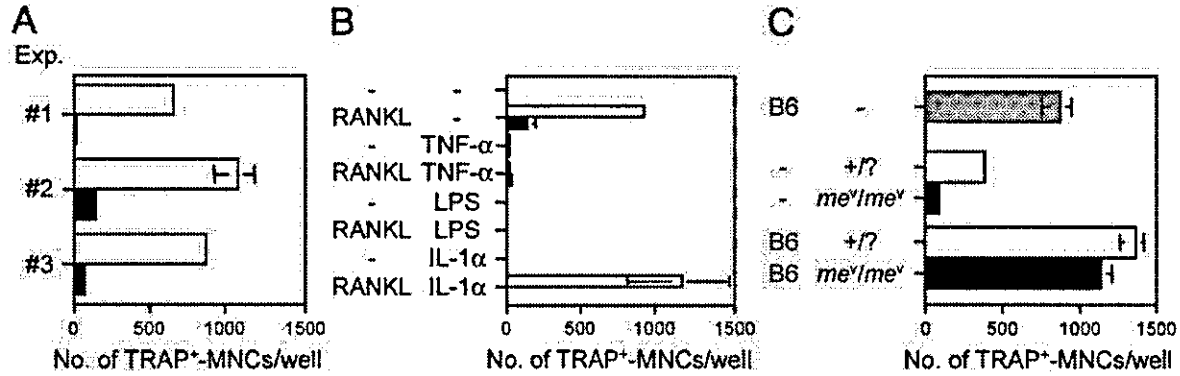

FIG. 7. Osteoclastogenesis from *me*^{v}/*me*^{v} PECs. A, PECs (10 × 10^{4}/well) from *me*^{v}/*me*^{v} mice (■) or +/? littermates (□) were cultured with M-CSF and RANKL for 6 d. The number of TRAP^{+} MNCs from three representative experiments in corresponding experiments in Fig. 1A were demonstrated. B, PECs were cultured with RANKL and/or TNFα, LPS, or IL-1α in the presence of M-CSF. C, PECs (5 × 10^{4}/well) from B6 mice were cultured without (▨) or with those from *me*^{v}/*me*^{v} (■) or +/? littermates (□) in the presence of M-CSF and RANKL. In all experiments, no TRAP^{+} cells were observed without M-CSF.

Previously, we reported that OCPs in the normal BM were enriched in Kit$^+$ cells (40, 42). In the current study OCPs in +/? BM responding to M-CSF plus RANKL were enriched in the Kit$^+$ cell-enriched population. Almost all TRAP$^+$ mononuclear cells induced by M-CSF and LPS were derived from this fraction. OCPs responding to both RANKL and LPS in *me*^{v}/*me*^{v} BM were also enriched in the magnetic bead column-bound fraction. Kit$^+$ cell-enriched populations from *me*^{v}/*me*^{v} BM cells expressed a higher level of Kit per cell than those from +/? cells. Few BM cells precultured with M-CSF for 3 d expressed Kit. In the presence of M-CSF and RANKL, osteoclasts were generated from both *me*^{v}/*me*^{v} and +/? precultured BM cells. In contrast, precultured BM cells gave rise to few TRAP$^+$ MNCs in the presence of M-CSF and LPS. Moreover, LPS inhibited osteoclastogenesis induced by M-CSF and RANKL (29). Corresponding to losing Kit expression of BM cells precultured with M-CSF, these cells from *me*^{v}/*me*^{v} mice lose the potential of LPS-responsive differentiation into osteoclasts. These results indicated that the majority of LPS-responsive OCPs as well as RANKL-responsive OCPs in freshly prepared *me*^{v}/*me*^{v} BM cell populations might be present in the Kit$^+$ cell fraction (29).

It is noted that a majority of c-Kit$^+$ cells in freshly prepared BM cells are immature before expressing RANK (41). Lam *et al.* (20) proposed that TNFα could induce osteoclast differentiation only in precursors simultaneously or previously exposed to RANKL. Their conclusion was based on the results that BM cells cultured for 3 d with M-CSF and OPG lost the potential of TNFα-induced osteoclastogenesis, although they also mentioned that overnight preincubation with M-CSF and OPG did not affect their potential of responsiveness to TNFα (20). A majority of OCPs that respond to LPS and differentiate into osteoclasts are Kit$^+$ cells, which might not express RANK. Even if previous exposure to RANKL is needed to maintain the responsiveness to LPS or TNFα in OCPs, OPG addition from 3 d before or from the initiation of culture must result in the same effect. We confirmed that overnight incubation with M-CSF and OPG did not affect the potential of LPS-induced osteoclastogenesis in *me*^{v}/*me*^{v} BM cells (data not shown). As Lam *et al.* (20) demonstrated, the presence of RANKL might be optimal for the maintenance of this potential; however, regardless of presence or absence of OPG, 3-d preculture with M-CSF reduced the potential to differentiate into osteoclasts induced by TNFα (29) or LPS. Moreover, several recent studies using RANK-KO mice showed that osteoclastogenesis is induced without RANK/RANKL signaling (18, 19). Therefore, LPS-induced osteoclastogenesis from *me*^{v}/*me*^{v} BM cells may be independent of RANK/RANKL signaling.

