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Measurement of annexin V uptake and lactadherin labeling for the quantification of apoptosis in adherent Tca8113 and ACC-2 cells

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Phosphatidylserine (PS) exposure occurs during the cell death program and fluorescein-labeled lactadherin permits the detection of PS exposure earlier than annexin V in suspended cell lines. Adherent cell lines were studied for this apoptosis-associated phenomenon to determine if PS probing methods are reliable because specific membrane damage may occur during harvesting. Apoptosis was induced in the human tongue squamous carcinoma cell line (Tca8113) and the adenoid cystic carcinoma cell line (ACC-2) by arsenic trioxide. Cells were harvested with a modified procedure and labeled with lactadherin and/or annexin V. PS exposure was localized by confocal microscopy and apoptosis was quantified by flow cytometry. The detachment procedure without trypsinization did not induce cell damage. In competition binding experiments, phospholipid vesicles competed for more than 95 and 90% of lactadherin but only about 75 and 70% of annexin V binding to Tca8113 and ACC-2 cells. These data indicate that PS exposure occurs in three stages during the cell death program and that fluorescein-labeled lactadherin permitted the detection of early PS exposure. A similar pattern of PS exposure has been observed in two malignant cell lines with different adherence, suggesting that this pattern of PS exposure is common in adherent cells. Both lactadherin and annexin V could be used in adherent Tca8113 and ACC-2 cell lines when an appropriate harvesting procedure was used. Lactadherin is more sensitive than annexin V for the detection of PS exposure as the physical structure of PS in these blebs and condensed apoptotic cell surface may be more conducive to binding lactadherin than annexin V.

Key words: Adherent cells; Annexin V; Apoptosis; Flow cytometry; Lactadherin

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Introduction

Sequential changes occur in the plasma membrane during apoptosis. In the early stage, the asymmetry in the distribution of the phospholipids is impaired and phosphatidylserine (PS), which normally is located on the internal leaflet of the membrane, is exposed on the cell surface. This event can be detected by acquisition of the ability of the cell to bind anticoagulant proteins such as lactadherin and annexin V (1-3). The various treatments used for the detachment of adherent cells may interfere with the annexin V binding to membrane PS. This creates a technical problem

in measuring apoptosis (4). To determine whether lactadherin or annexin V is more appropriate for the detection of apoptosis in adherent cell populations, a previously described modified procedure (5) was used to harvest cells. This approach circumvents the problem of measuring false-positive cells due to membrane damage inflicted upon healthy cells during their detachment from the culture flasks. Both lactadherin and annexin V can be used in the study of PS exposure on the surface of adherent cells during apoptosis.

Koopman et al. (1) were the first to describe the method using extrinsically applied hapten (i.e., fluorescein isothiocyanate, FITC, or biotin)-labeled annexin V to detect apoptosis, which has been extensively used since that time in a variety of cells (6). Binding of annexin V to a membrane is a complex function based on free annexin V and ambient Ca^{2+} concentration, membrane PS and phosphatidylethanolamine content (7-12). Commonly, in *in vitro* studies using annexin V affinity assay, the excess ambient Ca^{2+} concentration should reach a peak of 2.5 mM which is much higher than in the physiologic plasma range of 0.94-1.33 mM (13). Such a high calcium concentration makes it difficult to use the assay *in vivo*, especially in a circulation system. It would be very expensive to use this concentration for systemic injection into adult animals even as small as mice. Moreover, annexin V staining may vary in intensity and pattern between animals depending on the efficacy of the injection protocol used (14). In addition, the presence of calcium can also make phenotypic labeling more problematic. Annexin V also binds to oxidized phospholipids and to sulfatides (15,16). In addition, annexin V has also been shown to be incorporated into the membrane bilayer and to act as a calcium channel, which might cause additional changes (17).

Lactadherin is an abundant peripheral protein of the milk fat globule membrane but is also found in a wide range of tissues and is present in several body fluids (18,19). It contains discoidin-type domains with homology to the PS-binding domains of blood coagulation factor VIII and factor V (20). *In vitro*, lactadherin functions as a potent anticoagulant by competing with blood coagulation proteins for phospholipid binding sites and has been used as a detector of cells that express surface PS during apoptosis (2,21). In theory, lactadherin assay is more suitable for *in vivo* studies to determine the extent of PS exposure than annexin V because the lactadherin assay is independent of Ca^{2+}.

