ORIGINAL ARTICLE

Novel perspectives on the transcytotic route in osteoclasts

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We analyzed the characteristics of degraded bone matrix-delivering vesicles along the transcytotic route from the ruffled border to the functional secretory domain (FSD) in bone-penetrating osteoclasts. Cells of rat or human origin were cultured on bovine bone slices and analyzed via confocal microscopy. Helix pomatia lectin binding indicated that transcytotic vesicles expose aberrant N-acetylgalactosamine glycoconjugates, which is associated with a poor prognosis for a range of metastasizing human adenocarcinomas. Transcytotic vesicles fuse with the autophagosomal compartments and represent raft concentrates. Furthermore, the results of a vertical vesicle analysis suggest that multiple vesicle populations arise from the ruffled border and that some of these vesicles undergo a maturation process along the transcytotic route. Finally, our data suggest that the targeting of these membrane pathways may be determined by a novel F-actin-containing and FSD-circumscribing molecular barrier.

Introduction

Osteoclasts are highly specialized cells that are capable of bone resorption. They differentiate by the fusion of mononuclear precursors from the monocyte/macrophage lineage, which is a source of their multinuclear character.[1,2] When osteoclasts activate for bone resorption, they polarize and form several distinct plasma membrane domains.[3] A hallmark of this process is the formation of the ruffled border, the actual bone-degrading organelle facing the bone surface. Vesicles containing HCl and proteases fuse at the peripheral areas of the ruffled border, while degraded matrix is endocytosed at the central ruffled border.[4–7] The sealing zone circumscribes the ruffled border, mediates the tight attachment of the resorbing cell to the bone matrix and isolates the resorption milieu from the extracellular fluid.[8,9] The bone-antipodal membrane is divided into two functionally distinct compartments. The central functional secretory domain (FSD) that resembles the apical membrane of polarized epithelial cells and is labeled with influenza virus hemagglutinin (HA) serves as a site for secretion of the degraded bone matrix after endocytosis and transcytosis via microtubules.[7,10–12] A direct endocytic route from the peripheral non-bone-facing plasma membrane to the ruffled border contributes to the maintenance of the ruffled border. This route can be visualized by the labeling of the abundant transferrin receptors with iron-loaded transferrin (TfFe), a marker for the early endosomal recycling pathway.[13] Although the existence of the FSD has by now been long known as the FSD was shown to serve as the site of the secretion of transcytosed bone matrix,[10] we know still little about the mechanisms that maintain this specific membrane domain and what links the transcytotic vesicle trafficking to this subdomain of the non-bone-facing plasma membrane of the osteoclast. The transcytotic membrane pathway may be regulated by the neurotransmitter glutamate.[14] It has also been suggested that the transcytotic membrane pathway undertakes a maturation process, whereby cathepsin K activates the reactive oxygen species-generating activity of tartrate-resistant acid phosphatase (TRACP).[15] Both the fusion zone of the ruffled border and the FSD are lipid raft concentrates and labeled by fluorescent recombinant cholera toxin B subunit (recChlTx-B), a commonly used marker for lipid rafts, but the role of rafts along the post-lysosomal secretory pathway from the ruffled border to the FSD is not currently understood.[16,17] Autophagy proteins, including, Atg5, Atg7 and Atg4B, regulate the formation of the ruffled border in osteoclasts. Autolysosomal structures are abundant in osteoclasts, but they are not detected in the vicinity of the ruffled border. This finding suggests that the autophagosomes do not directly contribute to the formation or maintenance of the ruffled border.[18] The role of autophagosomes in bone degradation process is thus not currently understood.

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Helix pomatia (HPA) lectin recognizes oligosaccharides that bear terminal alpha-glycosidically linked *N*-acetylgalactosamine (GalNAc) glycoconjugates. These terminal monosaccharides usually form the core monosaccharide of *O*-linked glycans (GalNAc-O-Ser/Thr, or Tn-antigen), which may be capped by sialic acid or obscured by further chain extension. The presence of GalNAc glycoconjugates is associated with metastatic competence and poor prognosis in malignant melanoma and a range of human adenocarcinomas, including, cancers of the breast, esophagus, colorectum, thyroid and prostate.[19] In agreement with these findings, increased GalNAc expression has been reportedly associated with an increased capacity to invade basement membrane components.[20] Recent proteomic studies of metastatic colorectal and breast cell lines have revealed that integrin alpha 6 is the most abundant HPA-binding glycoprotein in these cells. In addition, integrin alpha v, annexin A2/A4, some heat shock proteins and a selection of transcription factors were identified as HPA-binding partners.[21,22] In addition, osteoclasts, which are highly invasive cells that penetrate into the bone matrix during resorption, stain positively for HPA lectin,[23] but the role of GalNac glycoconjugates in the tissue penetrative process of bone resorption is currently unknown.

The current study furthers our understanding of osteoclastic transcytosis through four major findings: first, we show the presence of GalNAc glycoconjugates in this post-lysosomal route; second, we show the presence of the autophagosomal machinery along this route by labeling with mono-dansylcadaverine (MDC), a marker for autophagic vacuoles; third, our results suggest the existence of multiple transcytotic routes rather than just one, supported by the lack of vertical polarity of all studied transcytotic markers (TRACP, cathepsin K, HPA lectin and lipid rafts), in which β_3-integrin may serve as a receptor; finally, our findings may indicate the existence of an F-actin-based molecular diffusion barrier between the FSD and the peripheral non-bone-facing plasma membrane.