To assess whether *me*^{v}/*me*^{v} BM contains more Kit$^+$ cells than +/? BM, flow cytometric analyses were performed repeatedly (data not shown). Some *me*^{v}/*me*^{v} BM contained a slightly higher ratio of Kit$^+$ cells than +/? BM, but others were comparable to the wild-type BM. As *me*^{v}/*me*^{v} Kit$^+$ cell-enriched populations still generated higher numbers of osteoclasts than Kit$^+$ cell-enriched +/? populations, the presence of more Kit$^+$ cells in *me*^{v}/*me*^{v} BM might not account for the accelerated osteoclastogenesis. Using a limiting dilution assay, we assessed the frequency of OCPs in BM (40). The *me*^{v}/*me*^{v} mice and +/? littermates contained, on the average, one OCP per 45.3 BM cells and one OCP per 34.8 BM cells, respectively. Single OCPs of *me*^{v}/*me*^{v} and +/? BM gave rise to 7.1 ± 9.6 and 11.8 ± 21.7 TRAP$^+$ cells, respectively. The frequency of OCPs in BM cells and the growth of OCPs in culture are comparable to those in +/? littermates.

LPS, but not PGN or CpG, induced osteoclastogenesis of *me*^{v}/*me*^{v} BM cells in the presence of M-CSF. These three TLRs (TLR2, -4, and -9) share the downstream signaling, MyD88, TRAF6, NF-κB, and MAPK, but only TLR4, a receptor for LPS, is known to be another signaling pathway independent of independent of MyD88 (23, 43, 44). LPS might mimic the function of RANKL/RANK signaling, but not that of M-CSF/Fms signaling, in *me*^{v}/*me*^{v} BM osteoclastogenesis. In the absence of M-CSF, *me*^{v}/*me*^{v} BM osteoclastogenesis was not observed even if RANKL and LPS were added to the culture (data not shown). SHP-1 is reported to negatively regulate signaling via receptor protein tyrosine kinases, but the ligands, such as stem cell factor, vascular endothelial growth factor 164, platelet-derived growth factor, or insulin could not replace M-CSF function (45) (Yamada, T., unpublished observation). In addition to M-CSF, at least 1 ng/ml RANKL or TNFα is necessary to induce osteoclastogenesis in both *me*^{v}/*me*^{v} and their littermate (+/?) BM cells. M-CSF plus LPS-induced osteoclastogenesis was not inhibited by either OPG or anti-TNFα Ab. A recent report demonstrated that enriched BM macrophages cultured with M-CSF and thioglycolate-activated peritoneal macrophages produced less than 400 pg/ml TNFα ((28). As we used whole BM cells in the steady state, less than 1/20th of the cell populations and approximately 1/50th of the LPS concentration were comparable to this report, and it is unlikely that our cultures contained more than 1 ng/ml TNFα. Therefore, production of RANKL or TNFα might not be involved in LPS-induced osteoclastogenesis of *me*^{v}/*me*^{v} BM cells.

Recently, it was shown that RANKL/RANK signaling activates SHP-1 recruitment to the complex containing TRAF6, and SHP-1 blocked the interaction of TRAF6 with the RANK signaling pathway (25). This suggests that SHP-1 might function in the TLR and TRAF6 signaling pathway. Mice lacking either triggering receptor expressed on myeloid cells 2 (TREM2) (46) or DAP12 are reported to develop osteopetrosis (47). These mice have fewer osteoclasts and lack the ability for bone resorption. DAP12, containing a cytoplasmic immunoreceptor tyrosine-based activation motif, is a TREM-related receptor, which recruits SHP-1. The *me*^{v}/*me*^{v} BM cells accelerate multinucleation (Fig. 1) and bone resorption (12, 13). DAP12 dephosphorylation may be delayed in *me*^{v}/*me*^{v} BM cells, resulting in an increase in multinucleated osteoclasts.

After addition of PD098059, a MAPK kinase inhibitor, to the culture for 6 d, the total number of TRAP$^+$ cells was relatively comparable to that in the absence of this reagent (data not shown). However, the number of TRAP$^+$ MNCs was significantly reduced. MEK/ERK signaling may be involved in multinucleation of osteoclasts, suggesting that SHP-1 regulates the MEK/ERK signaling pathway. Moreover, as it is known that downstream signaling of TREM and DAP12 activate ERK, SHP-1 deficiency may accelerate ERK activation, resulting in an increase in multinucleated osteoclasts.

Recently, we demonstrated that peritoneal OCPs lose the potential to differentiate into mature osteoclasts if they were exposed to TLR ligands, TNFα, or even RANKL before an encounter with M-CSF and RANKL as a differentiation signal (29). Because SHP-1 deficiency accelerates this signaling pathway, OCPs in the me^v/me^v peritoneal cavity may lose the potential by the exposure of their ligands or unknown natural ligands. Finally, experiments using me^v/me^v mice lacking RANK or RANKL will provide conclusive evidence as to whether LPS may induce osteoclastogenesis *in vivo*. If so, LPS injection should cure the osteopetrosis in the double-mutant mice.