PS exposure occurs slowly and progressively during the cell death program and fluorescein-labeled lactadherin permits the detection of the PS exposure in suspended cell lines earlier than annexin V. However, monitoring apoptosis-related PS exposure by adherent cell types also faces the problem of PS exposure by sample handling. In the present investigation, adherent cell lines were studied for this apoptosis-associated phenomenon to determine if PS probing methods are reliable because specific membrane damage may occur during harvesting.

Material and Methods

The human tongue squamous carcinoma cell line, Tca8113, and the adenoid cystic carcinoma cell line, ACC-2, were obtained from the Stomatology College of Shanghai Jiaotong University (China). RPMI 1640 was obtained from HyClone Biochemistry Products Ltd. (China) and arsenic trioxide (As_2O_3) was from Yida Pharmaceutical Ltd. (China). Annexin V-fluorescein was purchased from BD Biosciences Pharmingen (USA). The FluoReporter FITC Protein Labeling Kit and the Alexa Fluor 647 Protein Labeling Kit were from Molecular Probes (USA). Propidium iodide (PI) was purchased from Pharmingen.

Isolation of lactadherin

Lactadherin was isolated from fresh bovine milk, which was not homogenized (Daqing Firm Dairy, China). PMSF (1 mM) was added, and the cream was isolated by centrifugation at 2500 *g* for 25 min at 4°C. The cream was washed twice with deionized water and then diluted to 35% (w/v) with PBS containing 8 mM $NaHPO_4$, 2 mM KH_2PO_4, 0.14 mM NaCl, 3 mM KCl, 1 mM EDTA, pH 7.4, and churned. The mixture was filtered through cheesecloth to collect the buttermilk. The pH of the buttermilk was adjusted to 4.8 by the addition of 1 N HCl, and the preparation was stirred for 30 min at room temperature, and centrifuged at 20,000 *g* for 90 min. The precipitate was collected and homogenized in 50 mM $NaHCO_3$ containing 1 mM PMSF. A chloroform-methanol (2:1, v/v) mixture was added to the homogenate and the preparation was stirred for 10 min. The aqueous layer was collected and centrifuged at 20,000 *g* for 90 min, and the solid was suspended in 50 mL 0.2 mM NaCl, stirred for 30 min, and centrifuged at 18,000 *g* for 80 min. The precipitate was suspended in 100 mM Tris-HCl, pH 8.2, 6 M urea, 1 M KCl, 0.2 mM PMSF, and 0.02% NaN_3, and stirred overnight at 4°C. The supernatant, collected by centrifugation at 18,000 *g* for 80 min, was then concentrated with a Centricon YM-30 filter (molecular mass cut-off of 30,000 Da; Millipore, USA) and loaded onto a Sephacryl S-200 column (3 x 104 cm). The appropriate fractions containing lactadherin were pooled, concentrated, and rechromatographed through the same column (22).

Fluorescein labeling of proteins

Lactadherin or annexin V, 0.2 mg in 0.2 mL phosphate-buffered saline, was concentrated 10-fold by ultrafiltration

with a Centricon YM-10 filter (Millipore) followed by dilution to the starting concentration (1 mg/mL) with 0.1 M sodium carbonate buffer, pH 9.0. Six microliters of 10 mg/mL fluorescein isothiocyanate (Molecular Probes) in DMSO was added to the lactadherin and the mixture was incubated for 1 h at room temperature in the dark. Free fluorescein was removed by gel filtration using a microspin column equilibrated with 0.1 M sodium carbonate, 0.1 M betaine, and 0.004% Tween-80, pH 9.0 (FluoReporter FITC Protein Labeling Kit, Molecular Probes). Fluorescein-labeled lactadherin was concentrated approximately 10-fold by ultrafiltration through a Centricon YM-10 filter, and diluted to approximately the original concentration in 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5. Labeling efficiency was estimated by comparison of absorbance at 490 nm to the corrected absorbance at 280 nm (after correcting for fluorescein absorption at 280 nm).