Results

The association of HPA-labeled compartments with transcytotic compartments in rat osteoclasts

TRACP and cathepsin K have been previously shown to be important components of the transcytotic route in osteoclasts.[15] Although cathepsin K has been repeatedly observed to be present in the resorption lacuna as well, the localization pattern for TRACP remains controversial.[24–26] When osteoclasts cultured on pre-labeled bone were stained for HPA-lectin, we observed a clear intracellular and vesicular colocalization of internalized bone, HPA lectin and TRACP (**Figures 1a–d**); and HPA-lectin-binding sites TRACP and cathepsin K (**Figures 1e–h**) in both rat- and human-derived osteoclasts throughout the cells (**Figures 1i–k**). These results show that HPA-lectin-binding sites associate with the transcytotic vesicular compartments, which transport degraded bone matrix through the osteoclast for externalization. However, fluorescein isothiocyanate-labeled bone itself did not necessarily colocalize with TRACP, cathepsin K or HPA lectin, suggesting that some bone matrix is delivered via transcytotic routes bypassing TRACP, cathepsin K and/or HPA-lectin-binding vesicular compartments or that the markers used in the current study protocol did not meet the current phase of the maturation process of the transcytotic vesicles. None of these transcytotic vesicle markers showed vertical polarization

with respect to their association with endocytosed bone matrix, although the largest TRACP-containing vesicles were usually observed at the top of the osteoclast, as has been previously reported.[15] Furthermore, although HPA-lectin-binding sites were not present at the ruffled border in rat osteoclasts, we observed a clear localization pattern of TRACP at the fusion zone but not at the uptake zone of the ruffled border, as shown by the colocalization of TRACP with the previously shown fusion zone markers β_3-integrin and recChlTx-B (**Figures 1l–o**).[7]

To exclude the possibility that HPA-lectin-binding sites are also present along the endosomal membrane pathway from the non-bone-facing membrane to the fusion zone of the ruffled border, we incubated rat osteoclasts with human TfFe for 60 min. During this time, TfFe is delivered from the non-bone-facing plasma membrane to the ruffled border and then redelivered to the plasma membrane along a pathway that dissociates from degraded bone matrix.[13] We found little colocalization of TfFe with HPA-lectin-binding material. This finding suggests that HPA-lectin-binding sites dissociate from TfFe recycling vesicles trafficking back to the plasma membrane and that they are not present at the vesicular pathway from the plasma membrane to the ruffled border (**Figures 1p–r**).

### Association of lipid rafts and TRACP along the transcytotic route

As our previous data suggested that the delivery of HA from the Golgi apparatus to the FSD is a raft-dependent process, and as the integrity of rafts appeared to be a necessity of osteoclastic bone resorption,[17] we hypothesized that lipid rafts may also be associated with the transcytosis of degraded bone. For this purpose, we cultured osteoclasts on rhodamine-coated bone slices, fixed the cells and labeled them with antibodies against TRACP and fluorescent recChlTx-B. We studied 11 randomly selected representative cells by scanning 10 evenly spaced sections throughout each cell with a confocal laser scanning microscope. After a total of 723 transcytotic bone-containing vesicles had been examined, 17.6% of the vesicles contained TRACP but not recChlTx-B. Moreover, 44.3% of these vesicles bound recChlTx-B but were devoid of TRACP, and 17.0% contained both markers. Finally, 21.2% of the vesicles with pre-labeled bone colocalized neither with TRACP nor recChlTx-B. We expected to see some process suggestive of maturation where components of the trans-cytosed vesicles would change as they ascend to the FSD. However, these vesicle populations showed no vertical distinction. These vesicle populations were observed at all vertical levels of the osteoclast with no clear preference for any particular level (**Figures 2a–m**).

Involvement of $\alpha_v\beta_3$-integrin in the transcytosis of degraded bone matrix

