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Phosphatidylserine plasma membrane asymmetry *in vivo*: a pancellular phenomenon which alters during apoptosis

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Abstract

The distribution of phospholipids across the two leaflets of the plasma membrane is important for many cellular processes including phagocytosis and hemostasis. In the present study we investigated the *in vivo* plasma membrane distribution of the aminophospholipid phosphatidylserine in mouse embryos with a novel technique employing Annexin V, a Ca^{2+} dependent phosphatidylserine binding protein, conjugated to fluorescein isothiocyanate and biotin. Annexin V directly applied to cryostat sections labeled the plasma membrane of all cells at the interface. In contrast, Annexin V injected intracardially into viable mouse embryos labeled almost exclusively apoptotic cells. These apoptotic cells were visible in all tissues and derived from all germ layers. Our experiments demonstrate that phosphatidylserine is asymmetrically distributed between the two leaflets of the plasma membrane in virtually all cell types *in vivo* and that this asymmetry is lost early during apoptosis.

Keywords: Annexin V, cell death, mouse embryo, phagocytosis, phospholipids

Abbreviations: AnxV, Annexin V; PM, plasma membrane; ABC, ATP-binding cassette; PS, phosphatidylserine; AnxV-FITC, Annexin V conjugated to fluorescein isothiocyanate; PC, Post coitum; LM, light microscopical; EM, electron microscopical; PBS, phosphate buffered saline; DAB, 3,3′-diaminobenzidine tetrahydrochloride

Introduction

In vitro studies mainly of blood cells have shown that the two major classes of the plasma membrane (PM) phospholipids, the choline- and aminophospholipids, are distributed asymmetrically between the two leaflets of the PM. Sphingomyelin and phosphatidylcholine comprise the majority of the outer leaflet while ethanolamine and serine phospholipids reside predominantly in the leaflet facing the cytosol (Devaux, 1991).

The PM transverse translocation of phospholipids is mediated by so-called flippases (Higgins, 1994; Diaz and Schroit, 1996). The first flippase has recently been identified. This phosphatidylcholine translocase is the *mdr2* p-glycoprotein gene product (Mdr2) (Smit *et al*, 1993), which is a member of the phylogenetically old, ATP-binding cassette (ABC) transporter superfamily (Higgins, 1992). In an ATP/Mg^{2+} dependent manner, this translocase flips phosphatidylcholine from the cytoplasm-facing leaflet to the opposite orientation (Ruetz and Gros, 1994). A similar machinery seems to regulate the phosphatidylserine (PS) distribution across the PM (Tang *et al*, 1996). A PS specific energy dependent out-in translocase activity (Martin and Pagano, 1987; Seigneuret and Devaux, 1984; Zachowski *et al*, 1989) was measured in membranes of anucleated (Seigneuret and Devaux, 1984) and nucleated (Devaux, 1991; Tang *et al*, 1996) cell types *in vitro*. For blood platelets it was shown that the PS translocase activity can be enhanced under conditions that give rise to the appearance of PS in the outer leaflet (Tilly *et al*, 1990). This regulatory mechanism provides a steady state with low levels of surface exposed PS (Diaz and Schroit, 1996).

In vitro studies have also indicated the existence of molecular machineries that counteract the above regulation causing an increase of PS in the outer leaflet of the PM (Diaz and Schroit, 1996). Receptor/ligand activated platelets (Bevers *et al*, 1983), and ageing erythrocytes (Connor *et al*, 1994) as well as apoptotic hematopoietic cell lines (Fadok *et al*, 1992a; Martin *et al*, 1995; Vermes *et al*, 1995) express PS at their cell surface while keeping PM integrity intact. The surface exposed PS catalyses reactions of the coagulation system (Bevers *et al*, 1982) and mediates recognition and uptake by phagocytes (Connor *et al*, 1994; Fadok *et al*, 1992a, b). Receptor/ligand activated platelets exhibit a scramblase activity with concomitant inhibition of the PS translocase and redistribute PS symmetrically over the two leaflets of the PM (Williamson *et al*, 1995). Both the translocase and scramblase activity are regulated by cytosolic Ca^{2+} levels (Williamson *et al*, 1995). Nucleated cells regulate PS asymmetry of the PM similar to platelets. During apoptosis of lymphocytes *in vitro* PS translocase is inhibited and the scramblase is activated (Verhoven *et al*, 1995). Recently the scramblase present in erythrocytes has been isolated and purified (Basse *et al*, 1996).