Acknowledgments

We acknowledge Drs. Kensuke Miyake (Tokyo University, Tokyo, Japan), Masato Ogata (Mie University, Mie, Japan), and Nobuyuki Udagawa (Matsumoto Dental University, Nagano, Japan) for helpful suggestions, and Masayuki Takahashi and Takao Taki (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) for M-CSF. We also thank Drs. Tomohiro Kurosaki (RIKEN Yokohama Institute, Yokohama, Japan) for his warm encouragement, and Ms. Toshie Shinohara for her secretarial assistance.

Received February 10, 2004. Accepted February 17, 2004.

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This work was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science, and Technology; the Japanese government (to S.I.H. and H.Y.); the Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd.; and NIH Grant CA20408 (to L.D.S.).

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Bone 35 (2004) 1120–1126

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Detection of osteoclastic cell–cell fusion through retroviral vector packaging

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Received 30 April 2004; revised 14 June 2004; accepted 17 June 2004
Available online 9 September 2004

Abstract

Cell–cell fusion generates multinucleated cells such as osteoclasts in bone, myotubes in muscle, and trophoblasts in placenta. Molecular details governing these fusion processes are still largely unknown. As a step toward identification of fusogenic genes, we tested the concept that retroviral vectors can be packaged as a result of cell–cell fusion. First, we introduced replication-deficient retroviral vectors expressing mCAT-1, which mediates fusogenic interaction with the retroviral envelope protein Env, into Chinese hamster ovary (CHO) cells to generate vector cells. Plasmids expressing virion proteins Gag, Pol, and Env were introduced into a separate culture of CHO cells to generate packaging cells. Co-culturing vector and packaging cells resulted in production of infectious retroviruses carrying the mCAT-1 gene as a consequence of cell–cell fusion. Second, we introduced a retroviral vector into primary osteoclast precursors and co-cultured them with established osteoclast precursor RAW264.7 cells, which turned out to harbor packaging activity. Packaged retroviral vector was detected in culture supernatants only where the osteoclast differentiation factor receptor activator for NF-κB ligand (RANKL) induced fusion between these two cell types. These data suggest that retrovirus production can occur as a result of cell–cell fusion. This provides a novel approach for isolating and characterizing fusogenic genes using retroviral expression vectors.

Keywords: Retrovirus; Fusion; Osteoclasts

Introduction

Osteoclasts are cells that resorb bone [1]. Mononuclear osteoclast precursors of the monocyte-macrophage lineage fuse with each other in the presence of stromal factors such as macrophage-colony stimulating factor (M-CSF, also known as CSF-1) and receptor activator for NF-κB ligand (RANKL). The resulting multinucleated osteoclasts may contain from 3 to more than 20 nuclei. Macrophages also have the potential to form multinucleated cells called giant cells or polykarions at sites of chronic inflammation.

Various fusogenic molecules induce cell–cell fusion during mammalian development [2]. The gamete fusion process requires a disintegrin and metalloproteinase 2, fertilin β (ADAM 2) on the sperm [3], and integrin α6β1 and CD9 on the egg [4,5]. Multinucleated myotube formation involves a fertilin-related molecule, ADAM12 (meltrin α) [6], and other molecules [7]. Candidate molecules involved in fusion of osteoclasts or macrophages include ADAMs, especially ADAM9 [8], the purinergic $P2X_7$ receptor [9], terminal high mannose type oligosaccharide-mannose receptor [10], E-cadherin [11], CD98 (also known as fusion regulatory protein-1) [12], CD44, macrophage fusion receptor (MFR, also known as P84/SHPS-1/SIRP/BIT), and the ligand CD47 [13,14]. On the contrary, tetraspanin proteins CD9 and CD81 prevent the fusion of mononuclear phagocytes [15]. Most of these nonviral fusogenic and anti-fusion molecules have been character-

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doi:10.1016/j.bone.2004.06.011

ized by loss-of-function experiments where cell–cell fusion is blocked either by antibodies or soluble recombinant extracellular domains. Identification and analysis of fusogenic molecules in gain-of-function experiments should give further insights into fusion mechanisms in the osteoclast-macrophage lineage.

Enveloped viruses such as retrovirus and influenza virus express glycoproteins that induce fusion of viral and cellular membranes to initiate infection [16]. The envelope glycoprotein Env of Moloney murine leukemia viruses (MLV) is fusogenic and induces virus–cell fusion by interacting with the mouse ecotropic receptor mCAT-1, a cationic amino-acid transporter [17–19]. This interaction between Env and mCAT-1 can also induce cell–cell fusion. MLV usually does not infect CHO cells because CHO cells do not express receptors for MLV. However, when mCAT-1 is overexpressed in CHO cells, these cells become susceptible to MLV and form abundant syncytia in the presence of the viruses [20].

As an initial step to identify and analyze fusogenic genes by gain-of-function experiments, we developed model systems to demonstrate that retroviruses can be produced as a result of cell–cell fusion. Commonly used retroviral vectors lack coding capacity for viral proteins and thus cannot replicate as viruses once gene-transfer is completed. These vectors are usually prepared as plasmids and transfected into packaging cell lines, which provide retroviral proteins Gag (for group-specific antigens), Pol, and Env, and release infectious viral particles into the culture supernatant. In our system, retroviral genomes were introduced into packaging cells via cell–cell fusion. We observed infectious retroviral production as a consequence of cell–cell fusion, establishing the basis for a novel strategy to characterize osteoclast fusion mechanisms.

