Chapter 12

Apoptotic Bodies: Selective Detection in Extracellular Vesicles

Paul Hauser, Sha Wang, and Vladimir V. Didenko

Abstract

Normal and dying cells release various types of membrane-bound vesicles including microvesicles, exosomes, and apoptotic bodies. These vesicles play important roles in intercellular communication and signal transduction. However, their diverse forms and subtypes fluctuate in size and other properties. In result current purification approaches do not fully discriminate between different categories of extracellular vesicles. Here, we present a fluorescence technique that specifically identifies apoptotic bodies in preparations of microvesicles, exosomes, and other extracellular vesicles.

The approach exclusively labels the vesicles that contain DNA with 5′PO_4 blunt-ended DNA breaks, such as those produced by the apoptotic CAD nuclease during apoptotic DNA degradation. The technique can be useful in studies of apoptosis involving microvesicles and exosomes.

Key words Apoptotic bodies, Extracellular vesicles, Exosomes, Microvesicles, 5′P blunt-ended DNA breaks, In situ ligation technique, Apoptotic bodies labeling, Cell signaling in apoptosis, "find-me" signal, "eat-me" signal, Clearance of apoptotic cells

1 Introduction

Apoptotic bodies are characteristic membrane blebs released by cells undergoing apoptosis [1–3]. These vesicles contain membrane-enveloped fragments of apoptotic DNA and cytoplasm. Among their other functions, apoptotic bodies have an important signaling function. They carry "find-me" and "eat-me" molecular signals intended to attract phagocytes to apoptotic sites and promote apoptotic cell clearance [2, 4, 5].

Apoptotic bodies are released exclusively during apoptotic cell death. This separates them from other types of extracellular vesicles such as exosomes and microvesicles, which are constantly generated by normal viable cells [6].

Apoptotic bodes are produced during apoptotic cell disassembly when the nuclear and cytoplasmic parts are swiftly parceled into multiple and compact membrane-bound vesicles in a wide range of

Alexander E. Kalyuzhny (ed.), *Signal Transduction Immunohistochemistry: Methods and Protocols*, Methods in Molecular Biology, vol. 1554, DOI 10.1007/978-1-4939-6759-9_12,

sizes [3]. The cargo of an individual apoptotic body consists of the cellular components that happened to be in the cytoplasmic protrusion that created it. Because of that, some apoptotic bodies are almost entirely made of condensed nuclear chromatin, whereas others carry only cytoplasmic components [3].

This difference in composition is important because it changes the physical properties of the vesicles, which are used in the methods for their purification. The tightly packed DNA is denser, as compared to enclosed cytoplasm, and carries a negative charge. These differences can affect the pool of apoptotic bodies purified by methods that use density, gravity, or charge to separate the vesicles.

The additional important consideration, from the detection point of view, is that the cytoplasmic and nuclear apoptotic bodies differ in their ability to be stained by various markers because they contain either DNA or cytoplasmic proteins.

For these reasons, apoptotic bodies are subdivided into two groups based on their cargo. If they carry a fragment of an apoptotic nucleus, they are referred to as nuclear (DNA carrying) apoptotic bodies (NABs), if their cargo is cytoplasm, they are noted as cytoplasmic apoptotic bodies (CABs). These two groups require different labeling methods.

In addition to the differences in composition, apoptotic bodies significantly vary in size. Often visualized in sections as being 0.5–5 μm in diameter, i.e., larger than exosomes and microvesicles [2, 7, 8], they also exist as much smaller vesicles (50–500 nm) [1, 2, 6, 9, 10]. In this case, they are sometimes called apoptotic cell-derived microparticles [2] or apoptotic blebs [9].

Thus, the full range of apoptotic bodies spans two orders of magnitude 50–5000 nm [6], which is the widest spread compared to other extracellular vesicles, such as exosomes (40–120 nm) or microvesicles (100–1000 nm) [1].

The current purification methods do not fully discriminate between different categories of extracellular vesicles because of their variability [11]. In result, apoptotic bodies can be co-purified with the other extracellular vesicles. In studies they were detected among microvesicles even in the "apoptotic bodies-depleted medium" (ABDM) obtained after an 18,000×*g* spin [8], and in 10,000×*g* and 100,000×*g* fractions of microvesicles and exosomes [1, 12, 13].

An approach that can unquestionably identify apoptotic bodies in purified preparations of extracellular vesicles can be helpful in apoptosis research that involves microvesicles and exosomes.