Flow cytometry analysis of lactadherin and annexin V binding to cells

Phospholipid vesicles were prepared as described by Gilbert et al. (23). Cell samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with the CellQuest software and with all channels in log mode. Events were triggered based upon forward light scatter and a gate was placed on the forward scatter-side scatter plot that excluded cell debris but included all cells. The samples were excited using both 496- and 633-nm laser sources. For the PS exposure studies, cell suspensions were adjusted to 1 x 10^6 cells/mL. A 0.5-mL aliquot of the cell suspension was transferred to a microcentrifuge tube; 10 µL FITC-lactadherin and/or Alexa Fluor 647-annexin V was added to the cells to give a final concentration of 2 nM. Fluorescein emission intensity was evaluated via the 530 ± 30-nm band pass filter set (FI1) for fluorescein and the 660 ± 20-nm band pass (FI4) filter for Alexa 647.

Confocal microscopy

Samples were incubated with lactadherin and/or annexin V for 10 min at room temperature in the dark. Cells were centrifuged at 200 *g* for 1 min at room temperature, the supernatant was removed and the cell pellet gently resuspended in 0.5 mL Tyrode buffer with 1.5 mM $CaCl_2$ and analyzed immediately. Samples were imaged using a Zeiss LSM 510 META Laser Scanning system (Carl Zeiss GmbH, Germany). The samples were excited with 488, 568, and/or 647 emission lines of a krypton-argon laser, and narrow band pass filters were used to restrict emission wavelength overlap. A Zeiss Axiovert S100 inverted microscope equipped with a high-quality water immersion 40X/1.2 NA, a C-apochroma objective was used to image the cells in an epifluorescence mode. The images were collected using internal detectors at a pixel resolution of 0.240-0.484 µm with a Kalman 3 collection filter, and were reconstructed using the Zeiss LSM Image Examiner Version 3,5,0,223 (Carl Zeiss GmbH).

Results

Effect of detachment harvesting

After the induction of apoptosis, the adherent cells progressively became round, blebbed, and detached from the wall of the flask into the culture medium. Both mobilized and adherent Tca8113 and ACC-2 cells were collected, washed, and stained with FITC-lactadherin and Alexa-647-annexin V/PI or FITC-annexin V and PI. To avoid altering surface molecule expression, the cells were detached from the flask with EDTA without trypsin. To obtain an accurate quantitative analysis of apoptosis, the harvested cells were pooled in suspension. Flow cytometry analysis indicated that untreated Tca8113 and ACC-2 exhibited little staining with lactadherin/annexin V or PI (data not shown), indicating that the detachment procedure (unlike trypsinization or scraping) did not induce cell damage.

Findings of confocal microscopy

After 12 h of As_2O_3 treatment, some Tca8113 and ACC-2 cells showed changes in gross architecture while a similar number were condensed. The normal-sized cells had patches and small appendages that stained with lactadherin, but stained weakly or not at all with annexin V. After 24 h, some condensed cells showed diffuse lactadherin staining, detected as rings, whereas annexin V staining was punctate. Most cells exhibited staining of internal cell bodies by annexin V. In fact, the internalized bodies were the primary location of annexin V staining rather than the cell surface. These results indicate that an early response to As_2O_3 treatment is localized PS exposure and that the quantity of PS exposure generally remains below the annexin V threshold. After 48 h, the majority of the cells exposed sufficient PS to conventional staining with both annexin V and PI (Figure 1A). Some cells had fragmented nuclei and irregular contours, changes that are consistent with completed apoptosis. We wished to confirm that lactadherin could detect PS exposure on apoptotic cells. Co-staining cells with lactadherin and PI (Figure 1B) confirmed that PI-permeable cells expose sufficient PS to permit diffuse binding of lactadherin. The diffuse blebs that appear transiently during apoptosis were identified on a small number of cells (Figure 1B) (24). These vesicles stained diffusely with lactadherin. Staining by lactadherin and

annexin V showed significant internalization within 24 h, especially in the presence of 3 mM Ca^{2+} in competition binding experiments (Figure 1C), indicating the preferential binding of lactadherin to membrane vesicles and the preferential localization of annexin V to internal bodies.