Materials and methods

Retroviral vectors and cells

The retroviral vectors pBabe puro [21] and pFB neo (Stratagene) express inserted genes from the MLV long terminal repeat (LTR). pBabe puro-GFP and pFB neo-GFP were generated by inserting the 0.7-kb *Bam*HI–*Eco*RI fragment of phrGFP-1 (Stratagene) into pBabe puro and pFB neo, respectively. pFB neo-mCAT1-GFP was constructed by inserting the 2.7-kb *Bam*HI–*Not*I fragment of pmCAT1-GFP [22] into pFB neo. CHO cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). CHOpac was generated by sequential lipofection and antibiotic selection of CHO cells with pEnv-IRES-puro [23] in 5 μg/ml puromycin, and pGag-pol-IRES-bsr [23] in 3 μg/ml blasticidin, so that the expression of viral structural proteins Gag, Pol, and Env was driven by the *EF1*α promoter. CHOgfp and CHOmcat were generated by lipofection of CHO cells with pFB neo-GFP and pFB neo-mCAT1-GFP, respectively, followed by selection in 500 μg/ml G418. RAW264.7 cells [24] (ATCC TIB-71) were cultured in α-minimum essential medium (MEM) supplemented with 10% FCS. RAWpac cells were generated by sequential lipofection and selection of RAW264.7 cells with pEnv-IRES-puro in 5 μg/ml puromycin and pGag-pol-IRES-bsr in 1 μg/ml blasticidin. M-CSF-dependent murine bone marrow-derived macrophages were prepared from C57BL/6 mice as described [25] and maintained in α-MEM supplemented with 10% FCS and 30 ng/ml M-CSF (R&D). Mvec cells were generated by infecting these primary macrophages with pBabe puro-GFP or pFB neo-GFP viruses produced using Plat-E packaging cells [23]. Cell–cell fusion of RAW264.7 and Mvec cells was induced by adding 25 ng/ml recombinant mouse RANKL (R&D) to the culture medium. For titration of viruses, Rat-1A fibroblasts (ATCC JHU-25) were plated at 5000 cells/48 well, infected with serially diluted culture supernatants, and selected in 2.5 μg/ml puromycin or 500 μg/ml G418, and colonies were stained with 0.2% methylene blue in methanol.

Gene reporter-based cell fusion assay

p5G-luc-SV/Zeo was constructed by inserting a 150-bp *Sac*I–*Pvu*II fragment of 5G-OVEC containing five times multimerized binding sites for GAL4 [26] and an *Xho*I–*Bam*HI fragment containing the zeocin resistance gene of pSV40/Zeo2 (Invitrogen) into *Sac*I–*Pvu*II and *Bam*HI–*Sal*I of pGL3-Basic vector (Promega), respectively. pcDNA3.1/Zeo(+)-GAL4-VP16 was constructed by inserting a PCR-amplified coding region of GAL4(1–93)-VP16(413–490) [26] into pcDNA3.1/Zeo (Invitrogen) such that expression would be driven by the cytomegalovirus promoter. CHOpac+gluc was generated by transfecting p5G-luc-SV/Zeo into CHOpac cells and selecting under 400 μg/ml zeocin, 5 μg/ml puromycin, and 3 μg/ml blasticidin. CHOmcat+vp16 was generated by transfecting pcDNA3.1/Zeo(+)-Gal4-VP16 into CHOmcat cells and selecting under 400 μg/ml zeocin and 500 μg/ml G418. To monitor cell–cell fusion, cells were plated at 2000 cells/48 well and harvested in lysis buffer at 6, 12, and 24 h after the start of co-culture. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Other methods

Northern blotting was performed with *IRES*, *gag-pol*, and *env* probes, which were the 1-kb *Not*I–*Sal*I fragment of pGag-pol-IRES-bsr, the 2.1-kb *Sal*I–*Not*I fragment of pGag-pol-IRES-bsr, and the 2-kb *Eco*RI–*Not*I fragment of pEnv-IRES-puro, respectively. Southern blotting was performed using *Sal*I-digested genomic DNA of infected Rat-1A cells and a 765-bp hrGFP probe. Tartrate-resistant acid phosphatase (TRAP) staining was performed with the leukocyte acid phosphatase kit (SIGMA) after fixing in

3.7% formaldehyde for 30 min and in acetone/ethanol (50:50) for 30 s. Immunofluorescence microscopy was performed to identify F-actin microfilament. Briefly, cells were fixed in 4% paraformaldehyde for 10 min, washed twice with PBS for 5 min each, treated with 0.1% Triton X-100 in PBS for 10 min, stained with 5 U/ml phalloidin-conjugated rhodamine (Molecular Probes) for 45 min, washed twice for 5 min each, and mounted with DAPI to stain nuclei.