As $\alpha_v\beta_3$-integrin has been suggested to serve as a receptor for internalized bone matrix by binding to denatured collagen,[27] it was expected to associate with the transcytotic vesicles. Thus, we cultured rat osteoclasts on bone slices precoated with tetramethylrhodamine and recombinant osteopontin. We found that osteopontin was transcytosed from the ruffled border to the FSD in β_3-integrin-containing vesicles (**Figures 2n–p**), whereas tetramethylrhodamine-labeled bone constituents were not (**Figures 2q–s**). Altogether, these findings suggest that the early selection of cargo molecules into distinct vesicular pathways occurs at the ruffled border.
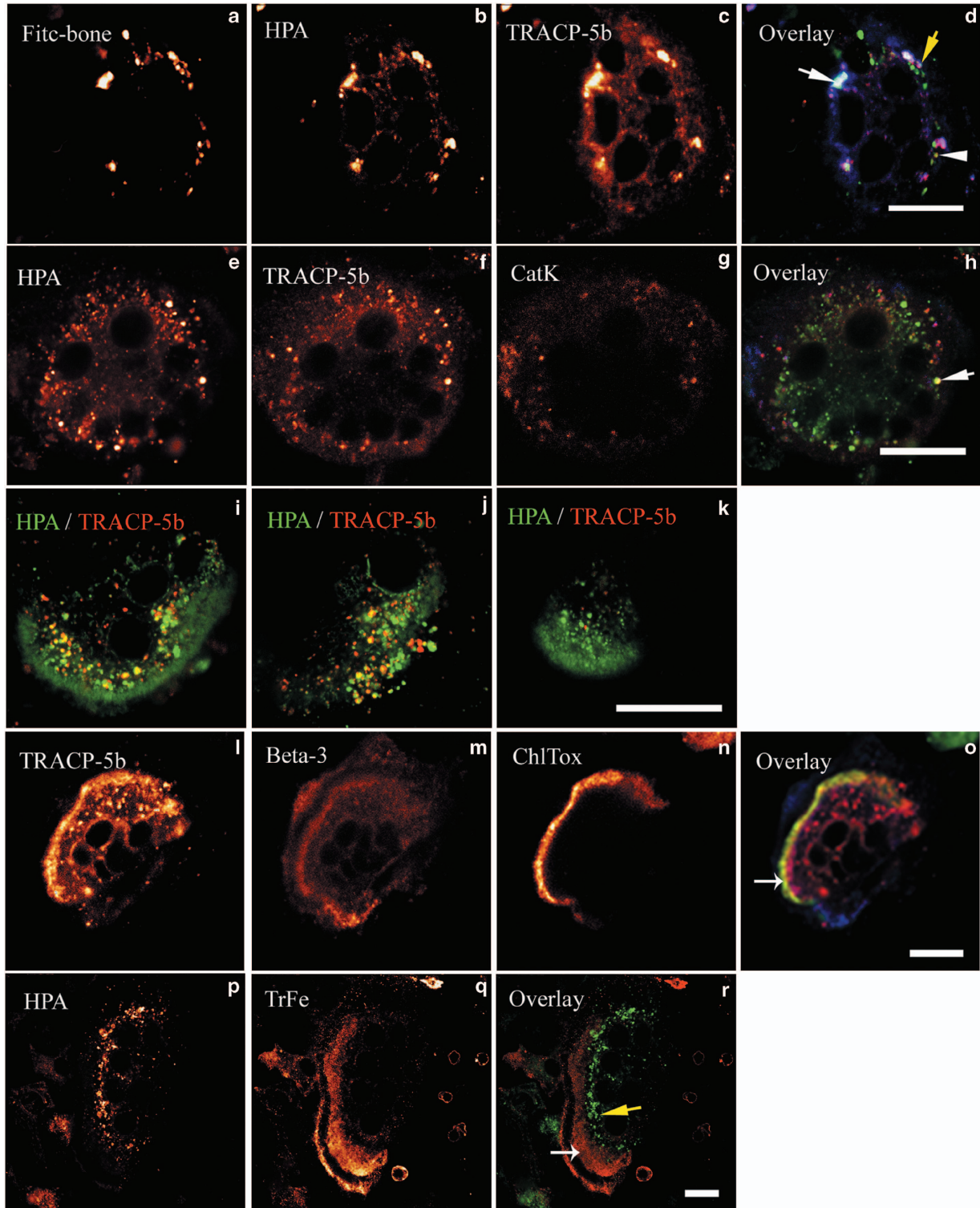
Figure 1 HPA-lectin-binding sites associate with the transcytotic route from the ruffled border to the FSD in rat and human osteoclasts. (**a–d**) When rat osteoclasts are cultured on fluorescein isothiocyanate-labeled bone slices, they internalize the label at the ruffled border and transcytose it via compartments that partly colocalize with either TRACP (yellow arrow) or HPA-lectin-binding sites (white arrowhead) or both (white arrow). Images represent the middle level of the cell. (**e–h**) HPA-lectin also partly colocalizes with cathepsin K together with TRACP (white arrow). Images represent the middle level of the cell. (**i–k**) HPA-binding site association with TRACP-positive compartments is also evident in human osteoclasts. In human osteoclasts, the colocalization can be observed at all vertical sections of the cell. (**i**) Ruffled border level, (**j**) middle level and (**k**) FSD level of the cell. (**l–o**) Although HPA-lectin-binding sites are not present at the ruffled border in rat osteoclasts, TRACP associates with the previously shown ruffled border fusion zone markers β_3-integrin and fluorescent recombinant cholera toxin subunit B (recChlTx-B). (**p–r**) When rat osteoclasts are incubated in the presence of transferrin for 