Microvesicles released from hormone-refractory prostate cancer cells facilitate mouse pre-osteoblast differentiation

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Abstract Bone metastasis is often occurs in patients with prostate cancer. There is a vicious cycle for bone metastases involving prostate cancer cells, osteoblasts, and osteoclasts. Acting among those cells during the process of metastasis are several molecules such as bone morphogenetic proteins, platelet-derived growth factor, endothelin-1, matrix metalloproteases, vascular endothelial growth factor, transforming growth factor-β, and insulin-like growth factors. Cell-derived microvesicles are endogenous carriers transporting proteins, mRNAs and miRNAs between cells, which is a candidate for participation in the bone metastasis of these cells. Here, we demonstrated that prostate cancer cells in vitro released microvesicles into the culture medium (PCa-MVs), which was shown by electron microscopic study and nanoparticle tracking analysis. In this study, we found for the first time that these PCa-MVs enhanced osteoblast differentiation mainly through the delivery of PCa cell-derived v-ets erythroblastosis virus E26 oncogene homolog 1, which is an osteoblast differentiation related-transcriptional factor.

Keywords Prostate cancer · Microvesicles · Osteoblast differentiation · Ets1 · Osteoblastic bone metastasis

Introduction

In many cell types, microvesicles (MVs) including shedding microvesicles (SMVs) and exosomes (EXOs) are released into the extracellular environment as a cell-to-cell communication tool (Bastida et al. 1984; Mack et al. 2000; Morel et al. 2004; Tesse et al. 2005; Martinez et al. 2006; Wysoczynski and Ratajczak 2009). In this study, we defined that MVs include SMVs and EXOs. These MVs contain receptor proteins, proteolytic enzymes, miRNAs, and mRNAs which are transferred into the target cell, and then affect various cell functions (Ratajczak et al. 2006; Bharadwaj et al. 2006). In tumor cells, α-disintegrin and metalloproteinase (ADAM) and matrix metalloprotease (MMP) in MVs enhance the matrix digestion, which action facilitates the migration and metastasis of tumor cells (Gutwein et al. 2003; Mochizuki and Okada 2007). Moreover, anti-cancer drugs such as a doxorubicin decrease the levels of SMVs (Shedden et al. 2003). Thus, the MV transfer system is one of the important systems for tumor cell proliferation and progression. Osteoblastic bone metastasis in prostate cancer (PCa) patients is frequently observed as the disease progresses, and is related to high patient mortality and morbidity (Coleman 1997; Bubendorf
et al. 2000; Roudier et al. 2003). In osteoblastic metastasis, a vicious cycle is established between the PCa cells and bone cells, i.e., osteoblasts and osteoclasts. PCa cells supply osteoblastic factors (e.g., bone morphogenetic proteins [BMPs], platelet-derived growth factor [PDGF], endothelin-1 [ET1]) and osteolytic factors (e.g., MMPs and vascular endothelial growth factor [VEGF]) to osteoblasts and osteoclasts, respectively, thereby allowing these cells to elaborate bone-derived growth factors (e.g., transforming growth factor-β [TGF-β], Insulin-like growth factors [IGFs]) for cell growth (Casimiro et al. 2009; Morrissey et al. 2006). Therefore, many signal exchanges are performed by direct or indirect contact between PCa cell and osteoblast during the process of bone metastasis. However, the effect of PCa-MVs on osteoblast function is not yet understood. In this study, we present evidence that PCa-MVs enhanced osteoblast differentiation mainly through the delivery of PCa-derived v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1), which is an osteoblast differentiation-related transcriptional factor.

Materials and methods

Reagents and materials

The 25× Complete® mixture of protease inhibitors purchased from Roche (Penzberg, Germany); and Phosphatase Inhibitor Cocktail® 1 and 2 from Sigma (St. Louis, MO, USA). Antibodies against human TSG101, CD9, CD81, PThrP, Ets1, GAPDH, and mouse Ets1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin as an internal standard was purchased from Sigma. Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit was obtained from GE Health Science (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, UK).

