Biochemical and Biophysical Research Communications 461 (2015) 76–79

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Low pH increases the yield of exosome isolation

Jae-Jun Ban [a, 1], Mijung Lee [b, 1], Wooseok Im [b, *], Manho Kim [b, c, **]

[a] Department of Bioscience and Biotechnology, College of Life Science, Institute of Biotechnology, Sejong University, Seoul, South Korea
[b] Department of Neurology, Seoul National University Hospital, Seoul, South Korea
[c] Protein Metabolism Medical Research Center, College of Medicine, Seoul National University, Seoul, South Korea

ARTICLE INFO

Article history:
Received 23 March 2015
Available online 4 April 2015

Keywords:
Exosome
pH
Acidic microenvironment

ABSTRACT

Exosomes are the extracellular vesicles secreted by various cells. Exosomes mediate intercellular communication by delivering a variety of molecules between cells. Cancer cell derived exosomes seem to be related with tumor progression and metastasis. Tumor microenvironment is thought to be acidic and this low pH controls exosome physiology, leading to tumor progression. Despite the importance of microenvironmental pH on exosome, most of exosome studies have been performed without regard to pH. Therefore, the difference of exosome stability and yield of isolation by different pH need to be studied. In this research, we investigated the yield of total exosomal protein and RNA after incubation in acidic, neutral and alkaline conditioned medium. Representative exosome markers were investigated by western blot after incubation of exosomes in different pH. As a result, the concentrations of exosomal protein and nucleic acid were significantly increased after incubation in the acidic medium compared with neutral medium. The higher levels of exosome markers including CD9, CD63 and HSP70 were observed after incubation in an acidic environment. On the other hand, no exosomal protein, exosomal RNA and exosome markers have been detected after incubation in an alkaline condition. In summary, our results indicate that the acidic condition is the favorable environment for existence and isolation of exosomes.

1. Introduction

Exosomes are small extracellular vesicles with 30–100 nm diameter that originate from many type of cells [1]. Exosomes are formed as part of the multivesicular body pathway in which intraluminal vesicles progressively accumulate during endosome maturation. Exosomes are formed by inward budding and scission of vesicles from the limiting endosomal membranes and released from the MVB lumen into the extracellular environment during exocytosis [1,2]. Secreted exosomes can be isolated and characterized in blood [3,4], urine [5], saliva [6] in vivo and conditioned medium [2] in vitro. They include various molecular constituents of their cell origin, including proteins, mRNA, and microRNA [7–9]. Because of their importance in normal physiology and disease progression, exosomes have been widely studied as a therapeutic agent [10–13], biomarker [3,6] and drug delivery vehicle [14].

Extracellular pH is various dependent on the tissues microenvironment and pathologic conditions, affecting the amount and characteristic of exosome. For example, the pH of gastric juice is severely low and there are RNAs in the stomach's acidic condition. These gastric juice-derived RNAs might be associated with gastric juice exosomes [15]. Abnormally proliferated cancer cells change their microenvironmental pH into acidic, resulting in cancer progression and metastasis [16–18]. Recent research of cancer exosomes revealed that acidic microenvironment promotes exosome traffic and uptake of cancer cells, contributing tumor favorable environment [16,19,20]. Exosomes seem to have a crucial role in propagation of Prion protein in Creutzfeldt–Jakob disease and low pH triggers Prion protein misfolding [21,22]. Considering these studies of exosome and pH on diseases, it is plausible that pH have some crucial role in exosome physiology.

Despite of this importance of pH condition in exosome and related diseases, most in vitro experiments of exosome isolation and characterization have been performed without regard to this situation. Thus, the effects of pH on the exosome isolation and

* Corresponding author. Department of Neurology, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea. Fax: +82 2 762 5178.
** Corresponding author. Department of Neurology, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea. Fax: +82 2 3672 7553.
E-mail addresses: imwooseok@gmail.com (W. Im), kimmanho@snu.ac.kr (M. Kim).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.03.172
0006-291X/

characterization should be thoroughly studied for future exosome researches. In this study, we isolated the exosomes after incubation in various pH conditions and investigated the yield of exosomes by measuring exosomal total protein and RNA concentration. Representative exosome markers were also investigated to confirm the yield of exosome isolation.