60 min, little colocalization with HPA can be observed. White arrow shows the fusion zone of the ruffled border. Yellow arrow shows HPA-lectin-binding site containing vesicles concentrated right above the matrix uptake zone of the ruffled border. (**l–r**) Images represent the ruffled border level and the middle level of the cell. Bars, 10 μm.
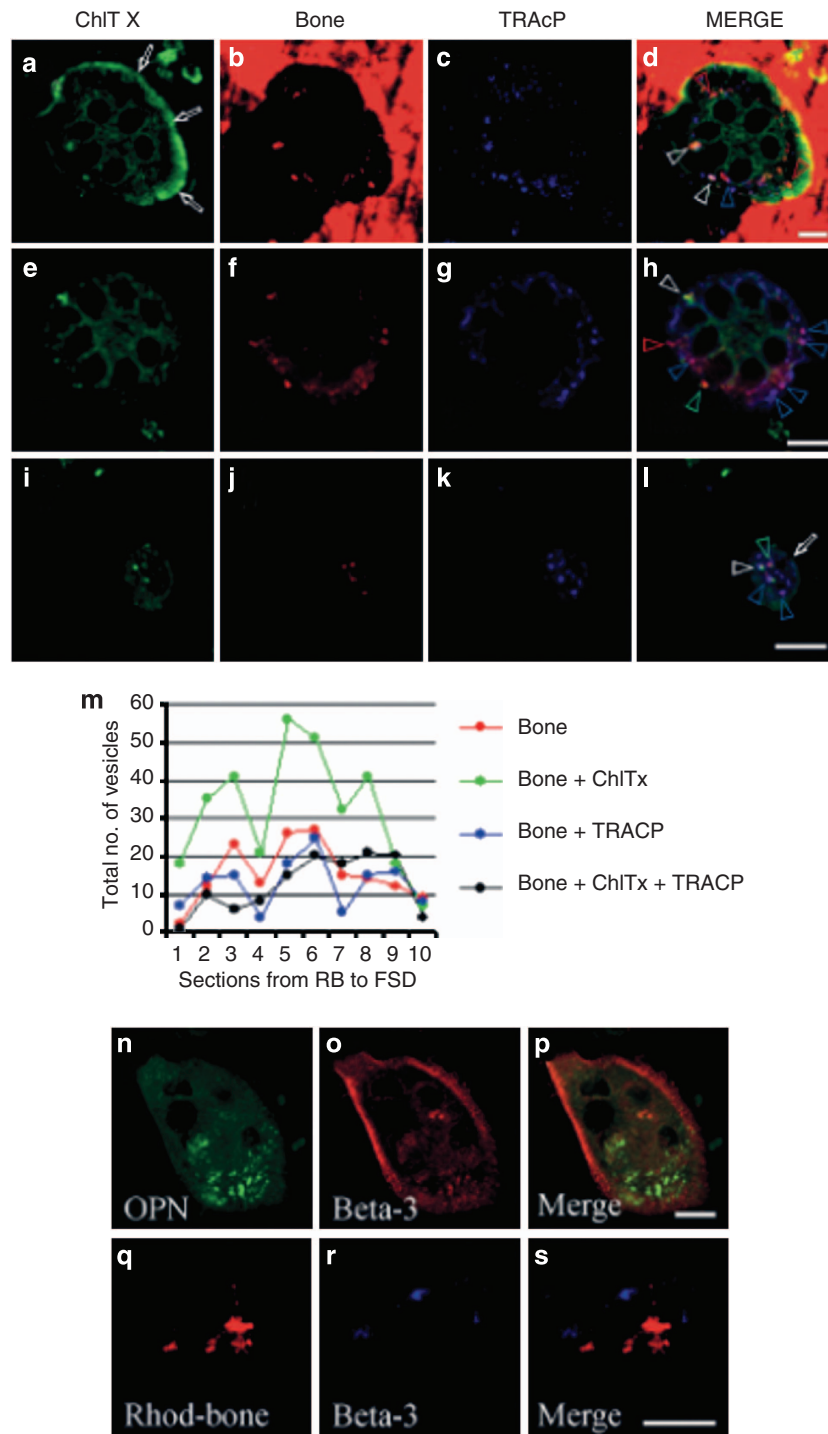
Figure 2 Existence of multiple transcytotic routes from the ruffled border to the FSD in osteoclasts. (a–l) Rat osteoclasts were cultured on 5-(6)-carboxytetramethylrhodamine-coated bone slices, fixed and labeled with antibodies against TRACP and Alexa Fluor 488 ChlTx-B. Vesicles containing the red-labeled bone matrix (b, f and j) colocalized with cholera toxin (a, e and i; green arrowheads in d, h and l), TRACP (c, g and k; blue arrowheads in d, h, l) or both (white arrowheads in d, h and l). (a–d) the ruffled border level, (e–h) the central level and (i–l) the upper part of the osteoclast. Note the dense labeling of the fusion zone of the ruffled border with ChlTx-B (a, arrows). Similarly, at the central (e–h) and at the upper part of the osteoclast (i–l), several different kinds of vesicle populations are observed. (m) The absolute number of distinct vesicles at different levels on their way from the ruffled border to the FSD. Ten sections with equal distances of 11 resorbing osteoclasts were scanned with confocal laser scanning microscope, and vesicles with differing contents were counted. (n–p) Osteoclasts cultured on recombinant osteopontin-coated bone slices and immunostained for recombinant osteopontin (n) and β_3-integrin (o) show colocalization (yellow) of the two markers in intracellular vesicles (p). (q–s) When osteoclasts were cultured on rhodamine-coated bone slices, no colocalization is observed between β_3-integrin (r) and internalized bone matrix (q). (n–s) Represents single optical sections through the nuclear level of osteoclasts. Bars, 10 μm.