Here, we present such a specific technique detecting only NABs, the type of apoptotic bodies that carry apoptotic chromatin cargo. The approach is based on in situ ligation (ISL) labeling [14–16].

ISL uses fluorescent hairpin-shaped oligoprobes to detect apoptotic DNA cleavage. During the labeling the blunt-ended fluorescent hairpins are attached by T4 DNA ligase to the blunt-ended 5′ phosphorylated DNA breaks present in apoptotic cell

chromatin. Such 5′ phosphorylated blunt-ended DNA breaks are selectively produced in apoptosis by the executioner nucleases and represent the signature apoptotic DNA cleavage [16–18].

There are only two processes in cells which selectively generate blunt-ended DNA: apoptosis and phagocytosis [19–21]. However, phagocytosis-derived blunt ends significantly differ from the blunt ends produced in apoptosis because, compared to each other, their end-groups are inverted. So both processes have unique configurations of their blunt-ended DNAs.

In apoptosis, DNA breaks produced by executioner nucleases have the 3′OH /5′PO_4 configuration, whereas DNA breaks generated in phagocytic digestion by DNase II have the inverted 3′ PO_4 /5′ OH configuration [17, 22]. These are very stable characteristics [20].

The ISL labeling exclusively detects the first (apoptotic), but not the second (phagocytic) type of DNA cleavage [14, 15, 17]. Moreover, it cannot label other DNA-end configurations such as nicked or single stranded DNA. Therefore, 5′ phosphorylated, blunt-ended DNA can be used as a distinctive marker of apoptotic bodies, because its presence inevitably points to the apoptotic origin of their cargo (*see* **Note 1**). ISL can label only NABs but not CABs, because these latter vesicles do not contain nuclear fragments with DNA (*see* **Note 2**).

In this chapter, we present the complete protocol for ISL-based detection of vesicles with apoptotic chromatin cargos (NABs). The procedure is performed on vesicles affixed to a glass slide. This fixation approach is presented at the beginning of the protocol (**steps 1–13**). The ISL labeling is detailed by **steps 14–20**. The protocol is applicable to various preparations of exosomes and microvesicles.

2 Materials

1. 30-Well Teflon-printed slides, 2 mm well diameter, cat. # 63434-02 (Electron Microscopy Sciences, Hatfield, PA).
2. Extracellular vesicles (microvesicles or exosomes), either purchased as a commercially purified sample, or isolated by available isolation kits (*see* **Note 3**).
3. Mix&Go™ Biosensor (Anteo Technologies) (*see* **Note 4**).
4. Coating buffer for Mix&Go™: 0.01 M MES buffer (pH 6.0). For 50 mL of MES buffer add 0.097 g of MES hydrate (195.23 MW) into 45 mL ddH_2O. Mix until dissolved. Adjust pH to 6.0 with 2 M NaOH, bring up to 50 mL with ddH_2O.
5. Blocking buffer for Mix&Go™: 0.5% BSA in coating buffer. Add 50 mg BSA to 10 mL coating buffer, mix.
6. PBS, pH 7.4.

7. PBS-T (PBS + 0.05 % tween).
8. Cold absolute methanol (stored at −20 °C). Caution: pure methanol is flammable and poisonous.
9. T4 DNA ligase 5 U/μL (Roche Life Science) (*see* **Note 5**).
10. 10× reaction buffer for T4 DNA ligase: 660 mM Tris–HCl, 50 mM $MgCl_2$, 50 mM dithioerythritol, 10 mM ATP, pH 7.5 (20 °C) (Roche Life Science).
11. FAM-labeled oligonucleotide hairpin probe (100 pmol/μL stock). The blunt-ended probe for the detection of 5′PO_4 blunt-ended DNA breaks:

5′ AAG GGA CCT GC**t** GCA GGT CCC TT 3′ **t**=FAM dt

12. Bovine Serum Albumin (BSA), 20 % solution in distilled water. Store at −20 °C in frozen aliquots.
13. Vectashield without DAPI (Vector Laboratories, Burlingame, CA).
14. 24 × 60 mm glass coverslips (VWR) (*see* **Note 6**).
15. Humidified chamber.
16. Fluorescent microscope with appropriate filters and objectives.