Results

Experimental design

To examine whether retroviral particles can be produced as a result of cell–cell fusion, we developed a novel strategy that requires "vector cells" and "packaging cells" (Fig. 1). The vector cells carry an integrated retroviral vector containing an intact packaging signal (ψ) and an antibiotic resistant gene (R) but no retroviral proteins, while the packaging cells express retroviral proteins, Gag, Pol, and Env. We examined whether production of infectious retroviruses occurs when these two types of cells fuse with each other. In the following experiments, cell–cell fusion was induced in two different ways: by expressing a fusion-inducing gene (F) from the retroviral vector or by adding the osteoclastogenic cytokine RANKL to the culture media of osteoclast precursors.

Fusion of CHO cells

We chose the ecotropic receptor mCAT-1 as a model fusion-inducing gene based on the observation that over-expression of mCAT-1 in CHO cells not only makes CHO cells susceptible to MLV infection, but also induces

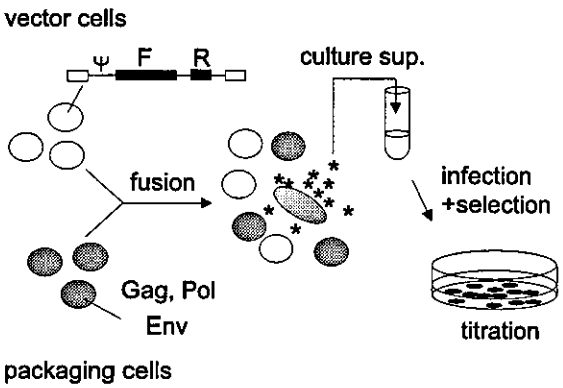

Fig. 1. Experimental design for detecting retroviral production upon cell–cell fusion. Vector cells (white) carry integrated proviral DNAs, and packaging cells (gray) express viral proteins, Gag, Pol, and Env. Fusion of these two cell types results in release of infectious retroviral particles into culture supernatant. F: a fusion-inducing gene (mCAT-1-GFP) or a green fluorescent protein (GFP) gene. R: puromycin- or G418-resistance gene. These genes are used to select cells infected with culture supernatants.

syncytia formation through the interaction between mCAT-1 and the fusogenic viral glycoprotein Env [20]. We generated vector cells by introducing pFB neo-mCAT1-GFP into CHO cells so that the resulting CHOmcat cells expressed the receptor mCAT-1 fused to GFP [22]. CHO cells carrying pFB neo-GFP (CHOgfp) served as a negative control. We then generated CHOpac by introducing expression vectors for Gag, Pol, and Env into CHO cells. CHOmcat, CHOgfp, and parental CHO cells were cultured alone or co-cultured with CHOpac. These cells were morphologically indistinguishable and do not fuse spontaneously (data not shown). As expected, fused cells were observed under the microscope when CHOmcat cells were co-cultured with CHOpac (Fig. 2). The multinucleated cells were GFP-positive, indicating that they expressed mCAT-1-GFP protein. To demonstrate that these cells were not aggregated cells and that cell–cell fusion was occurring between CHOmcat and CHOpac cells, we devised a gene reporter-based cell fusion assay. This assay makes use of the synthetic transcription factor GAL4-VP16 containing a DNA-binding domain of the yeast transcription factor GAL4 linked to a transactivation domain of the herpes simplex virus transcription factor VP16. As a reporter, we used the luciferase gene driven by a promoter containing GAL4 binding sites. We expressed GAL4-VP16 in CHOpac and the luciferase reporter in CHOmcat cells. As shown in Fig. 3, luciferase activity was significantly elevated 24 h after co-culturing CHOmcat and CHOpac cells, suggesting that fusion had occurred between these two types of cells. We tested for the presence of infectious retroviral vectors pFB neo-mCAT1-GFP in culture supernatants by infection of Rat-1A fibroblasts followed by G418 selection. Significant numbers of G418-resistant and GFP-positive Rat-1A cell colonies were observed when the co-culture supernatants of CHOmcat and CHOpac were titrated (175 ± 35 cfu/ml). Such colonies were not observed with supernatants of CHO, CHOgfp, CHOmcat, or CHOpac cells alone, or co-cultures of all combinations of the two cell populations other than CHOmcat and CHOpac. These data show that cell–cell fusion can result in the production of infectious retroviruses carrying a fusion-inducing gene.

RAW264.7 cells harbor endogenous packaging activity

The murine macrophage cell line RAW264.7 was originally established from a tumor induced by Abelson-murine leukemia virus (A-MLV) [24]. These cells form multinucleated osteoclast-like cells via cell–cell fusion in response to RANKL and are extensively used in the study of osteoclast biology. Although RAW264.7 cells do not produce infectious retroviruses by themselves [24], we unexpectedly detected infectious retroviruses in the culture supernatant after transfecting the retroviral vector pBabe puro-GFP into RAW264.7 cells. The titer measured in fibroblasts was 2500 and 770 cfu/ml for transient and stable transfection, respectively. Control NIH3T3 fibroblasts pro-