Involvement of autophagosomal machinery with the transcytotic route in osteoclasts. Next, we studied the association of transcytotic vesicles with autophagy, which is one of the major pathways for the degradation of intracellular macromolecules in lysosomes. Rat osteoclasts were cultured on fluorescein-coated bovine bone slices, and autophagic

vacuoles were detected with MDC. Cells were then fixed and labeled with antibodies against Cathepsin K and TRACP, and analyzed by confocal fluorescence microscopy. MDC-binding vesicular structures colocalized with fluorescein-labeled bone, TRACP and cathepsin K (white arrowheads in **Figure 3e**), but only in the case of a colocalization of MDC with TRACP was a vertical polarization pattern of labeling observed, as these vesicles were visible at the middle (**Figure 3**) and superior levels (not shown) of the cells. This finding suggests that the autophagic vacuoles fuse with the transcytotic vesicles during transcytosis. We could not observe MDC staining at the ruffled border (not shown).

Role of cytoskeleton in targeting of the transcytotic route to the FSD—does a molecular barrier between the FSD and the peripheral non-bone-facing plasma membrane exist? We have previously reported that when osteoclasts are infected with Influenza virus, and the behavior of hae-magglutinin at the FSD is monitored, a strictly demarcated border between the HA-labeled FSD and the peripheral non-bone-facing plasma membrane is present in 66.7% ($n = 120$) of the resorbing osteoclasts.[17] In 31.7% of the resorptive cells, there is a clear increase in the number of budding virions toward the FSD but no distinct borderline was observed. In the rest of the cells (1.6%), HA is spread throughout the non-bone-facing plasma membrane and shows no preference for the FSD. Similar results were obtained with human osteoclasts (our unpublished data). This pattern of FSD diversity is also observed with scanning electron microscopy, which reveals osteoclasts similarly with and without fully developed FSD as well as osteoclasts with microvilli throughout the non-bone-facing plasma membrane.[10] The existence of osteoclasts with a strictly bordered FSD in most resorbing osteoclasts suggest the presence of a cytoskeletal machinery maintaining such a membranous structure (**Figure 4a**). Indeed, during numerous independent experiments, it became apparent that a thin actin circle structure at the top of a subset of resorbing osteoclasts exists (**Figures 4b, d and f**). WGA-lectin, which labels transcytotic degraded bone matrix and has been previously used to determine the FSD in osteoclasts,[11] accumulates inside this structure (**Figure 4c**). There are only few vimentin filaments at this area (**Figure 4e**), but dense bundles of microtubules, which we have previously suggested to be involved in transcytosis of degraded bone matrix, are abundantly present (**Figure 4g**). The osteoclast thus harbors two actin ring structures, namely, a robust ring structure surrounding the ruffled border[28–30] and a less prominent ring-like structure surrounding the FSD.

Discussion

Previous data have indicated the existence of a maturation process in which one enzyme pool along the transcytotic route is replaced by another.[15] Our current study argues that although some stepwise maturation may occur with respect to the fusion of the autophagosomal system with the transcytotic machinery, there are multiple transcytotic pathways in osteoclasts. Previously shown markers of the transcytotic route, such as TRACP and cathepsin K, which associate with internalized bone matrix did not show a vertical gradient, nor did recChlTx-B or HPA-lectin, two novel markers described in this study. It must, however, be noted that this conclusion is based on static immunofluoresence techniques and is only applicable to

vesicles in which markers meet transcytosed bone matrix, not the entire vesicle population containing the marker (for example, the signal intensity of TRACP itself is misleadingly bright at the upper part of the cell, where large and bright TRACP-containing vesicles are commonly located). The association of TRACP with transcytotic vesicles was observed along the transcytotic pathway, and no vertical polarity could be observed. There was also a great variability in the contents of transcytotic vesicles, suggesting that these vesicles exhibit marked heterogeneity at various sites along the pathway toward FSD. Furthermore, when we cultured osteoclasts on recombinant osteopontin- or rhodamine-coated bone slices, we found that only osteopontin was transcytosed in β_3-containing vesicles and that there was a distinct vesicle population delivering rhodamine-labeled bone. This initial sorting of different cargoes to different carrier vesicles in this post-lysosomal secretory pathway may indicate the occurrence of selective processing of various matrix components for transcytosis. These data not only strongly suggest that different vesicle families follow their own trafficking pathways to the FSD but also support the suggestion that $\alpha_v\beta_3$-integrin may act as a receptor for some degraded bone matrix components.[27]

There is a direct relation between the aggressiveness of metastatic cancer tissues and their HPA-lectin-binding intensity in histological samples. Nevertheless, little is known about the function of HPA-lectin receptors, exposed GalNAc glycoconjugates, in this process. Recent studies have revealed that in highly metastatic colorectal and breast cancer cell lines, an array of proteins is glycosylated by HPA-binding epitopes. These proteins include integrins alpha 6 and v, heat shock proteins and transcription factors. However, HPA-binding proteins identified in these two different cell lines were not identical, indicating that there may be great variability in HPA glycoproteins between cell types. Integrin alpha 6 was identified as the most abundant HPA glycoprotein in both cell types.[21,22] Metastatic cancer cells invade through extracellular matrix structures by triggering proteinase synthesis and secretion to the extracellular space, which leads to matrix degradation. However, the mechanisms that lead to this pathogenic process of vesicle fusion to the plasma membrane remain unknown. We analyzed the distribution of HPA-lectin-binding sites in another physiologically relevant matrix-degrading cell, the osteoclast. Our results show that osteoclasts express high numbers of HPA-lectin-binding sites, and GalNAc glycoconjugates are localized in vesicular structures along the transcytotic pathway and, in the case of human osteoclasts, at the ruffled border. Although the expression of GalNAc glycoconjugates in both metastatic cancer cells and along the transcytotic route of the osteoclast may indicate their common functional role in both cell types, their occurrence may also simply be a consequence of enhanced enzymatic activity and disruption in normal glycan extension, which would then result in the exposure of normally cryptic GalNAc. Identification of the HPA-binding glycoproteins in osteoclasts is a subject of future studies.