In the present study we have assessed the PS asymmetry of the PM *in vivo* in whole mouse embryos utilizing Annexin V. This protein is a member of the Annexin family of structural and functional related proteins (Van Heerde *et al*, 1995) and engages specifically with PS in a calcium-dependent manner through a putative binding

pocket for the serine headgroup (Swairjo *et al*, 1995). The presence of PS in cells was monitored by applying Annexin V conjugated to fluorescein isothiocyanate (AnxV-FITC) directly to cryostat sections of mouse embryos. The presence and extent of PS at the outer PM leaflet was assessed by intracardiac injection of biotinylated Annexin V (AnxV-biotin) into viable mouse embryos.

Results

All cells with exposed interior bind Annexin V

Applying AnxV-FITC to cryostat sections through limbs of day 13 mouse embryos showed that all cells contain Annexin V binding sites (Figure 1). The PM was especially intensely labeled. To a lesser degree, labeling was also observed at the nuclear membrane.

Distribution of intracardially administered Annexin V

AnxV-biotin was injected intracardially in vital mouse embryos such that the entire circulatory system was thoroughly perfused and widespread interstitial distribution was achieved. AnxV-biotin and heat inactivated AnxV-biotin, (i.e. AnxV-biotin with a destroyed phospholipid binding activity; Reutelingsperger *et al*, 1985), were administered to embryos of 11, 12 and 13 days post coitum (PC). While embryos injected with heat inactivated AnxV-biotin did not show any cell labeling, those that were injected with active AnxV-biotin showed cell labeling at specific locations in the whole organism (Figure 2).

Cells derived from all three germ layers were labeled at the many sites where cell death appears during morphogenesis (Glücksmann, 1951). Depending on embryonic age, AnxV-biotin binding was present with left-right symmetry and cranially in the more differentiated organs (Poelmann and Vermeij-Keers, 1976), e.g. eye (Figure 2a), in both the soma and axons of neurons of the central (Figure 2a, b) and peripheral (Figure 2c) nervous system, bronchi (Figure 2d), and caudally in primitive organs, e.g. somites and degenerating tailgut (Figure 2e) (Nievelstein *et al*, 1993). In addition, many labeled cells were observed in the degenerating interdigital tissue (Figure 2f).

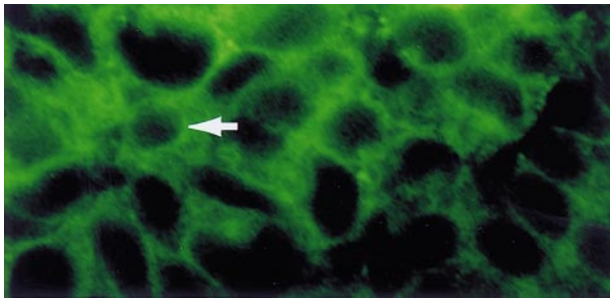

Figure 1 Cryostat section through day 13 mouse embryo limb, showing Annexin V binding sites in cells with exposed interior. AnxV-FITC binding was present in all cells, both at the nuclear membrane and PM (arrow).

Apoptotic cells bind Annexin V

The above results indicate that Annexin V binding cells are located in regions where cell death occurs during morphogenesis. To verify whether these cells were apoptotic, labeled cells in the interdigital mesenchymal tissue of day 13 mouse embryos were investigated for their ultrastructural characteristics.

AnxV-biotin positive cells showed the ultrastructural characteristics of apoptosis (Wyllie *et al*, 1980). Throughout the sections examples were found of AnxV-biotin labeled cells, undergoing phagocytosis including engulfment by neighboring cells, and more advanced stages of intraphagolysosomal degradation (Figure 3a and b). Other positive cells had more condensed chromatin (Figure 3c and d) and loss of electron density of the cytoplasm which contained an increased proportion of free ribosomes and dilated endoplasmatic reticulum while other organelles appeared unchanged (Figure 3c). The AnxV-biotin binding cells in later stages of apoptosis showed advanced chromatin condensation (Figure 3e), nuclear pyknosis (Figure 3f), and cell fragmentation.

Exceptions

In all the cases the Annexin V binding cells showed the morphology of apoptosis. However, two evidently non-apoptotic cell types also appeared to bind AnxV-biotin at their PM: (i) myoblasts differentiating into myotubes composed of a sarcolemma that closely ensheaths several aligned nuclei (Figure 4a) and myocardioblasts forming cardiac muscle (data not shown), and (ii) megakaryoblast and megakaryocytes in the liver, recognisable for their large size, polymorphic nucleus and the many thrombocyte-like attachments (Figure 4b).