Flow cytometry results

Experiments were conducted to determine whether lactadherin and annexin V could be used simultaneously to detect PS exposure. Prior to As_2O_3 exposure, dot plots of staining by lactadherin and annexin V (Figure 2) showed that only 3.5 and 3.0% of Tca8113 and ACC-2 cells, respectively, stained with lactadherin. After 24-h exposure to As_2O_3, more than 60% of the cells were lactadherin+ annexin V+, indicating that both agents can be used simultaneously or separately to detect apoptosis. After 48 h, 87% of cells were strongly positive for both lactadherin and annexin V. Conventional staining with both annexin V and PI showed that one population is annexin V+ and the other is annexin V+PI+ (Figure 3, upper panel). Staining with both lactadherin and PI showed three cell populations, indicating the progressive pathway of PS exposure and PI impermeability (Figure 3, lower panel). Approximately 25% of the cells remained in the left lower quadrant, but PS exposure was greater than that of untreated cells. Approximately 25% were positive and 25% strongly positive for

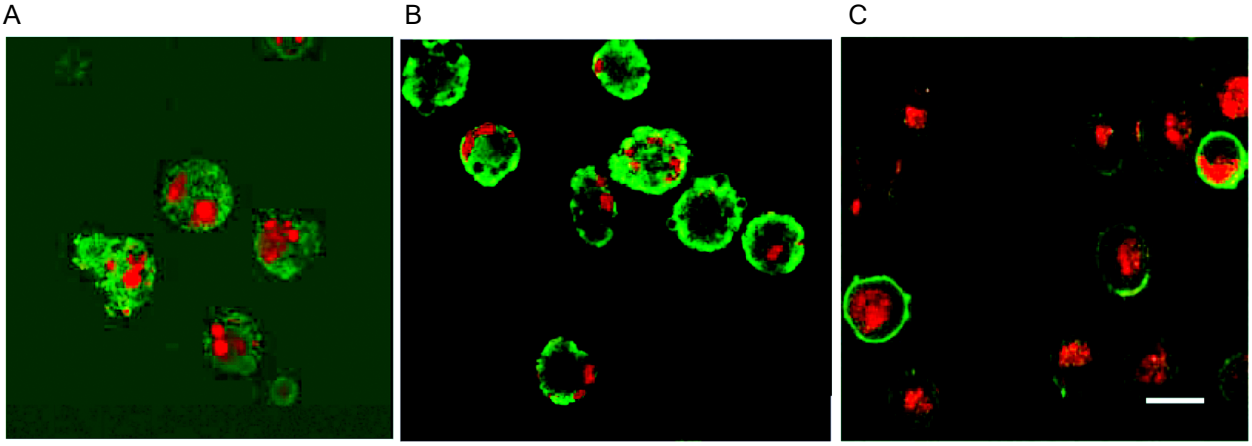

Figure 1. Confocal microscopy of Tca8113 cells after treatment with As_2O_3 from 24 to 48 h. Cells were stained green with annexin V and red with propidium iodide (PI) (A). Most cells were stained diffusely with lactadherin (green) and fragmented nuclei were PI+ (red) (B). Co-staining with lactadherin (green) and annexin V (red) showed that annexin V was identified in intracellular bodies and in discrete surface patches (C) and lactadherin staining was detected diffusely on membrane as rings. Bar = 10 µm in all panels.

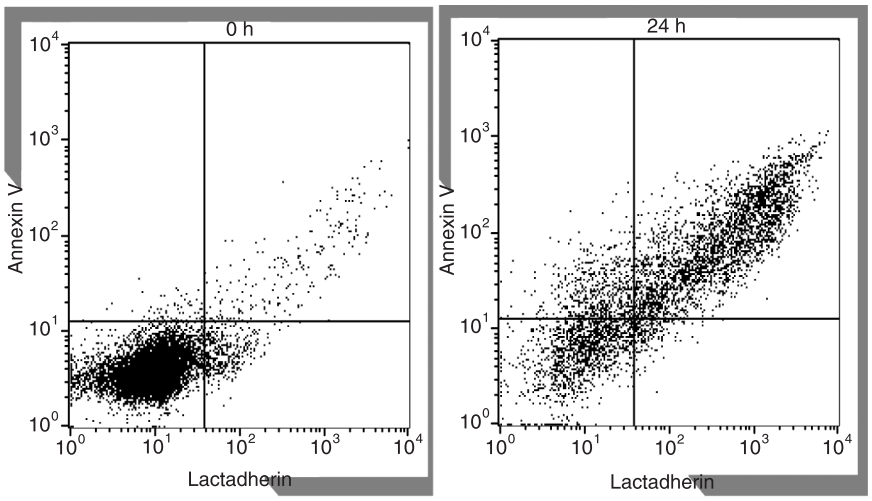

Figure 2. Flow cytometry analysis of lactadherin and annexin V binding to apoptotic Tca8113 cells. Phosphatidylserine exposure on the cells was evaluated by co-staining with lactadherin and annexin V at 0 and 24 h of As_2O_3 exposure. The fraction of lactadherin+annexin V+ cells in the quadrants increased from 3.5 to 67%.