The mechanisms of determining the formation and maintenance of the FSD and how the transcytotic route is targeted to the FSD are not currently understood. We have observed that in 66.7% of resorbing osteoclasts there is a strict border between the FSD and the peripheral non-bone-facing plasma membrane in bone-resorbing osteoclasts. Furthermore, both Influenza virus HA and recombinant mutant G-proteins are also frequently
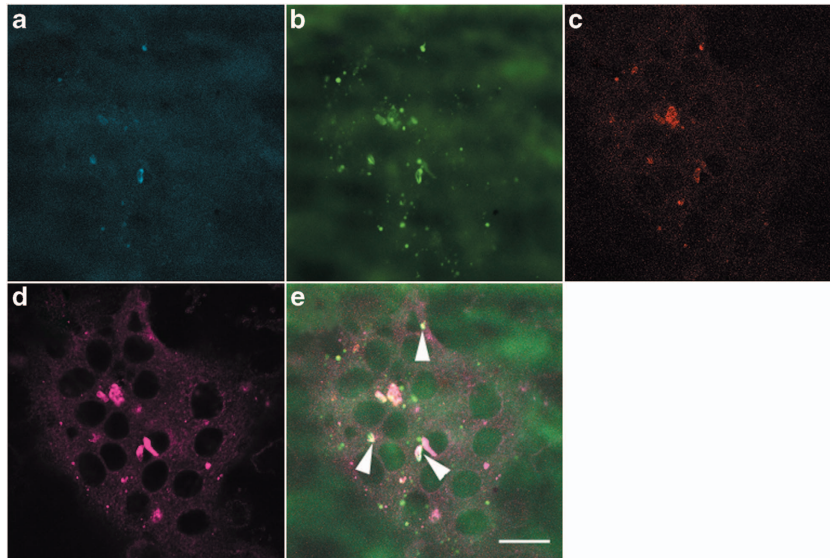
Figure 3 Involvement of autophagosomal machinery with the transcytotic route in osteoclasts. Rat osteoclasts were cultured on fluorescein-coated bovine bone slices and autophagic vacuoles were detected with MDC. Cells were fixed and labeled with antibodies against Cathepsin K (Cat K) and TRACP. At the nuclear level of rat osteoclasts, MDC-binding vesicular structures (**a**) colocalized with fluorescein-labeled bone (**b**), cathepsin K (**c**) and TRACP (**d**) (**e**, white arrowheads). Bars, 10 μm.