Cell culture

PC3 and DU145 hormone-refractory human prostate cancer cells, and hormone-sensitive LNCaP cells were purchased from ATCC and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml streptomycin. MVs in FBS were excluded by ultra-centrifugation (250,000×g, 3 h) and filtration (0.45 μm). PrEC cells were used as normal human prostate epithelial cells. Murine pre-osteoblast cell line MC3T3-E1 was obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and cultured in phenol-red free α-MEM supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 μg/ml streptomycin. These cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

Electron microscopic observation

The PC3 and DU145 cells were harvested and rinsed with PBS, after which they were fixed for 30 min in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, PB), rinsed in PB, and postfixed in 1% osmium tetroxide for 30 min. After having been washed with PB, the cells were progressively dehydrated by passage through a 10% graded series of 50–100% ethanol and then cleared in QY-1 (Nissin EM, Tokyo, Japan). They were then embedded in Epon 812 resin (TAAB Laboratories Equipment, Reading, UK); subsequently, thin sections (70 nm thickness) were cut, stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy using an Hitachi-7650 (Hitachi, Tokyo, Japan).

Isolation of microvesicles from medium of PC3 or DU145 cell cultures

For preparation of MVs from PC3 or DU145 cells, the medium from either source was centrifuged at 1,500×g for 10 min to remove cells and other debris. These supernatants were then centrifuged at 250,000×g for 3 h at 4 °C. The centrifuged-microvesicles were resuspended in serum-free α-MEM and then filtered (0.45 μm). The filtered samples were quantified based on the protein levels by using the method of Bradford (BioRad, Hercules, CA, USA).

Nanoparticle tracking analysis (NTA)

Microvesicles were purified from the medium of PC3 cell cultures, as described above. The microvesicle samples after passage through the 1st filter (0.22 μm) of an ExoMir kit (Bioo Scientific, Austin, TX) were used for analysis. The Nanosight LM10 nanoparticle characterization system (NanoSight, NanoSight Ltd, UK) equipped with a blue laser (638 nm) illumination was used for real-time characterization of the vesicles. The results were presented at the average value of 2 independent experiments.

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MC3T3-E1 cells were inoculated into 96-well plates (1 × 10^5 cells/ml, 100 μl/well; Nunc, Roskilde, Denmark) and cultured with or without PCa-MVs (2/100 μl of MEM/well, equivalent protein conc. 20 μg/μl) for 3 days. After incubation, the treated cells were washed twice with PBS, and then fixed with EtOH for 10 min. The ALP activity was estimated by the using a TRAP & ALP double-staining kit (Takara Bio Inc. Ohtsu, Japan) according to the manufacturer’s protocol. As a positive control, MC3T3-E1 cells were treated with 100 ng/ml of BMP-2 (R&D Systems, Minneapolis, MN, USA).

Results and discussion

As shown in Fig. 1a, the cells of hormone-refractory PCa cell lines PC3 and DU145 cells in logarithmic growth phase shed MVs from their plasma membrane (Fig. 1a; upper panel and middle panel, respectively). The diameters of these MVs were approximately 50–100 nm (Fig. 1a, lower panel). NTA (nanoparticle tracking analysis) indicated that the microvesicles from PC3 cells were 139 nm in diameter, as shown by the peak in the size-distribution graph (Fig. 1b). The biochemical characterization of MVs indicated a difference in expression levels of MV-related TSG101, tetraspanin CD9 and CD81 between the cells and MVs (Fig. 1c). CD81 was relatively specific for MV among them. Thus, we confirmed that the PC3 and DU145 cells released MVs into their culture medium. To examine the effect of PCa-MVs on osteoblast differentiation, we added PCa-MVs in suspension to murine pre-osteoblast MC3T3-E1 cell cultures and then incubated the cells for 72 h at 37 °C. Thereafter, the induction of differentiation was estimated by ALP staining. The number of the MVs incubated in a well was approximately 1 × 10^7 particles. PCa-MVs prepared from either PC3 or DU145 cell cultures significantly facilitated osteoblast differentiation, but the PCa-MVs from LNCaP cells did not (Fig. 2a, b). The differentiating activity of these PCa-MVs was in the order of BMP-2 (100 ng/ml, positive control) > DU145 > PC3.