2. Materials and methods

2.1. HEK 293 cell culture

To deplete bovine exosomes in the culture medium, Dulbecco's modified eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin was centrifuged at 100,000 g for 15 h at 4 °C and supernatant was used for cell culture. HEK 293 cells were seeded at a density of 1×10^5 cells in 15 ml of exosome free medium into T-75 culture flask and cultured for 5 days at 37 °C in a 5% CO_2 incubator.

2.2. Adjustment of pH in conditioned medium

Cells and cellular debris were eliminated from conditioned medium (CM) by centrifugation at 3000 g for 10 min at room temperature. pH in CM was adjusted with 1 M hydrogen chloride (HCl) or 1 M sodium hydroxide (NaOH) solution and measured by a pH meter (Thermo Fisher Scientific, MA, USA) for making pH4, 7 and 11 CM. Exosomes were isolated from pH4, 7 or 11 CM after incubating for 30 min at room temperature.

2.3. Exosomes isolation

Exosome isolation using Exo-quick exosome precipitation kit (System Biosciences, CA, USA) was performed according to manufacturer's specifications. Briefly, the 3 ml CM was mixed thoroughly with 0.6 ml of Exo-Quick exosome precipitation solution and incubated for 24 h at 4 °C. CM complex were centrifuged at 1500 g for 30 min, and then the supernatant was removed and centrifuged at 1500 g for 5 min again. The remaining exosome pellet was used for protein or RNA isolation.

2.4. Protein isolation and western blot

Proteins in exosomes were extracted by 100 μl of Radio Immuno Precipitation Assay buffer (Pierce, IL, USA) with freshly added protease inhibitor (Roche, USA) and exosomal proteins were measured by BCA protein assay kit (Pierce, IL, USA). Proteins were loaded on sodium dodecyl sulfateopolyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane (Millipore, MA, USA). The blots were probed with primary antibodies: CD9 (1:200; Santa Cruz, CA, USA), CD63 (1:200; Santa Cruz, CA, USA), HSP70 (1:200; Santa Cruz, CA, USA), followed by horseradish peroxidase conjugated secondary anti-mouse or rabbit antibody (1:3000; GE Healthcare, USA). The relative protein levels were analyzed by ImageJ software.

2.5. RNA isolation

Isolation of RNA from exosomes was carried out by the mirVana isolation kit (Ambion, TX, USA) according to the manufacturer's protocol. Briefly, exosomes pellet was lysed with lysis buffer and exosomal RNA was extracted by miRNA homogenate solution and chloroform. The exosomal RNA was suspended with 100 μl of DEPC water after purifying by the filter cartridge and the concentration of RNA was quantified using the Nano Drop ND-1000 Spectrophotometer (Nano-Drop Technologies, DE, USA).

2.6. Statistical analysis

All values shown in the figures are presented as mean ± standard error. Western blot results were analyzed by student's t-test. A 2-tailed probability value below 0.05 was considered statistically significant. Data were analyzed using SPSS version 17.0 (SPSS Inc., USA).

3. Results

3.1. Exosomes isolation and characterization

HEK 293 cells were cultured for 3 days and exosomes were isolated by Exoquick isolation kit. After isolation, proteins were extracted by protein extraction buffer and western blot was performed. As a result, representative exosome markers including CD9, CD63 and HSP70 were examined in the conditioned medium derived exosomal protein (Fig. 1A). Exosome-free media was examined as a negative control and didn't show any exosome markers. The result of this experiment indicates that our exosome-free media and isolation method is suitable for cell line-derived exosome research.

3.2. Effect of pH on the representative exosome markers

To investigate the level of representative exosome markers in exosomes of CM in different pH conditions, the pH of CM was adjusted to pH4, 7 and 11 by HCl or NaOH, and exosomal proteins of pH4, pH7 or pH11 CM were immunoblotted with CD9, CD63 and HSP70 antibodies. As a result, pH4 CM shows higher level of exosome markers than pH7 CM. On the contrary, the sample which had been incubated in pH11 showed no exosomal markers (Fig. 1B). The effect of pH on exosomal HSP70 level was measured in a range from ph3 to 10, and result also showed inverse correlation of pH and protein level (S2 Fig.).