enriched at the periphery of the FSD.[17] This finding suggests that there is a mechanism that inhibits free protein diffusion during the continuous membrane flow to the FSD. Neurons have no cell contacts to support their membrane polarity, but their free lipid diffusion is restricted at the axon hillock and axonal initial segment by a barrier formed by transmembrane proteins anchored to the actin-based membrane skeleton.[31,32] Furthermore, the polarized distribution of membrane proteins, including the Influenza virus HA and vesicular stomatitis virus G proteins at the axonal and dendritic/cell body surfaces, is disturbed by actin-disrupting drugs.[33,34] It is likely that an actin-based barrier exists between the FSD and the peripheral non-bone-facing plasma membrane in osteoclasts during some phases of bone resorption. Our data support the view that highly regulated control of cytoskeletal organization has a role in maintaining the FSD. However, the presence of this actin-based barrier at ultrastructural level still remains to be demonstrated. Unlike neurons and epithelial cells, the morphology of osteoclasts changes from a non-polarized to a polarized cell according to the resorptive activity, making it possible that several co-existing mechanisms create and maintain the FSD. One such mechanism could be based on lipid rafts, which may also maintain the peripheral fusion zone of the ruffled border.[7] Dense bundles of microtubules were observed inside the actin ring-like structure around the FSD, which is in agreement with their role in vesicle transcytosis. Furthermore, this area was practically devoid of vimentin filaments, which characteristically formed a dense network of filaments stretching from the actin ring of the ruffled border to that of the FSD between nuclei and peripheral non-bone-facing plasma membrane (data not shown).
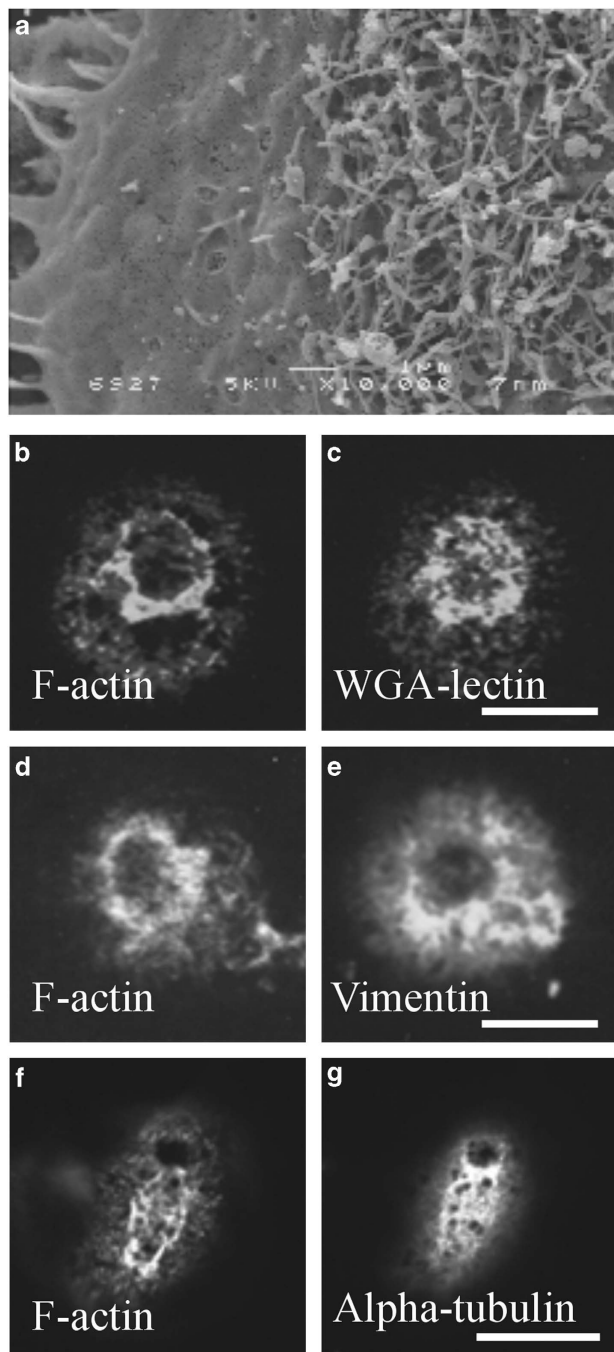
In conclusion, the osteoclastic transcytotic route from the ruffled border to the FSD is thus far poorly characterized. We demonstrated that multiple types of transcytotic vesicles arise from the ruffled border and deliver the bone degradation products for exocytosis. These vesicles are transported to the FSD that is circumscribed and separated from the peripheral non-bone-facing plasma membrane by an actin-based molecular barrier (**Figure 5**). Specific details of transcytosis in osteoclasts, for example, the regulatory molecules, such as small GTPases, however, remain yet to be elucidated.
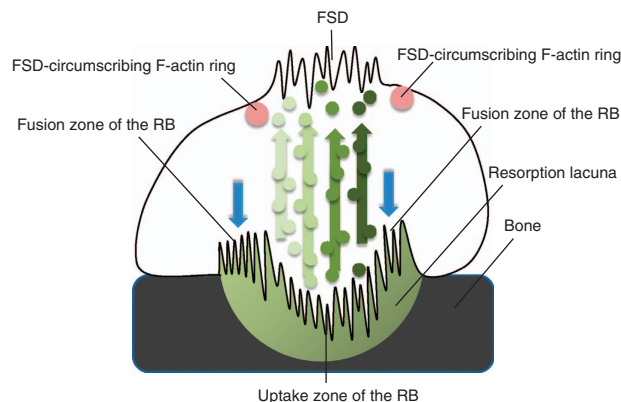
Materials and Methods

Osteoclast cultures. All animal experiments were carried out in accordance to the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes, the Statute 1076/85 § 3 and 1360/90 of the Law on Animal Protection in Finland, and the EU Directive 86/609. Rat osteoclasts were cultured according to previously described procedures.[28] Briefly, rats (Spraque–Dawley) were humanely killed at the age of 1–2 days. Osteoclasts were mechanically harvested from the bone marrow of tibiae, femora and humeri, after which they were allowed to attach to bovine bone slices (100–150 μm thick) for 60 min in a moisture chamber. After a quick rinse in phosphate-buffered saline (PBS), the cells were cultured in α-MEM (Gibco, Invitrogen Corporation, Glasgow, UK) with Glutamax (Gibco), 20 mM HEPES (Gibco), 10% heat inactivated FCS (Gibco) and 100 IU ml^{-1} of penicillin (Gibco) (pH 7.2) for 48 h at 37 °C and 5% CO_2/95% air.

Human osteoclasts were prepared as follows from a volunteer donor blood sample: heparinized blood was collected, and monocytes were isolated by Ficoll–Pague Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation. A total of 10^6 cells per well were plated and cultured in α-MEM supplemented with 10% FCS, 20 mM HEPES, 30 ng ml^{-1} RANK-L (PeproTech Inc., Rocky Hill, NJ, USA) and 10 ng ml^{-1} M-CSF (R&D Systems, Abingdon, UK) for 14 days. Half of the medium was changed and supplemented with 60 ng ml^{-1} RANKL and 20 ng ml^{-1} M-CSF every 3 days. Mature human osteoclasts expressed TRACP and formed resorption pits on bone slices.