lactadherin staining but negative for PI. Another 25% exposed the same quantity of PS but were also stained with PI. Maximal staining of most cells with both PI and lactadherin occurred after 48 h. Samples studied at intermediate times confirmed that most cells progressed through the two intermediate stages identified after 24 h (data not shown). These findings indicate that PS exposure begins prior to PI permeability. Comparative staining with lactadherin and PI versus annexin V and PI indicated that the two proteins have distinct staining patterns prior to PI staining. Lactadherin stained most cells with a continuous intensity from negative to highly positive. In contrast, staining with annexin V showed most cells with either minimal staining or bright staining immediately prior to permeability to PI. Very few cells demonstrated intermediate levels of annexin V staining, indicating that most cells had not exposed sufficient PS to reach the annexin V threshold.

The engagement of PS-binding motifs in staining by lactadherin and annexin V was evaluated in competition binding experiments (Figure 4A,B). Phospholipid vesicles containing 16% PS competed with As_2O_3-treated Tca8113 and ACC-2 cells for both lactadherin and annexin V binding. These vesicles competed for more than 95 and 90% of lactadherin but only about 75 and 70% of annexin V binding to Tca8113 and ACC-2 cells. This difference was rationalized by assuming that a larger fraction of annexin V was internalized by the cells. The results confirmed that the PS-binding motifs of both lactadherin and annexin V participate in binding to the As_2O_3-treated apoptotic cells. Addition of EDTA at twice the Ca^{2+} concentration reduced