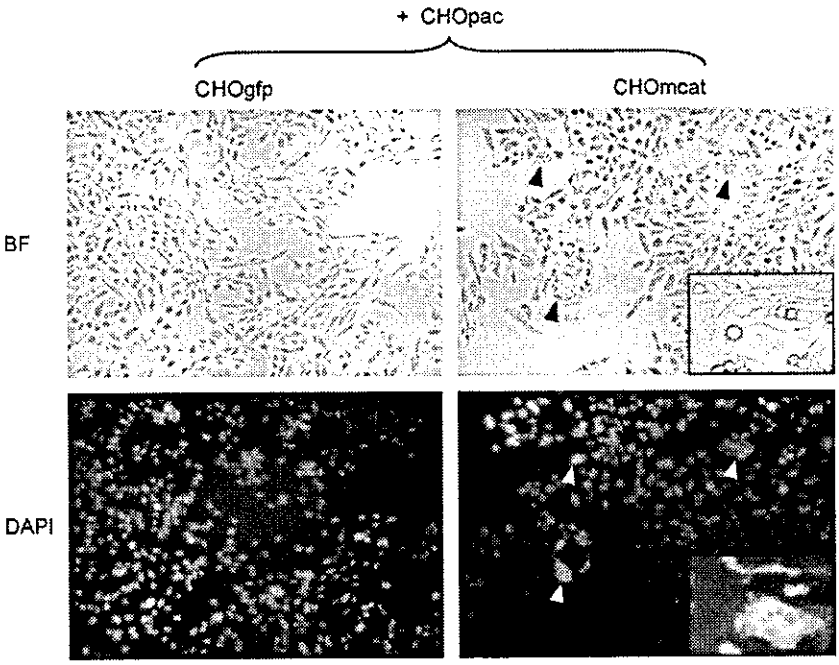

Fig. 2. Fusion of CHO-derived cells. CHOgft or CHOmcat was co-cultured with CHOpac cells. Bright field (BF) and DAPI staining of nuclei are shown. Arrowheads indicate multinucleated cells. The insets show that multinucleated cells in bright field are GFP-positive under fluorescence.

duced no infectious retrovirus upon transfection, as expected (data not shown). In an attempt to boost packaging activity of RAW264.7 cells, we stably introduced two plasmids that were originally used to establish the Plat-E conventional packaging cell line [23] expressing Gag, Pol, and Env into RAW264.7 cells. Three independent subclones of RAW264.7, designated RAWpac cells, were obtained, but the viral titers of their supernatants (2700 cfu/ml for transient transfection) were not significantly higher than that of parental RAW264.7 cells. Consistent with the observation that RAW264.7 cells could package retroviruses, Northern blot analysis showed that RNA from parental RAW264.7 cells hybridized with *gag-pol* and *env* probes (Fig. 4). These transcripts were not observed in other murine cell lines, such as NIH3T3 fibroblasts and Ba/F3 pro-B cells. Therefore, we concluded that the parental RAW264.7 cells harbor packaging activity and used these cells as packaging cells in the following experiments.

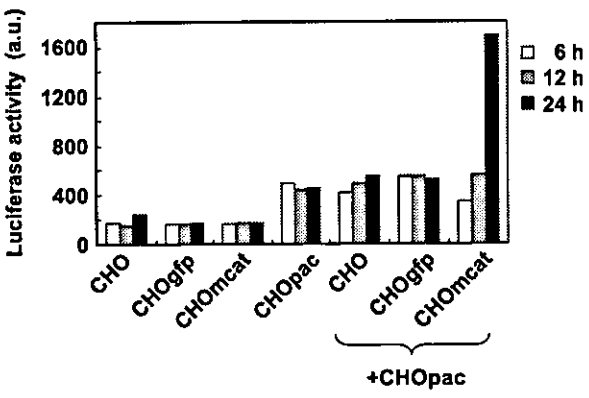

Fig. 3. Gene reporter-based cell fusion assay. A GAL4-VP16 transcription factor was expressed in CHOmcat (CHOmcat + vp16) cells and a GAL4-dependent luciferase reporter gene was introduced into CHOpac (CHOpac + gluc) cells. Cell lysates were prepared at the indicated times after the start of co-culture. a.u., arbitrary units.

Fusion-induced packaging of retroviral vectors

Because RAW264.7 cells have an intrinsic packaging activity, they are not suitable to generate vector cells, which should carry defective retroviral vectors without any viral production. Therefore, we prepared primary bone marrow

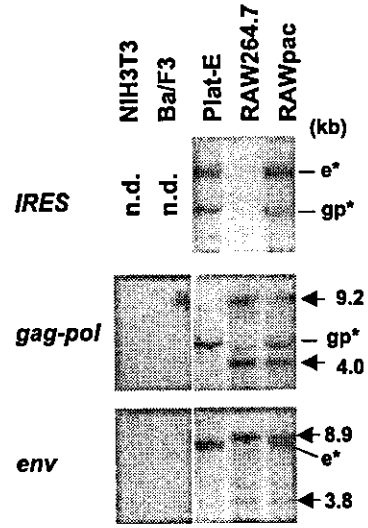

Fig. 4. RAW264.7 cells express endogenous *gag-pol* and *env* transcripts. Estimated sizes of endogenous transcripts are indicated. Exogenous *gag-pol* (gp*) and *env* (e*) transcripts were detected with the *IRES* probe. Plat-E is a packaging cell line.