Figure 5 A schematic summarizing the major findings of the current paper. Osteoclast degrades bone by secretion of acid and proteolytic enzymes to the resorption lacuna at the peripheral fusion zone of the ruffled border (blue arrows). Bone degradation products are endocytozed from the resorption lacuna at the central uptake zone of the ruffled border and delivered to the FSD via transcytosis. Multiple types of transcytotic vesicles that are formed initially at the ruffled border level follow their distinct routes to the FSD (green arrows). FSD is circumscribed by a thin actin-based barrier (red circles).

and polyclonal antibody against TRACP was kindly provided by S Alatalo (University of Turku, Finland). Alexa Fluor 488 conjugate recombinant cholera toxin subunit B, Alexa Fluor 488 and -568 phalloidin as well as Alexa Fluor 488, − 568 and − 647 conjugated secondary antibodies were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Fluorescently conjugated HPA lectin and 5-(6)-carboxytetramethylrhodamine succimidyl ester were purchased from Molecular Probes Europe BV. Monoclonal vimentin antibody (clone V9), monoclonal α-tubulin antibody (clone DM 1A) and polyclonal GST antibodies and fluorescently conjugated WGA-lectin were obtained from Sigma-Aldrich (St Louis, MO, USA). The rabbit anti-human transferrin was purchased from Zymed Laboratories Inc. (South San Fransisco, CA, USA).

Immunofluorescent procedures. Cell cultures were fixed with 3% paraformaldehyde (2% sucrose, PBS) for 15 min. Samples were then washed in PBS and permeabilized with cold 0.1% Triton X-100 (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, pH 7.0) for 4 min. After blocking nonspecific binding with 0.2% (w/v) gelatin in PBS (pH 7.1), the cells were incubated with primary antibodies in a moisture chamber for 30 min at 37 °C. The samples were then washed and incubated with fluorescently labeled secondary antibodies, recombinant cholera toxin subunit B and phalloidin for 30 min at 37 °C. Finally, after mounting in glycerol, the samples were examined with Leica fluorescent microscope and Leica TCS-SP confocal laser scanning microscope equipped with Argon–Krypton laser (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). Images were processed with Adobe Photoshop 7.0 and CorelDRAW 9.0.

Coating the bone slice surfaces with fluorescent labels and recombinant osteopontin. To coat the surface of the bone slices with fluorescent labels, thin bone slices were incubated in 0.1 M bicarbonate buffer (pH 8.3) containing 5-(6)-carboxytetramethylrhodamine succimidyl ester and 5-(6)-carboxyfluorescein succimidyl ester (0.25 and 1 mg ml^{-1}, respectively,

Figure 4 The FSD is surrounded by F-actin and is colonized by microtubules but not by vimentin filaments. (**a**) A field emission scanning electron microscopy image of a rat osteoclast viewed from above. Villous FSD is observed on the right and the peripheral non-bone-facing plasma membrane on the left. Note the clearly demarcated border between the two membrane domains. (**b**, **d** and **f**) Represent single horizontal sections from the FSD level of three separate osteoclasts. Phalloidin staining reveals a ring-like F-actin structure at the top of the osteoclast. (**c**) WGA-lectin that binds to the transcytosed bone matrix accumulates inside the actin boundaries. (**e**) Vimentin filaments are absent from the interior of the ring structure, while numerous microtubules (**g**) are present. Immunolabelings were performed after permeabilization with Triton X-100. Bars, 10 μm.

Antibodies and fluorescent conjugates. Monoclonal antibody against the β3 subunit of vitronectin receptor was a generous gift from Dr MA Horton (University College London),

Molecular Probes) for 2 h and then washed several times with PBS.

To coat the surface of the bone slices with recombinant osteopontin, thin bone slices were incubated overnight at $+4\,^{\circ}\text{C}$ with GST-osteopontin in coating buffer (5.1 ng ml^{-1} Na_2CO_3 in dH$_2$O, pH 9.6) and washed with PBS before use. Recombinant osteopontin was provided by Merck Laboratories (West Point, PA, USA) and was visualized by anti-GST antibody immunostaining. Resorption activity of osteoclasts on coated bone slices was equal to that on non-coated bone slices (data not shown).

Transferrin experiments. After 48 h of culture, iron-loaded human transferrin (0.1 mg ml^{-1}) was added into the culture medium for 60 min, washed in prewarmed PBS for 20 s and fixed with 3% paraformaldehyde for 20 min. Then, the cells were permeabilized with 0.1% Triton X-100 in PBS and immunostained with polyclonal antibodies against human transferrin.

Scanning electron microscopy. To visualize the FSD with field emission scanning electron microscope, samples were first fixed with 5% glutaraldehyde in s-collidin-HCl buffer (pH 7.4, 0.16 M) for 45 min, then dehydrated in an ascending ethanol series, processed for critical point drying (Balzers Union, Balzers, Liechtenstein), Au/Pg sputtered (Polaron Equipment Ltd., E-5100) and finally examined with field emission scanning electron microscope (JEOL JSM-6300F, JEOL, Tokyo, Japan) at 5 kV.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

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