Discussion

This paper demonstrates that regulation of PS topography and, consequently asymmetric membrane architecture, is a ubiquitous process during morphogenesis. All cell types confine PS to the cytoplasm-facing leaflets when viable. The process of apoptotic cell death results in PS exposure at the outer leaflet of the PM.

Annexin V is a protein that strongly binds to PS-containing membranes in the presence of Ca^{2+} (Van Heerde *et al*, 1995). Utilizing this capacity, *in vitro* studies indicated that Annexin V specifically marks apoptotic human neutrophils (Homburg *et al*, 1995), germinal B-lymphocytes (Koopman *et al*, 1994) and peripheral blood lymphocytes (Vermes *et al*, 1995). A study with hematopoietic cell lines and Annexin V demonstrated that these cells expose PS at the cell surface during apoptosis in culture, from an early stage onwards and regardless of the initiating stimulus (Martin *et al*, 1995).

We have applied Annexin V to study loss of PM asymmetry *in vivo*. Mouse embryos were used as a model, because they carry spontaneous, spatiotemporally consistent cell death patterns (Glücksmann, 1951). While all cells possess Annexin V binding sites, as we

demonstrated by cryostat sections, perfusion of viable embryos with AnxV-biotin revealed that only specific cell populations bear these binding sites at their cell surface. The interaction of AnxV-biotin with the embryonic cells relies on its phospholipid binding property, as was shown from our control experiment with heat inactivated AnxV-biotin.

Active AnxV-biotin identified cells at locations where cell death serves morphogenesis; in cells derived from all three germlayers, and both in primitive (Nievelstein *et al*, 1993; Vermeij-Keers and Poelmann, 1980) and in more differentiated tissues (Glücksmann, 1951; Poelmann and Vermeij-Keers, 1976). The whole cells were labeled, which was best visualized in dying neurons, with their labeling of both the soma and axons. At both the light microscopical (LM) and electron microscopical (EM) level, the AnxV-biotin labeled cells fitted into the sequence of morphological stages that apoptotic cells undergo (Poelmann and Vermeij-Keers, 1976; Wyllie, 1992; Wyllie *et al*, 1980), from the earliest stages, hardly distinguishable from viable neighboring cells, up to intraphagolysosomal degradation.

The consistent presence of PS on the outer layer of the PM of apoptotic cells and the common absence of PS exposure by viable cells suggests a tight regulation of PS PM asymmetry. This predicts an important physiological role for this process *in vivo*. One such a role may exist in the process of phagocytosis. It is this process that largely determines the elimination of apoptotic cells without any inflammatory reaction (Kerr *et al*, 1972; Martin *et al*, 1994; Savill *et al*, 1993). In effecting this, apoptotic cells must be recognized and ingested rapidly by phagocytes before membrane integrity is lost and the cells eventually succumb