3 Method

1. Activate a 30-well Teflon-printed glass slide for the attachment of extracellular vesicles by applying 5 μL/well of Mix&Go Biosensor reagent. The Teflon coat on the slide prevents the uncontrollable spread of the reagent.
2. Incubate 30 min at room temperature (23 °C) in a humidified chamber.
3. Wash off the reagent with distilled water by using a squirt bottle.
4. Tap slide to remove liquid, then air-dry for 5 min at room temperature (23 °C). The activated glass surface is now sticky and has a high binding capacity. Do not touch it and protect from dust and spills.
5. While the slide is drying, dilute extracellular vesicles in Coating Buffer to the final concentration of 10 ng/μL.
6. Add 5 μL of the diluted sample into each well. Be careful not to touch the slide with the micropipette tip. It can produce micro scratches and other artifacts interfering with detection.
7. Air-dry the slide for 50 min at room temperature (23 °C). Cover loosely to prevent accidental contamination of the slide surface.
8. Gently rinse the surface with PBS-T using a 1000 μL micropipette. Be careful not to touch the slide. Tap excess solution off slide.

9. Add 5 µL of pure ice-cold methanol to each well to fix the extracellular vesicles. Be careful not to touch slide. Incubate for 5 min at room temperature. Cover the slide to prevent accidental contamination.
10. Gently rinse with PBS by using a 1000 µL micropipette. Be careful not to touch the slide with the pipet tip. Tap excess solution off slide.
11. Add 5 µL of Mix&Go™ Blocking Buffer. Be careful not to touch the slide with the pipet tip.
12. Incubate 30 min at room temperature (23 °C) in a humidified chamber.
13. Gently wash the slide with PBS-T by using a 1000 µL micropipette. Tap excess solution off slide.
14. To each well add 5 µL of preblocking solution containing 10% BSA in ligase buffer.

For a five-well experiment (25 µL of labeling solution), mix on ice in this order:

10 µL—distilled water.

12.5 µL—20% BSA in water.

2.5 µL—10× buffer for T4 DNA ligase.

15. Incubate for 15 min at room temperature (23 °C) in a humidified chamber.
16. In the meantime prepare the in situ ligation labeling solution (5 µL per well).

For a five-well experiment (25 µL of labeling solution), mix on ice in this order:

8.2 µL—distilled water.

12.5 µL—20% BSA in water.

2.5 µL—10× buffer for T4 DNA ligase.

0.9 µL—FAM-labeled hairpin probe.

0.9 µL—T4 DNA ligase (5 U/µL) (*see* **Note 5**).

The total volume of the labeling solution can be scaled up to accommodate bigger wells.

17. Add 5 µL in situ ligation labeling solution to each well. Be careful not to touch the slide with the pipet tip. Incubate for 18 h (overnight) at room temperature (23 °C) (*see* **Note 7**) in a humidified chamber (*see* **Note 8**). Protect from light.
18. Next day, gently rinse the slide with PBS three times using a 1000 µL micropipette. Be careful not to touch the wells with the pipette tip.
19. Add 2 µL of Vectashield (without DAPI) to each well, add coverslip, and seal with nail polish.

20. Analyze the signal using a fluorescent microscope. Vesicles containing apoptotic double stranded DNA breaks with $5'P0_4$ will fluoresce green.

4 Notes

1. Although the larger DNA-carrying apoptotic bodies can be visualized in tissue sections by standard DNA stains, such as propidium iodide (PI) or DAPI, such labeling cannot produce a reliable signal with most preparations of smaller extracellular vesicles attached to the glass slide. Besides, these generic DNA stains cannot verify the apoptotic origin of the cargo.
2. Depending on the probe used in ISL (blunt or with an overhang), the technique can selectively detect either blunt-ended DNA breaks or breaks with single nucleotide 3′ overhangs. Here, we only describe the detection of blunt-ended DNA breaks with $5'PO_4$ groups. More information about ISL labeling of other configurations of DNA breaks and about combining ISL with additional methods is presented in [23–25].
3. Apoptotic bodies are not always present in samples and their concentrations depend on the purification technique and on occurrence of cell death in the biological source of the sample.
4. Mix&Go™ Biosensor reagent binds proteins and other organic molecules to a variety of surfaces including glass and plastics. It is an effective and easy to use approach. Alternatively, EDC/NHS chemistry can be used for the attachment.
5. We found that for ISL labeling the more concentrated preparations of T4 DNA ligase are preferable, such as the highly concentrated (5 U/μL) (Roche) ligase. Lower concentrations of the enzyme can be used, but can sometimes result in weaker labeling.
6. The large 24 × 60 mm glass coverslips cover the entire 30-well slide and can be permanently attached with nail polish.
7. Temperature is important for efficient in situ ligation. It can be lower than room temperature (16 °C) with only a slight loss of the labeling signal. However, increasing the temperature to 37 °C completely eliminates the signal.
8. Coverslips are not needed at this stage since the solution will not evaporate when kept in a humidified chamber.