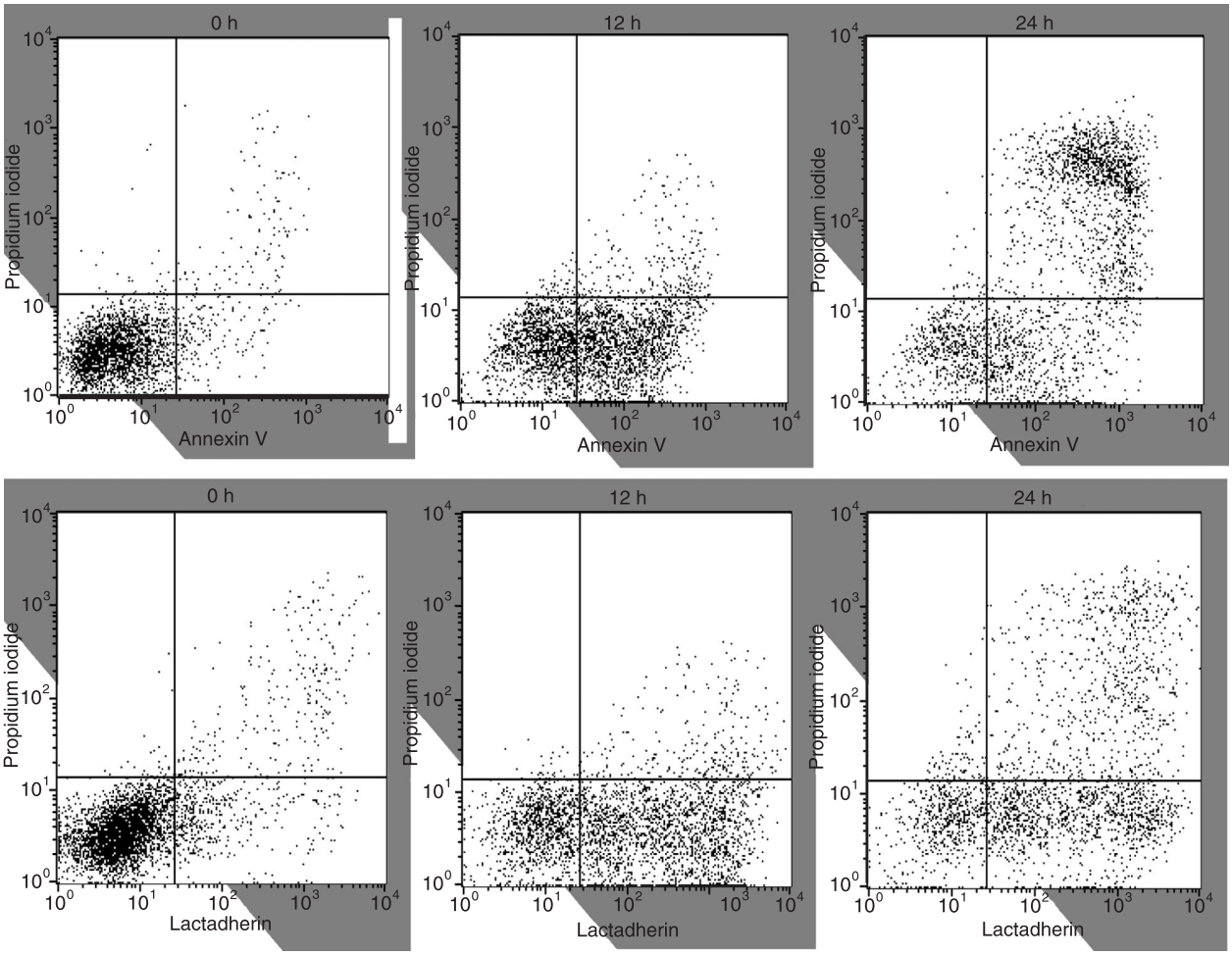

Figure 3. Flow cytometry analysis of annexin V + propidium iodide (PI) and lactadherin + PI binding to apoptotic Tca8113 cells. Some cells were stained with annexin V (upper panels) or lactadherin (lower panels) 12 h after treatment. Some cells showed annexin V+PI+ (upper panels) or lactadherin+PI+ (lower panels) staining 24 h after the treatments. Two cell populations with dim lactadherin staining and bright but PI-negative staining were observed (lower right panel).

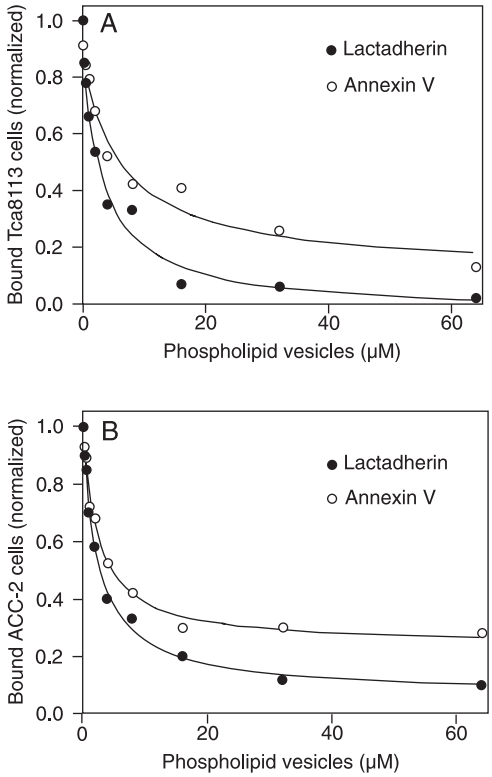

Figure 4. Phospholipid vesicles compete with lactadherin or annexin V binding to apoptotic Tca8113 (A) and ACC-2 (B) cells. Phosphatidylserine (PS)-containing vesicles were mixed with treated cells prior to the addition of 4 nM lactadherin-FITC or annexin V-FITC. Binding of each protein to cells was measured by flow cytometry. The phospholipid vesicle composition was PS:PE:PC (16:20:64). Results are representative of at least two experiments. PE = phosphatidylethanolamine; PC = phosphatidylcholine.

Table 1. Effect of Ca^{2+} concentration on lactadherin and annexin V binding to Tca8113 and ACC-2 cells.