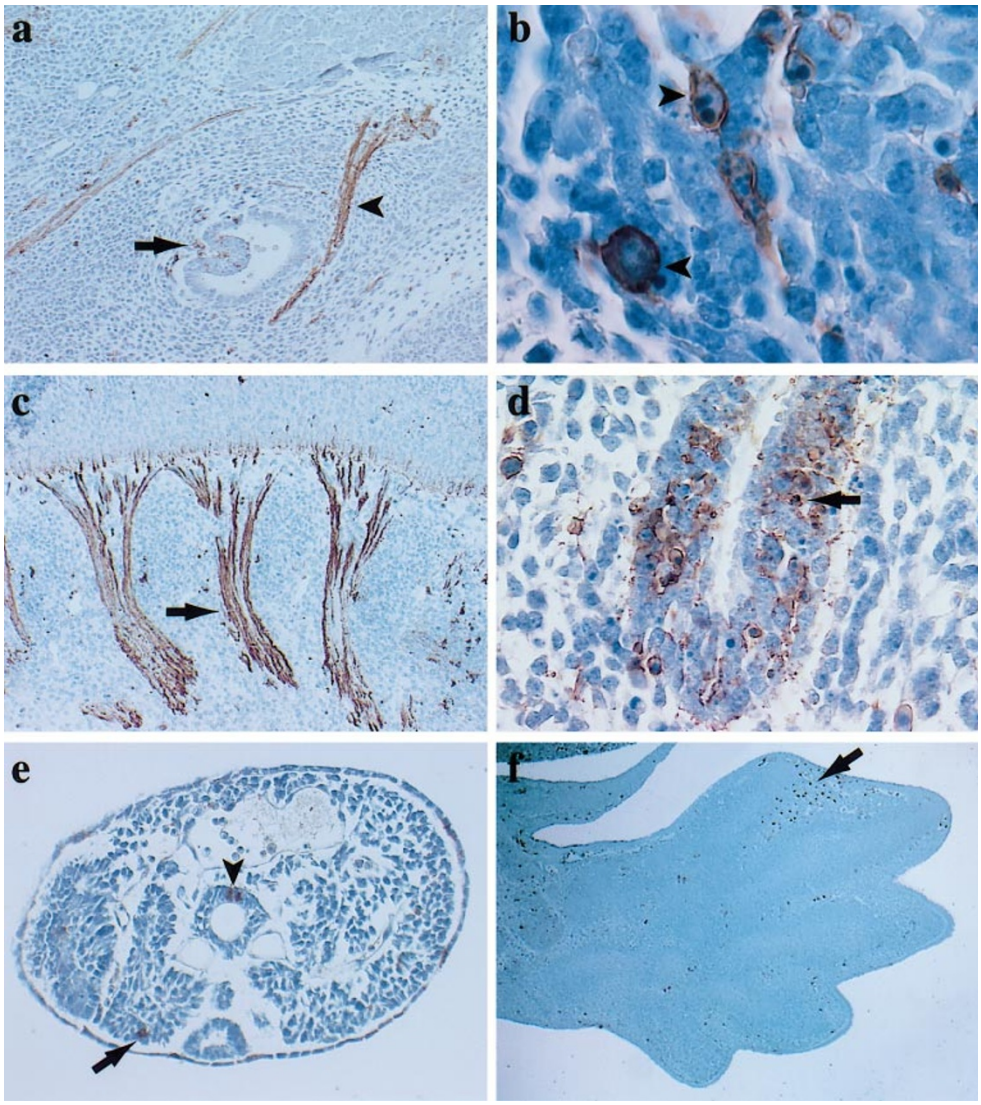

Figure 2 Examples of AnxV-biotin labeling in day 11 (**a, b, d, e**), day 12 (**c**) and day 13 (**f**) mouse embryos: eye (**a:** arrow), both the soma and axons of neurons of the central (**a, b:** arrowhead) and peripheral (**c:** arrow) nervous system, bronchi (**d:** arrow), somites (**e:** arrow) and degenerating tailgut (**e:** arrowhead). In between digits also the cells were marked (**f:** arrow).

to lysis (Majno and Joris, 1995; Wyllie, 1992). Exposure of PS after loss of PM asymmetry is one of three general structural changes in the PM of apoptotic neutrophils and lymphocytes that have been identified *in vitro*, together with formation of thrombospondin binding sites, and exposure of side chain sugars after loss of sialic acid (Savill *et al*, 1993). Like in the latter two, PS exposure may well permit recognition by phagocytes, which remove the dying cell by phagocytosis.

From our study it appears that the presence of PS at the outer PM marks the entire period of cell progression through apoptosis from the earliest stages, hardly distinguishable from viable cells, up to intraphagolysosomal degradation. The involvement of PS in phagocytosis of apoptotic cells may then explain why in *in vitro* phagocytic assays, next to phagocytes containing recognizable apoptotic cells, phagocytes have also been observed containing apparently viable cells (Savill *et al*, 1989). Moreover, it also explains the clearance of apoptotic cell fragments during morphogenesis. These cell fragments too still bear the surface structures necessary for recognition and removal by phagocytes.

In addition to apoptotic cells, two types of viable cells were observed to express Annexin V-binding sites at their cell surface. This may reflect a PS-dependent pathway for intercellular recognition shared by apoptotic cells and invoked by viable cells under specific conditions. The AnxV-biotin positive myoblast must promote homotypic recognition to fuse into myotubes and heart muscle syncytium. The AnxV-biotin positive megakaryocytes and megakaryoblasts may invoke the PS-dependent recognition pathway to keep the entities of already distinguishable platelet structure together until maturation is completed and platelets are dispersed. Within this concept of intercellular recognition an independently regulated but associated mechanism of phagocytosis should be considered. Whereas the apoptotic cells drive phagocytes to phagocytose them, myoblasts, megakaryocytes and megakaryoblasts should be inert in this respect or even discourage phagocytes to approach and phagocytose. Involvement of PS exposure in attachment of cells to each other without ending up in phagocytosis may indeed take place in certain circumstances, such as was observed in recent studies on the scavenger receptor present on mouse peritoneal macrophages. These macrophages bind erythrocytes exposing PS without subsequent engulfment (Ottnad *et al*, 1995; Sambrano and Steinberg, 1995).