3.3. Effect of pH on the exosomal protein and RNA concentration

To investigate exosomal protein and RNA concentration in different pH conditions, proteins and total RNA were extracted by protein extraction buffer and total RNA isolation kit respectively after isolating exosomes from each pH4, pH7 or pH11 CM. As a result, total exosomal protein concentration in pH4 CM is fivefold higher than that of pH7 CM (Fig. 2A) and RNA concentration of pH4 CM also showed five times increased level compared to pH7 CM (Fig. 2B). Two representative methods for exosome isolation are serial ultracentrifugation and Exoquick reagent. We identified increased yield of exosomal protein by low pH using ultracentrifugation method (S1 Fig.). These results suggest that the yield of isolation of representative exosome components, such as proteins and nucleic acids, could be increased by acidic environment.

4. Discussion

In this study, we demonstrated that acidic pH could increase the stability of exosomes in vitro, resulting in higher yield of exosome isolation. Incubation in acidic medium showed increased exosomal protein and RNA concentration. Immunoblot of representative exosome markers also showed increased isolation of intact exosomes by acidic condition. On the contrary, exosome isolation failed after incubation in an alkaline medium.

Cancer cell derived exosomes seem to induce cancer favorable microenvironment and tumor core is relatively acidic [16–18]. Recent study shows that precipitation of exosomes from tumor cells has improved after treatment of acetate [23]. Parolini et al.

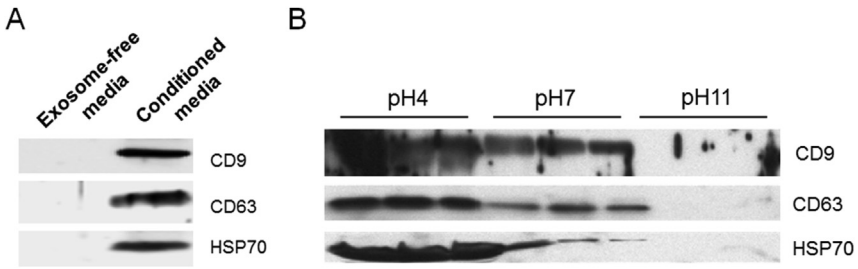

Fig. 1. Western blot analysis with exosome markers. Exosome isolated from exosome-free media or conditioned media were immunoblotted with CD9, CD63 and HSP70 (A). Exosomes were incubated with pH4, pH7 or pH11 medium and tested with western blot for analyzing the level of CD9, CD63 and HSP70 (B).

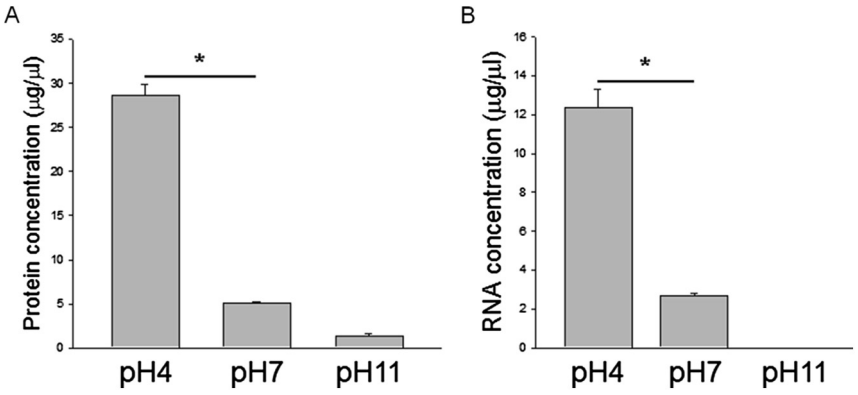

Fig. 2. Measurement of exosomal protein and RNA concentration. Exosomal protein and RNA were extracted from each exosomes incubated with pH4, pH7 or pH11 and protein concentration was measured by BCA protein quantification (A) and Nano Drop for RNA concentration (B). *$p < 0.01$.