To disclose the mechanisms underlying the stimulation of osteoblast differentiation by PCa-MVs, we further focused on the protein levels of Ets1 and parathyroid hormone-related protein (PTHrP) in PCa cells and PCa-MVs. Tumor-derived PTHrP up-regulates bone remodeling, leading to the release of numerous bone-derived growth factors from osteoblasts or osteoclasts for PCa cell growth (Casimiro et al. 2009; Morrissey et al. 2010; Ibrahim et al. 2010). Ets1 is a proto-oncogene protein that is highly expressed tumor cells and regulates the expression of MMP-1, -3, -9, and uPA (Sementchenko and Watson 2000). In osteogenesis, Ets1 also regulates the expression of several proteins such as osteopontin (OPN), tenascin-C, and procollagen (Sato et al. 1998; Raouf and Seth 2000). Moreover, Ets1 activates PTHrP gene expression via the binding of PTHrP P3 promoter region with cAMP response element binding protein (CREB) (Hamzaoui et al. 2007). Thus, PTHrP and Ets1 are important molecules in tumor metastasis. The data presented in Fig. 3a show that both hormone-refractory PCa cell lines, PC3 and DU145, highly expressed Ets1 and PTHrP but that neither protein was detected in PrEC normal human prostate epithelial cells. Interestingly, LNCaP human hormone-sensitive PCa cells did not express Ets1 but did express PTHrP. On the other hand, Ets1 was detected in MVs from both hormone-refractory PCa cell lines: but PTHrP was detected in
neither of them (Fig. 3b). From these results, we propose that the bone-derived growth factors such as BMPs, Ets1, and PTHrP were wrapped in PCa-MVs in accordance with some certain rules rather than enclosed randomly. Ets1 acts as a transcription factor in osteoblasts during their differentiation. Therefore, the PCa-MVs delivery system, which affords fusion of MVs to the plasma membrane of target cells, is a reasonable transfer model for Ets1. In contrast to Ets1, PTHrP binds to the PTH receptor (PTHrP) expressed on the surface of osteoblasts, which cells produce receptor activator of nuclear factor kappa-B ligand (RANKL) and monocyte chemoattractant protein-1 (MCP-1) (Lu et al. 2007; Liao et al. 2008). Hence, PTHrP needs to interact with PTHR for osteoblast differentiation. Thus, PC3 and DU145 cells highly expressed Ets1 and PTHrP, but in their MVs PTHrP was hardly detected by western blot analysis (Fig. 3). Also, LNCaP cells expressed PTHrP but not Ets1, the MVs from LNCaP cells were not able to induce the differentiation (Fig. 2). These findings indicate that the higher expressed Ets1 is a possible candidate inducer of osteoblast differentiation.

In order to investigate intracellular location of the Ets1 delivered to the osteoblast via PCa-MVs, we used immunofluorescence staining. Although much of the Ets1 from the PCa-MVs adhered to the osteoblast cell surface, a part of it was transferred into the nuclei (Fig. 4). However, we
did not measure the expression levels of Ets1-regulated molecules such as PTHrP, OPN, tenascin-C, and procollagen. Previously, we reported that miR-208 targets Ets1, which leads to attenuation of the differentiation with downregulation of OPN and Runx2 (Itoh et al. 2010). So, the question still remains as to whether or not the Ets1 transferred via PCa-MVs acts in nuclei as a transcription factor. We cannot deny the possibility that not only Ets1 protein but also other proteins, mRNAs and miRNAs related to osteoblast differentiation may be transferred into the cells via the PCa-MVs. Further study is required to answer these questions.

In summary, Ets1-containing MVs from hormone-refractory PCa cells were transferred into osteoblasts, and the Ets1 discharged into the cytoplasm functioned to induce differentiation. Our findings thus suggest that PCa-MVs acted as a cell-to-cell communication tool in osteoblastic metastasis.
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