macrophages and infected them with pBabe puro-GFP virus to generate vector cells, which we designated Mvec. Either Mvec or RAW264.7 cells alone, or 50:50 mixture of Mvec and RAW264.7 cells was cultured in the absence or presence of RANKL (Fig. 5). On day 4, the culture supernatants were harvested and the viral titer was determined using Rat-1A fibroblasts. Either Mvec or RAW264.7 cells alone formed multinucleated cells dependent on RANKL (Figs. 5D, E, G, and H). Infection experiments using the supernatant from Mvec or RAW264.7 cells did not produce puromycin-resistant fibroblast colonies regardless of cell–cell fusion events. When Mvec and RAW264.7 cells were co-cultured, many irregularly shaped multinucleated cells were formed (Figs. 5F and I). These multinucleated cells were likely a result of fusion events between Mvec and RAW264.7 cells. The co-culture supernatant of Mvec and RAW264.7 cells in the presence of RANKL produced puromycin-resistant colonies that were GFP-positive. No such colonies were obtained using supernatant of Mvec or RAW264.7 cells alone, or in the absence of RANKL (Fig. 5). These observations suggest that infectious retroviruses were produced as a consequence of cell–cell fusion between Mvec and RAW264.7 cells. We repeated the experiment by replacing pBabe puro-GFP with another retroviral vector pFB neo-GFP. G418-resistant Rat-1A fibroblast colonies were obtained only when supernatant from co-culture in the presence of RANKL was used. Southern blotting showed that genomic DNA prepared from these G418-resistant colonies contained retroviral DNA, indicating the successful transfer of retroviral vector to fibroblasts (data not shown). These observations demonstrate that cell–cell fusion during osteoclast differentiation can result in retrovirus production.

Discussion

Cells of multicellular organisms, in principle, do not usually fuse with each other. Osteoclasts, however, possess abilities to fuse efficiently with each other during differentiation. Multinucleated osteoclasts seem to provide an advantage in resorption, because the area of the resorption pit surface is linearly correlated to the number of nuclei per osteoclast [27]. We described here model systems demonstrating that cell–cell fusion results in retrovirus production when one cell contains defective retroviral vector and the other produces proteins for retroviral packaging.

In the first model, cell–cell fusion is triggered by interaction between Env expressed on CHOpac cells and the receptor mCAT-1 on CHOmcat cells [20]: CHOpac produced Env as well as Gag and Pol, and a specialized vector cell, CHOmcat, carried a retroviral vector expressing mCAT-1-GFP. Fusion between CHOpac and CHOmcat was detected by a novel cell fusion assay. A luciferase gene driven by a GAL4-dependent promoter was introduced into CHOpac and a chimeric GAL4-VP16 transcription factor

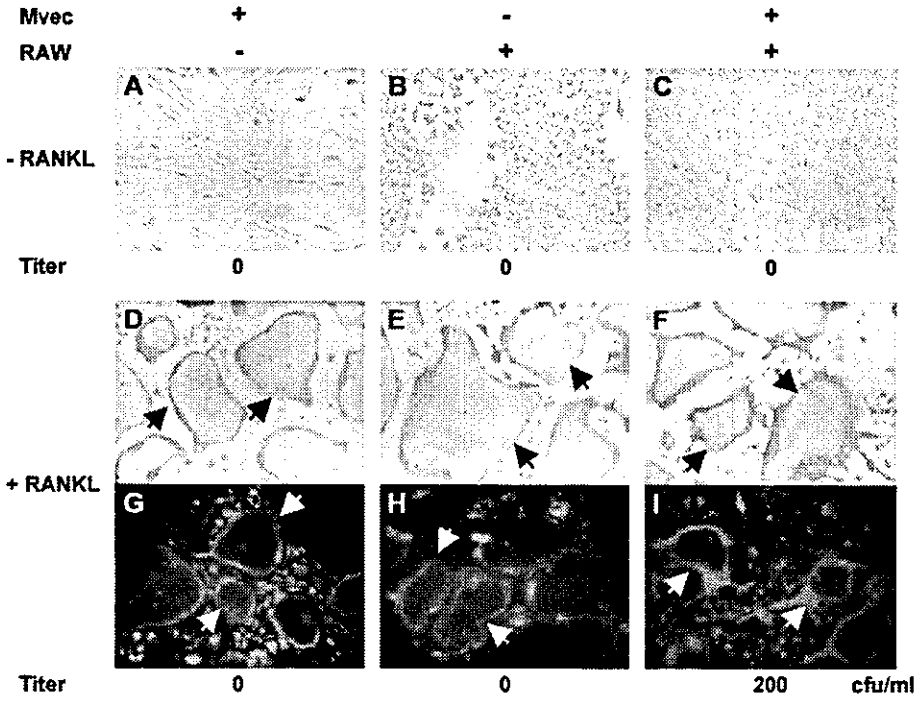

Fig. 5. Production of retroviruses upon osteoclast fusion. Mvec and RAW264.7 (RAW) cells were cultured separately or together (2×10^4/24 well) in the absence or presence of 25 ng/ml RANKL for 3 days. A–C, bright field. D–F, stained for TRAP activity (red). G–I, phalloidin staining for actin (red) and DAPI staining for nuclei (blue). Arrowheads indicate multinucleated osteoclast-like cells. Titer of pBabe puro-GFP virus (cfu/ml) was measured using Rat-1A fibroblasts.