found that intercellular acidic pH could increase cancer exosome secretion and uptake, leading to tumor progression and metastasis [19]. Prion is propagated by extracellular exosomes in Creutzfeldt–Jakob disease and misfolding of Prion protein is related with low pH [21,22]. Based on these exosome researches, it is plausible that pH could affect stability of exosomes. Our study showed that acidic condition could increase the amount of isolation of cell-derived exosomes, which enables to extract exosomal proteins and nucleic acids from the small amount of the specimen. Proteins and nucleic acids in exosomes are protected against low pH condition of gastric fluid by showing that they are present in high concentration in gastric fluid while there are almost no exosomes after incubation in alkaline condition.

Exosome mediates genesis and progression of diseases including cancer, Creutzfeldt–Jakob disease and Alzhermer's disease [20,21,24]. Despite of their promising potential in medical field, the exact mechanism of biogenesis, secretion and uptake of exosome is still unknown. In addition to this, clear protocol for handling, storage and isolation method for exosome experiment need to be established. In summary, our results show that low pH increases the yield of exosome isolation and high pH degrades the exosome, suggesting that environmental pH of exosome needs to be carefully considered for exosome researches.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) (2014R1A2A1A11051520), Korea Health 21 R & D Project (HI14C2348) by the Ministry of Health & Welfare, Republic of Korea, and National Research Foundation of Korea (2011–0012728).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.172.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.172.

References

[1] A. Lakkaraju, E. Rodriguez-Boulan, Itinerant exosomes: emerging roles in cell and tissue polarity, Trends Cell Biol. 18 (2008) 199–209.
[2] C. Thery, S. Amigorena, G. Raposo, A. Clayton, Isolation and characterization of exosomes from cell culture supernatants and biological fluids, Curr. Protoc. Cell Biol. (2006). Chapter 3 Unit 3.22.
[3] S.K. Gupta, C. Bang, T. Thum, Circulating microRNAs as biomarkers and potential paracrine mediators of cardiovascular disease, Circ. Cardiovasc. Genet. 3 (2010) 484–488.
[4] M. Tsujiura, D. Ichikawa, S. Komatsu, A. Shiozaki, H. Takeshita, T. Kosuga, H. Konishi, R. Morimura, K. Deguchi, H. Fujiwara, K. Okamoto, E. Otsuji, Circulating microRNAs in plasma of patients with gastric cancers, Br. J. Cancer 102 (2010) 1174–1179.
[5] H. Zhou, P.S. Yuen, T. Pisitkun, P.A. Gonzales, H. Yasuda, J.W. Dear, P. Gross, M.A. Knepper, R.A. Star, Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery, Kidney Int. 69 (2006) 1471–1476.
[6] A. Michael, S.D. Bajracharya, P.S. Yuen, H. Zhou, R.A. Star, G.G. Illei, I. Alevizos, Exosomes from human saliva as a source of microRNA biomarkers, Oral Dis. 16 (2010) 34–38.
[7] D.M. Pegtel, K. Cosmopoulos, D.A. Thorley-Lawson, M.A. van Eijndhoven, E.S. Hopmans, J.L. Lindenberg, T.D. de Gruijl, T. Wurdinger, J.M. Middeldorp, Functional delivery of viral miRNAs via exosomes, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 6328–6333.