Acknowledgment

This research was supported by grant R01 NS082553 from the National Institute of Neurological Disorders and Stroke, National Institutes of Health and by grants R21 CA178965 from the National Cancer Institute, National Institutes of Health and R21 AR066931 National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health (all to V.V.D.).

References

1. Zaborowski MP, Balaj L, Breakefield XO et al (2015) Extracellular vesicles: composition, biological relevance, and methods of study. Bioscience 65(8):783–797
2. Poon IK, Lucas CD, Rossi AG et al (2014) Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 14(3):166–180. doi:10.1038/nri3607
3. Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
4. Depraetere V (2000) "Eat me" signals of apoptotic bodies. Nat Cell Biol 2(6):E104
5. Ravichandran KS (2010) Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. J Exp Med 207:1807–1817
6. Akers JC, Gonda D, Kim R et al (2013) Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol 113(1):1–11. doi:10.1007/s11060-013-1084-8
7. Ihara T, Yamamoto T, Sugamata M et al (1998) The process of ultrastructural changes from nuclei to apoptotic body. Virchows Arch 433(5):443–447
8. Hristov M, Erl W, Linder S et al (2004) Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. Blood 104(9):2761–2766
9. Mathivanan S, Ji H, Simpson RJ (2010) Exosomes: extracellular organelles important in intercellular communication. J Proteomics 73(10):1907–1920. doi:10.1016/j.jprot.2010.06.006
10. Simpson RJ, Mathivanan S (2012) Extracellular microvesicles: the need for internationally recognised nomenclature and stringent purification criteria. J Proteomics Bioinform 5:ii. doi:10.4172/jpb.10000e10
11. Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200(4):373–383
12. Thery C, Boussac M, Veron P et al (2001) Proteomic analysis of dendritic cell–derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol 166:7309–7318
13. Crescitelli R, Lässer C, Szabo TG et al (2013) Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles 2 (art. 20677). doi:10.3402/jev.v2i0.20677
14. Didenko VV (2002) Detection of specific double-strand DNA breaks and apoptosis in situ using T4 DNA ligase. Methods Mol Biol 203:143–151
15. Didenko VV, Minchew CL, Shuman S et al (2004) Semi-artificial fluorescent molecular machine for DNA damage detection. Nano Lett 4(12):2461–2466
16. Staley K, Blaschke A, Chun J (1997) Apoptotic DNA fragmentation is detected by a semiquantitative ligation-mediated PCR of blunt DNA ends. Cell Death Differ 4:66–75
17. Hornsby PJ, Didenko VV (2011) In situ ligation: a decade and a half of experience. Methods Mol Biol 682:49–63. doi:10.1007/978-1-60327-409-8_5
18. Widlak P, Li P, Wang X et al (2000) Cleavage preferences of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease) on naked DNA and chromatin substrates. J Biol Chem 275(11):8226–8232
19. Minchew CL, Didenko VV (2014) Nanoblinker: Brownian motion powered bio-nanomachine for FRET detection of phagocytic phase of apopto-

sis. PLoS One 9(9):e108734. doi:10.1371/journal.pone.0108734
20. Minchew CL, Didenko VV (2011) Fluorescent probes detecting the phagocytic phase of apoptosis: enzyme-substrate complexes of topoisomerase and DNA. Molecules 16(6):4599–4614. doi:10.3390/molecules16064599
21. Didenko VV (2011) 5′OH DNA breaks in apoptosis and their labeling by topoisomerase-based approach. Methods Mol Biol 682:77–87. doi:10.1007/978-1-60327-409-8_7
22. Samejima K, Earnshaw WC (2005) Trashing the genome: the role of nucleases during apoptosis. Nat Rev Mol Cell Biol 6:677–688
23. Minchew CL, Didenko VV (2012) In vitro assembly of semi-artificial molecular machine and its use for detection of DNA damage. J Vis Exp 59:e3628. doi:10.3791/3628
24. Didenko VV, Ngo H, Minchew CL et al (2002) Caspase-3-dependent and -independent apoptosis in focal brain ischemia. Mol Med 8(7):347–352
25. Didenko VV, Ngo H, Baskin DS (2003) Early necrotic DNA degradation: presence of blunt-ended DNA breaks, 3′ and 5′ overhangs in apoptosis, but only 5′ overhangs in early necrosis. Am J Pathol 162(5):1571–1578