In conclusion, our results indicate that confinement of PS to the inner leaflet of the PM is a ubiquitous process of

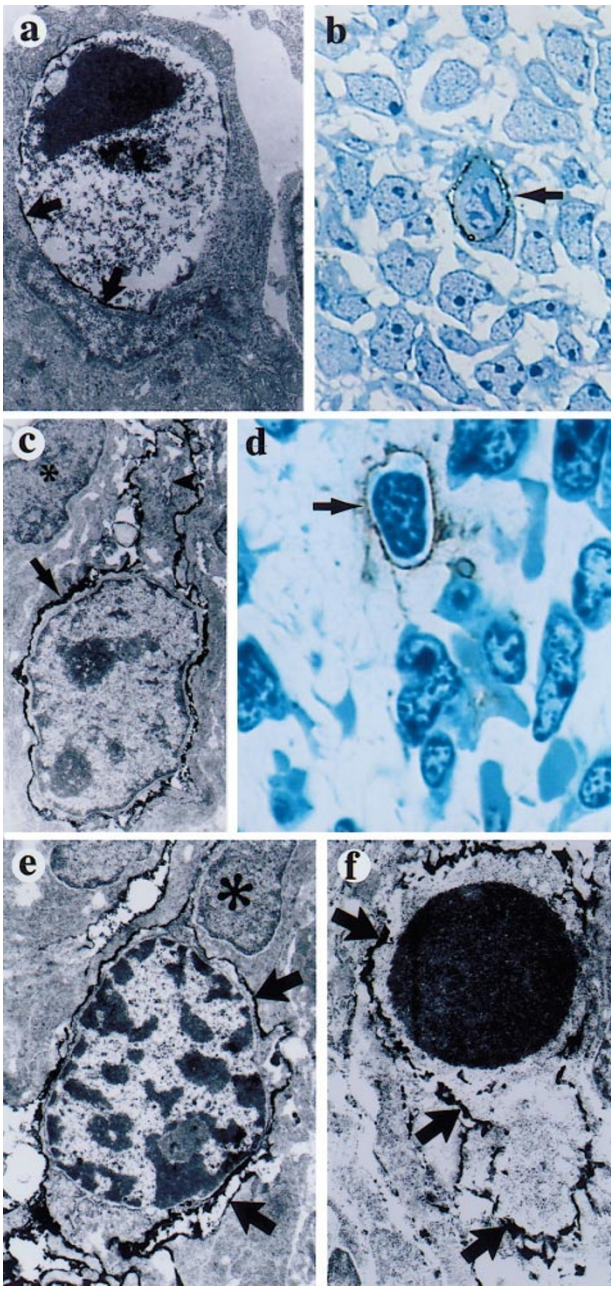

Figure 3 AnxV-biotin labeled cells (arrows) in day 13 mouse embryo interdigital mesenchyme in successive stages of apoptosis (EM: **a, c, e, f;** LM: **b, d**): ingested by phagocytes (**a, b**); early apoptotic, showing loss of electron density of the protoplasm, a dilated endoplasmatic reticulum (**c:** arrowhead; viable cell: asterix) and starting chromatin condensation (**c, d**); with advanced chromatin condensation (**e**) pyknotic, c.q. late apoptotic cell (**f**).