[8] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell. Biol. 9 (2007) 654–659.
[9] I.S. Zeelenberg, M. Ostrowski, S. Krumeich, A. Bobrie, C. Jancic, A. Boissonnas, A. Delcayre, J.B. Le Pecq, B. Combadiere, S. Amigorena, C. Thery, Targeting tumor antigens to secreted membrane vesicles in vivo induces efficient antitumor immune responses, Cancer Res. 68 (2008) 1228–1235.
[10] F. Arslan, R.C. Lai, M.B. Smeets, L. Akeroyd, A. Choo, E.N. Aguor, L. Timmers, H.V. van Rijen, P.A. Doevendans, G. Pasterkamp, S.K. Lim, D.P. de Kleijn, Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury, Stem cell Res. 10 (2013) 301–312.
[11] M.C. Deregibus, V. Cantaluppi, R. Calogero, M. Lo Iacono, C. Tetta, L. Biancone, S. Bruno, B. Bussolati, G. Camussi, Endothelial progenitor cell derived micro-vesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA, Blood 110 (2007) 2440–2448.
[12] T. Katsuda, R. Tsuchiya, N. Kosaka, Y. Yoshioka, K. Takagaki, K. Oki, F. Takeshita, Y. Sakai, M. Kuroda, T. Ochiya, Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes, Sci. Reports 3 (2013) 1197.
[13] S. Sahoo, E. Klychko, T. Thorne, S. Misener, K.M. Schultz, M. Millay, A. Ito, T. Liu, C. Kamide, H. Agrawal, H. Perlman, G. Qin, R. Kishore, D.W. Losordo, Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity, Circ. Res. 109 (2011) 724–728.
[14] S. Ohno, M. Takanashi, K. Sudo, S. Ueda, A. Ishikawa, N. Matsuyama, K. Fujita, T. Mizutani, T. Ohgi, T. Ochiya, N. Gotoh, M. Kuroda, Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells, Mol. Ther. J. Am. Soc. Gene Ther. 21 (2013) 185–191.
[15] Y. Shao, M. Ye, X. Jiang, W. Sun, X. Ding, Z. Liu, G. Ye, X. Zhang, B. Xiao, J. Guo, Gastric juice long noncoding RNA used as a tumor marker for screening gastric cancer, Cancer 120 (2014) 3320–3328.
[16] V. Estrella, T. Chen, M. Lloyd, J. Wojtkowiak, H.H. Cornnell, A. Ibrahim-Hashim, K. Bailey, Y. Balagurunathan, J.M. Rothberg, B.F. Sloane, J. Johnson, R.A. Gatenby, R.J. Gillies, Acidity generated by the tumor microenvironment drives local invasion, Cancer Res. 73 (2013) 1524–1535.
[17] A. Riemann, B. Schneider, A. Ihling, M. Nowak, C. Sauvant, O. Thews, M. Gekle, Acidic environment leads to ROS-induced MAPK signaling in cancer cells, PloS One 6 (2011) e22445.
[18] I.F. Tannock, D. Rotin, Acid pH in tumors and its potential for therapeutic exploitation, Cancer Res. 49 (1989) 4373–4384.
[19] I. Parolini, C. Federici, C. Raggi, L. Lugini, S. Palleschi, A. De Milito, C. Coscia, E. Iessi, M. Logozzi, A. Molinari, M. Colone, M. Tatti, M. Sargiacomo, S. Fais, Microenvironmental pH is a key factor for exosome traffic in tumor cells, J. Biol. Chem. 284 (2009) 34211–34222.
[20] G. Taraboletti, S. D'Ascenzo, I. Giusti, D. Marchetti, P. Borsotti, D. Millimaggi, R. Giavazzi, A. Pavan, V. Dolo, Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH, Neoplasia 8 (2006) 96–103.
[21] B. Fevrier, D. Vilette, F. Archer, D. Loew, W. Faigle, M. Vidal, H. Laude, G. Raposo, Cells release prions in association with exosomes, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 9683–9688.
[22] M.L. DeMarco, V. Daggett, Molecular mechanism for low pH triggered mis-folding of the human prion protein, Biochemistry 46 (2007) 3045–3054.
[23] Z. Brownlee, K.D. Lynn, P.E. Thorpe, A.J. Schroit, A novel "salting-out" procedure for the isolation of tumor-derived exosomes, J. Immunol. Methods 407 (2014) 120–126.
[24] S. Saman, W. Kim, M. Raya, Y. Visnick, S. Miro, S. Saman, B. Jackson, A.C. McKee, V.E. Alvarez, N.C. Lee, G.F. Hall, Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease, J. Biol. Chem. 287 (2012) 3842–3849.