Research Article

Dictyostelium discoideum cells shed vesicles with associated DNA and vital stain Hoechst 33342

I. Tatischeff a,*, M. Bomsel b, C. de Paillerets c, H. Durand b, B. Geny b, D. Segretain d, E. Turpin b and A. Alfsen c

a Laboratoire de Physicochimie Biomoléculaire et Cellulaire, CNRS URA 2056, Université Pierre et Marie Curie, 4, Place Jussieu, Case 138, F-75252 Paris Cedex 05 (France), e-mail: tati@lpbc.jussieu.fr
b Signalisation, Inflammation et Transformation Cellulaire, U. 332, Institut Cochin de Génétique Moléculaire, 22, rue Méchain, F-75014 Paris (France)
c Etats Liés Moléculaires, Université René Descartes, 45, rue des Saints-Pères, F-75270 Paris Cedex 06 (France)
d Laboratoire d’Histologie et Embryologie, CHU Paris-Ouest, Faculté de Médecine, 45, rue des Saints-Pères, F-75270 Paris Cedex 06 (France)

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Abstract. Dictyostelium discoideum cells are highly resistant to xenobiotics. We previously observed that these primitive eukaryotic cells contain a 170-kDa P-glycoprotein, mediating multidrug resistance in mammalian cells, but nonfunctional in Dictyostelium cells. We show here that D. discoideum cells vitally stained with the DNA-specific dye, Hoechst 33342, release fluorescent material in their culture medium. Electron microscopy and lipid analysis demonstrate the vesicular nature of this material. Moreover, nucleic acids associate with these extracellular vesicles independently of Hoechst vital staining. The main vesicular DNA component exhibits a size >21 kb. Shedding of microvesicles during cell growth is not concomitant with programmed cell death. We propose that these extracellular vesicles are involved in a new cellular resistance mechanism against xenobiotics. Furthermore, since the association of DNA with vesicles occurs in physiological growth conditions and independently of vital staining, the new shedding process might be involved in a more general intercellular mechanism.

Key words. Multidrug resistance; extracellular vesicles; Hoechst 33342; DNA; Dictyostelium discoideum.

* Corresponding author.

Dictyostelium discoideum cells are highly resistant to structurally unrelated xenobiotics such as the carcino- genic hydrocarbon benzo(a)pyrene [B(a)P] [1], the antitumoral antibiotic daunorubicin (DAU), DNA vital stains Dapi and Hoechst 33342 (HO342), mitochondrial vital stains Rhodamine 123 (Rh123) [2] and a carbocya-nine dye JC-1. All these xenobiotics except Dapi are known substrates of the so-called P-glycoprotein (P-gp), a 170-kDa protein that mediates classical multidrug resistance (reviewed in refs 3–5).

We have previously demonstrated biochemically the presence of a constitutive P-gp in D. discoideum cells by Western blot analysis with the anti-P-gp monoclonal antibody (mAb) JSB-1 [6] and, more recently, by flow cytometry analysis using two other anti-P-gp mAbs, C219 and MRK-16. Furthermore, hybridization of complementary DNA (cDNA), prepared from D. discoideum cell RNA and polymerase chain reaction (PCR)-amplified, with a human multidrug MDR1 gene probe confirmed the presence of P-gp in D. discoideum cells (unpublished results). Despite the presence of a constitutive 170-kDa P-gp expressed by an MDR1-like
gene, the classical multidrug resistance mediated by this P-gp appeared nonfunctional, or the reversion process functions differently from the usual mechanism in D. discoideum cells [2]. Searching for a new resistance mechanism to explain the high endogeneous resistance of D. discoideum cells, we observed that, when cells were vitally stained with the DNA-targeted fluorescent HO342, a fluorescent material was released in the culture medium. The spectral characteristics of the fluorescence allowed us to identify HO342 as the emitting compound. After concentration, the vesicular nature of this fluorescent extracellular material was revealed by electron microscopy (EM) and lipid analysis. The new extracellular organelles were biochemically characterized by protein analysis and phospholipid identification. Nucleic acids were also found to be associated with these vesicles. A major vesicle DNA component exhibited a size >21 kb. Importantly, production of the extracellular organelles as well as presence of associated DNA did not rely on HO342 vital staining of the cells.