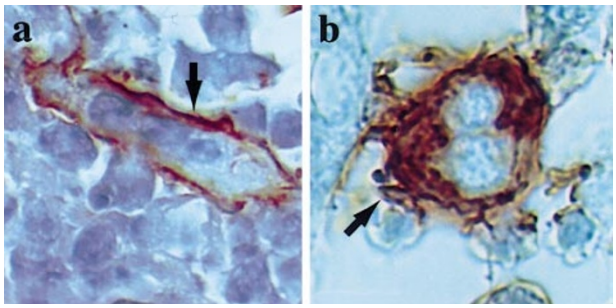

Figure 4 Annexin V labeled non-apoptotic cells (arrows) in paraffin embedded serially sectioned mouse embryos. (**a**) Section through a day 12 embryo showing membrane labeling of a myotube. (**b**) Section through a day 13 embryo showing a membrane labeled megakaryocyte.

viable cells *in vivo*. This PS PM asymmetry changes in surface exposure of PS early during apoptosis, irrespective from which germ layer the cells are derived. The general commitment of cells to this concept of regulation of PS PM topography makes it possible to visualize the many sites of apoptosis that are present during embryogenesis *in situ*, both at the topographical and at the ultrastructural level.

Materials and Methods

Experimental animals

Eighteen pregnant FVB-mice, from 11–13 days PC (plug=day 0), were killed by cervical dislocation after ether anesthesia. The uteri were dissected out and from the embryos collected, 116 were used for this study. From these embryos; nine were directly processed for cryostat sectioning and 107 were temporarily cultured for control experiments using heat inactivated AnxV-biotin (n=16) and for detection of loss of PS asymmetry of the PM by microinjection of AnxV-biotin (LM, n=54; EM, n=37).

Binding of FITC conjugated Annexin V to cryostat sectioned embryos

Day 13 mouse embryo limbs were sectioned on a cryostat (CM3000, Reichert Jung, Germany) at 10 μm. Directly thereafter, sections were washed with HEPES buffer, incubated for one minute with AnxV-FITC (APOPTEST^{TM}-FITC kit, a product from NeXins Research BV, The Netherlands), washed again with HEPES buffer and mounted with anti fading agent. The slides were stored at -4°C until examination. Sections were examined under a microscope using standard settings for FITC fluorescence-detection.

Heat inactivated Annexin V-biotin

AnxV-biotin was inactivated by heating it for 10 min at 56°C (Reutelingsperger *et al*, 1985). Microinjection and staining procedures were identical to those used for active Annexin V-biotin. Non-specific binding of Annexin V and quenching of endogenous peroxidase activity was tested for in this manner.

Microinjection of Annexin V-biotin

Embryos were perfused by microinjection using a Hamilton-Syringe pipetting system with glass needles (tip diameter $\sim$20 μm). Per embryo, a volume of approximately 3 μl AnxV-biotin (660 μg/ml), purchased from NeXins Research BV, The Netherlands, (APOPTEST^{TM}-biotin kit), was injected through the ventricle of the heart under a surgical microscope while the embryo was kept in HEPES buffer (20 mM HEPES (pH 7.4), 132 mM NaCl, 2.5 mM $CaCl_2$, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, 0.5% BSA) at 37°C. When injected, a temporary blanching of the umbilical vein could be seen. Successfully injected embryos that showed heart activity after 30 min of incubation were fixed overnight in HEPES buffer containing 4% formalin at 4°C and further processed for LM.

Detection of Annexin V binding-biotin, LM

Following fixation embryos were dehydrated, embedded in paraffin and serially sectioned at 3 μm. Endogenous peroxidase activity was blocked by incubation in methanol/H_2O_2 (9 : 1 v/v) for 20 min. Sections were washed in phosphate buffered saline (PBS). Bound AnxV-biotin was visualized using the avidin-biotin complex method with horseradish peroxidase conjugated avidin (ABC Elite kit, Vector Laboratories, USA) at room temperature. After washing with PBS, staining was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.05%), and counterstained with Hematoxylin.

Detection of Annexin V-biotin binding, EM

Day 13 embryos were processed for EM. The embryos were dissected out of the uterus, microinjected with Annexin V as described above, and subsequently intracardially perfused with 0.5 ml 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The limbs were removed, postfixed overnight in the same fixative, and cut on a Vibratome into 50 μm sections, which were processed to visualize the biotinylated Annexin V as described for LM. After the reaction with DAB, the sections were postfixed in 1.5% OsO_4 in a 8% glucose solution, rinsed in aquadest, stained *en bloc* in 3% uranyl acetate, dehydrated in dimethoxypropane and embedded in Durcupan (for details of EM procedures see: De Zeeuw *et al*, 1988, 1989). Semithin and ultrathin tissue sections were cut on an ultratome (Ultracut S, Reichert Jung, Germany); semithin and ultrathin sections were counterstained with Toluidine blue and with lead citrate, respectively. The ultrathin sections were examined in a Philips electron microscope (CM 10).

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