Ca^{2+} (mM)	Annexin V		Lactadherin	
	Tca8113	ACC-2	Tca8113	ACC-2
0	10	3	63	62
1.5	70	64	67	65
3.0	80	72	64	68

Cells were labeled with lactadherin or annexin V 24 h after exposure to As_2O_3 at a concentration of 10 μM. Data are reported as percent of positive-stained cells. Lactadherin staining was stable at the concentrations of Ca^{2+} studied. In contrast, the percent of annexin V+ cell staining was much lower in the absence of Ca^{2+} and it increased markedly to a high level with increasing Ca^{2+} concentrations.

staining by annexin V but did not decrease lactadherin binding as detected by flow cytometry or by microscopy (data not shown). Binding increased with increasing Ca^{2+} concentration but did not differ from lactadherin binding (Table 1). These results confirm the Ca^{2+}-dependent PS binding of annexin V and the Ca^{2+}-independent PS binding of lactadherin. Furthermore, they exclude the possibility that lactadherin binding was mediated by interaction with the $\alpha v\beta 3$ or $\alpha v\beta 5$ integrins of the dying cells.

Discussion

Loss of plasma membrane asymmetry is an early event and independent of the cell type in apoptosis, resulting in the exposure of PS residues at the outer plasma membrane leaflet (6) and marks those cells for clearance by macrophages (25). PS exposure is frequently detected by the binding of fluorescein-labeled annexin V (1), which is a Ca^{2+}-dependent, phospholipid-binding protein. The complexity of annexin V binding and the internalization of annexin V by stressed cells leaves uncertainty about the extent to which annexin V fluorescence reflects plasma membrane PS exposure in early apoptosis.

Lactadherin, a newly discovered PS-binding protein, has two lectin-type domains that are homologous with the PS-binging domains of blood clotting factor VIII (26). In contrast to annexin V, binding appears to be proportional to PS content and is independent of Ca^{2+} concentration or membrane phosphatidylethanolamine content. The contrasting membrane binding properties of lactadherin and annexin V suggested that they could be used as complementary probes of membrane PS content.

Lactadherin and annexin V were used to identify PS exposure on Tca8113 and ACC-2 cells undergoing apoptosis. The results of lactadherin staining of both Tca8113 and ACC-2 were consistent with progressive PS exposure after treatment with As_2O_3. PS exposure is first localized and later generalized. The ability of apoptotic cells to bind lactadherin increased evenly within 24 h after As_2O_3 treatment. The results indicate that, in the early stage of apoptosis, exposed PS remains regulated and its quantity remains below the annexin V threshold (3). The time window of annexin V+PI- is narrow. Membrane integrity loss (the cause of PI uptake) may occur when the plasma membrane is exposed to more than 2.5% of PS content. Lactadherin binds preferentially to regions of sharp curvature while annexin V binds to the flat membrane (9,20). During apoptosis PS is localized in small blebs and the condensed cell membrane (apoptotic cell) has a more sharp curvature than swelling (necrotic cell). Therefore, the physical structure of PS in these blebs and condensed cell

surface may be more conducive to binding lactadherin than annexin V (27).

Our results agree with reports of PS-dependent binding of lactadherin (28) and annexin V (1) to apoptotic suspended cells. However, the studies of lactadherin binding have been very limited and the cell features recognized by the two proteins have not been compared. Our results of preferential internalization of annexin V by cells undergoing apoptosis agree with previous reports (3,29).

Our data indicate that PS exposure occurs in three stages during the cell death program and that fluorescein-labeled lactadherin permits the detection of early PS exposure. In the first stage, PS is exposed on scattered vesicles, membrane-bound projections, and ruffled regions. In the second stage, diffuse PS exposure is evident over the plasma membrane. The intensity of PS exposure increases as the cytoplasm condenses. In the third stage, the plasma membrane phospholipid asymmetry collapses and the cells expose sufficient PS to permit intense staining by both lactadherin and annexin V, and the plasma membrane becomes permeable to PI. The slow, progressive PS exposure suggests that cells have a mechanism for graded PS exposure that is distinct from the Ca^{2+}-dependent "scramblase" that mediates the collapse of phospholipid asymmetry in the final stage of apoptosis (30). We have observed a similar pattern of PS exposure in two differently adherent oral malignant cell lines, suggesting that this pattern of PS exposure is common in adherent cells.

In conclusion, both lactadherin and annexin V can be used in adherent Tca8113 and ACC-2 cell lines after an appropriate harvesting procedure. Lactadherin is more sensitive than annexin V for the detection of PS exposure. Further studies should be conducted to compare lactadherin with other documented methods for detecting apoptosis *in vitro* and *in vivo*.

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