Materials and methods

Culture conditions. D. discoideum (Dd) cells, cloned Ax-2 strain [7], were grown in suspension in HL5 semi-defined medium [8], containing 7 g/l of yeast extract (Oxoid, Unipath, Dardilly, France), on a gyratory shaker (175 rpm) at 22 °C. For proper oxygenation, each suspension was grown in an Erlenmeyer containing five times the suspension volume. Cells are able to grow and divide at the expense of soluble or particulate nutrients to produce vegetative cells. Cells were generally used in the exponential growth phase. Cell viability was controlled either by trypan blue (0.05% w/v) or propidium iodide exclusion (2 μg/ml final concentration).

Vital staining of D. discoideum cells by HO342. Vital staining of Dd cells was performed in HL5 medium. The cellular density and the morphological appearance of the cells were controlled on a hemocytometer before and after HO342 vital staining. In the case of preparation of extracellular vesicles from vitally stained cells, HO342 (3 μg/ml) was added to a cell suspension at an initial density of 2 x 10^6 cells/ml. Cells were grown for 23 h in the continuous presence of HO342, reaching a still exponential density of 7 x 10^6 cells/ml. In the case of spectrophotometric studies, cells were incubated with indicated concentration of HO342 either for 0.5 to 5 h, a short incubation time relative to the mean generation time of amoebae (11 h), or for 72 h in long-term experiments. In order to eliminate extracellular HO342, cells were harvested by centrifugation at 700g for 5 min, washed twice in ice-cold buffer A [17 mM potassium phosphate (pH 6.8)] and resuspended in the same buffer at a density of 2 x 10^7 cells/ml.

Light microscopy of D. discoideum cells and extracellular material. After vital staining, cells were either kept alive at 4 °C until observation by light microscopy or fixed at 2 x 10^7 cells/ml in methanol:buffer A (70:30 v/v), which improved nuclear access to the previously intracellularly accumulated HO342. Alternatively, cells fixed after vital staining were further prepared for long-term conservation on glass slides as follows. Ten-microlitre deposits of the fixed cell suspension were allowed to air-dry on methanol-washed slides before addition of 25 μl of pure methanol that was further air-dried. After mounting in 15 μl of glycerol:buffer A (20:80 v/v), slides were kept at 4 °C and remained observable for several weeks. Phase contrast and ultraviolet (UV) fluorescence microscopy were performed with a BHA Olympus microscope (Scop, Rungis, France), equipped with a 100-W mercury lamp and a UV-blue fluorescence excitation block. Pictures were taken with either Kodak Ektachrome Professional P800/1600 film or FUJI 400 ASA film for color slides.

Preparation of D. discoideum extracellular material. Extracellular material was prepared from at most 400 ml of culture medium from Dd cells. Cells grown with or without vital staining were centrifuged at 700g for 5 min, and cell-free supernatant was centrifuged at 105,000g for 1 h, using a refrigerated L5-50DB Beckman centrifuge with a Ti 60 rotor. Clarified supernatant was discarded, after an aliquot had been kept for protein concentration measurement. The pellet was re-suspended in 1/100 of initial volume with buffer B [10 mM Tris (pH 7.05)] and kept at 4 °C. This extracellular material was analysed by a density gradient as previously described for intracellular clathrin-coated vesicles [9]. Briefly, 1 ml was diluted twice in buffer B and layered on top of the D2O-sucrose step gradient between 1.050 and 1.210 g/cm³ density. After centrifugation at 4 °C in an SW28 rotor at 90,000g for 2 h, the pellet and five fractions were collected and extensively dialysed overnight at 4 °C against buffer B. Samples were stored at 4 °C.

HL5 medium alone was treated in the same way as the 700g cell-free supernatant. The 105,000g pellet was suspended in 1/100 of initial volume with buffer B and used as a control for further protein, lipid and nucleic acid analyses.

EM of D. discoideum extracellular material. Dd concentrated extracellular material has been observed by a negative staining technique. The material was washed after fixation in 2.5% (v/v) glutaraldehyde, deposited on a formvar (2%)-coated grid, stained with phosphotungstic acid (2% in water) and air-dried. Samples were...