was introduced into CHOmcat. Fusion of these two types of cells provided the reporter gene with the transcription activator and was measured as luciferase activity. Similar complementation-based fusion assays were previously reported using T7 promoter-luciferase construct in one cell and T7 polymerase in the other [28], and an enzymatic *lacZ* complementation [29]. Infectious retroviruses were specifically demonstrated in the supernatant from the fusion-induced co-culture of CHOpac and CHOmcat. The relatively low titer observed suggests that a rescued retroviral vector does not efficiently spread among vec cells, presumably due to competition with "empty" virions produced by pac cells. It is also possible that membrane fusion mediated by mCAT-1 in CHO cells was inefficient due to limited mCAT-1 expression levels [20]. Therefore, cloned CHOmcat sublines expressing mCAT-1 at high levels might result in more efficient cell–cell fusion and higher viral titer than the bulk population of CHOmcat cells used in the experiment.

In the second model, we used the osteoclastogenic cytokine RANKL to induce fusion of RAW264.7 cells with primary macrophages. We observed packaging activity in RAW264.7 cells, which was unexpected because these cells carry a defective A-MLV, and thus do not produce virus [24]. In hindsight, the packaging activity of RAW264.7 cells is consistent with the observations that RAW264.7 cells are highly resistant to retroviral gene transfer, unlike primary osteoclast precursors, which are susceptible to retroviral vectors [30,31]. It is conceivable that Env proteins expressed in RAW264.7 cells may fully occupy the receptor mCAT-1. At the mRNA level, RAW264.7 cells express mCAT-1 as abundantly as NIH3T3 cells, and additional introduction of a mCAT-1 expression vector did not improve susceptibility of RAW264.7 cells to murine retroviral infection (our unpublished data). Furthermore, Northern blot experiments showed that *gag-pol* and *env* sequences were transcribed in RAW264.7 cells, and a "signal sequence trap" experiment, which detects signal sequences in cDNA fragments [32], revealed *env* cDNA sequences in RAW264.7 cells (K. Mizuno and K. Watanabe, personal communication). These observations are consistent with the notion that RAW264.7 cells can produce principal retroviral proteins. It should be noted that the use of retroviral vectors in RAW264.7 cells requires extra caution because infectious retroviruses may be produced in culture supernatants. We conclude that the retroviral production is a consequence of cell–cell fusion between Mvec and RAW264.7 cells induced by RANKL. Although unlikely, there may be other explanations. RANKL treatment might induce RAW264.7 cells to produce endogenous retroviruses, which then infect Mvec cells to rescue retroviral vector production. Transwell culture methods separating RAW264.7 cells from Mvec cells should allow us to examine this possibility. Alternatively, Gag, Pol, and Env proteins might be transferred from RAW264.7 cells to Mvec cells without cell–cell fusion, as in the case of retroviral protein Tat or the herpes simplex virus structural protein VP22 [33–35]. However, considering the production of infectious retroviruses in the CHO model, we believe that fusion of osteoclasts is the cause of retroviral production.

Collectively, these observations suggest that retroviral packaging rescue can occur upon cell–cell fusion. Cell–cell fusion can be induced by the cDNA carried by a retroviral vector as in the mCAT-1 model. Therefore, fusion potentials of various putative fusogenic genes and their mutants can be monitored by the release of retroviruses. Furthermore, this provides a new strategy for screening fusogenic genes inserted in retroviral cDNA expression libraries. The cDNA library is constructed in "vector cells" and the fusogenic cDNA can be isolated in infectious retroviruses, which are released via fusion-induced retroviral packaging. With conventional expression vectors, it may be difficult to recover cDNA from a few fused cells because multinucleated cells usually do not proliferate. However, retroviral rescue allows us to amplify cDNA by further infecting proliferating cells. One might think that Env expressed on the surface of "packaging cells" would disturb the natural cell–cell fusion process. However, Env expression does not induce fusion in NIH3T3 cells, which express mCAT-1 abundantly. Therefore, retroviral packaging rescue seems to be applicable to define additional host cell factors facilitating fusion processes. These factors should include both fusion molecules and their positive regulators.

In conclusion, retroviral packaging rescue as described here is a novel approach for producing retroviruses as a consequence of cell–cell fusion and may allow expression cloning and functional analysis of genes responsible for osteoclastic cell–cell fusion.

Acknowledgments

We thank T. Kitamura for pGag-pol-IRES-bsr and pEnv-IRES-puro, M. Masuda for pmCAT1-GFP, H. Hara for CHO cells, and K. Mizuno and K. Watanabe for unpublished sequence information. We thank J. Silver, S. Kato, K. Segawa, N. Ray, and C. Zhao for critical reading of the manuscript. We also thank M. Suzuki for technical support. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan to K.I.

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