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# Transient expression of phosphatidylserine at cell-cell contact areas is required for myotube formation

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## SUMMARY

Cell surface exposure of phosphatidylserine (PS) is shown to be part of normal physiology of skeletal muscle development and to mediate myotube formation. A transient exposure of PS was observed on mouse embryonic myotubes at E13, at a stage of development when primary myotubes are formed. The study of this process in cell cultures of differentiating C2C12 and H9C2 myoblasts also reveals a transient expression of PS at the cell surface. This exposure of PS locates mainly at cell-cell contact areas and takes place at a stage when the structural organization of the sarcomeric protein titin is initiated, prior to actual fusion of individual myoblast into multinucleated myotubes. Myotube formation *in vitro* can be inhibited by the PS binding protein annexin V, in contrast to its mutant

M1234, which lacks the ability to bind to PS. Although apoptotic myoblasts also expose PS, differentiating muscle cells show neither loss of mitochondrial membrane potential nor detectable levels of active caspase-3 protein. Moreover, myotube formation and exposure of PS cannot be blocked by the caspase inhibitor zVAD(OMe)-fmk. Our findings indicate that different mechanisms regulate PS exposure during apoptosis and muscle cell differentiation, and that surface exposed PS plays a crucial role in the process of myotube formation.

Key words: Myotube formation, Skeletal muscle development, Heart development, Apoptosis, Mouse embryo

## INTRODUCTION

Hallmarks of myoblast differentiation are the assembly of the sarcomeres, the mechanical-electrical coupling of cells, and in the case of skeletal muscle, fusion of individual myoblasts into multinucleated myotubes. Phosphatidylserine (PS) has recently been identified as a novel factor related to myoblast differentiation *in vivo* during embryogenesis (van den Eijnde et al., 1997a; van den Eijnde et al., 1999), using the Ca<sup>2+</sup>-dependent PS binding protein annexin V (Swairjo et al., 1995) to detect cell surface exposure of PS. The embryo studies revealed PS exposure at the cell surface of apparently viable myoblasts in the developing heart and skeletal muscle. This has led us to the hypothesis that PS can mediate homotypic recognition between cardiac and skeletal muscle cells in the process of intercalated disk- and myotube formation, respectively (van den Eijnde et al., 1997a).

At present, the molecular control of myogenesis has been studied in most detail in *Drosophila*, in particular for skeletal muscle (Dobberstein et al., 1997). In this species the genes *myoblast city*, *blown fuse*, *rolling stone* and *Drac1<sup>G12V</sup>* have been shown to be essential to myotube formation. These genes encode proteins that mediate key processes of recognition and

adhesion and formation of a pre-fusion complex, as well as plaque, cell alignment and plasma membrane apposition and plasma membrane breakdown, respectively. Molecules that have been implicated in mammalian skeletal muscle differentiation include active protease nexin, Ca<sup>2+</sup>, cathepsin B, desmin, GRP49, ERK6, m-calpain, NCAM, N-cadherin, proteasomes and the H145 antigen (Crescenzi et al., 1994; Dourdin et al., 1999; Dourdin et al., 1997; Gogos et al., 1996; Gorza and Vitadello, 2000; Hyodo and Kim, 1994; Lechner et al., 1996; Li et al., 1994; Moncman and Wang, 1998; Peck and Walsh, 1993; Seigneurin-Venin et al., 1996). Extending our knowledge of intercellular interactions in vertebrate muscle development may aid in the understanding of muscle tissue repair, which includes the reassembling of intercalated disks in the infarcted or hibernating heart (Kaprielian et al., 1998; Matsushita et al., 1999), and the fusion of satellite cells with damaged myotubes in skeletal muscle after exercise (Anderson, 1998).

The aim of the present study was to explore in greater detail the role of cell surface exposure of PS in myoblast differentiation, both in mouse embryos *in vivo*, and in established muscle cell lines C2C12 and H9C2 *in vitro*. Because PS exposure is predominantly considered a hallmark

of apoptosis, occurring downstream of changes in the mitochondrion and after caspase activation (Martin et al., 1995; Verhoven et al., 1999), we compared maturation-induced and apoptosis-associated PS exposure. To achieve this, the spatiotemporal pattern of annexin V binding was determined during myoblast differentiation in relation to a panel of differentiation and cell death markers. In addition, the function of surface-exposed PS in myotube formation was studied by fusion-inhibition studies using annexin V.

## MATERIALS AND METHODS

### Embryo studies

The procedures for in vivo labeling of PS-exposing cells in mouse embryos have been described previously (van den Eijnde et al., 1999; van den Eijnde et al., 1997b). Briefly, vital embryos were collected from pregnant mice at E10-14 (plug=day 0) without damaging the extraembryonic membranes. The embryos were injected with 2-3  $\mu$ l of annexin V conjugated to biotin (annexin V-biotin B-500; NeXins BV, Kattendijke, The Netherlands) into the sigmoid vein in the head and kept alive in annexin V binding buffer (20 mM Hepes (pH 7.4), 132 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 6 mM KCl, 1 mM MgSO<sub>4</sub>, 1.2 mM K<sub>2</sub>HPO<sub>4</sub>, 5.5 mM glucose, 0.5% BSA) for 30 minutes at 37°C. Subsequently, the embryos were fixed with 4% formaldehyde in binding buffer overnight at 4°C, and stored in 70% ethanol at -20°C until further processing for routine paraffin embedding and sectioning. Sections (5  $\mu$ m thick) were dewaxed in xylol and hydrated in a descending alcohol series. Annexin V-biotin binding was detected by a streptavidin-HRP conjugate (ABC Elite kit, Vector Laboratories, Burlingame, CA) and an H<sub>2</sub>O<sub>2</sub>/3,3'-diaminobenzidine tetrahydrochloride (DAB) reaction. Differentiating muscle cells, including smooth muscle, skeletal muscle and cardiomyocytes were detected using the anti  $\alpha$ -smooth-muscle actin antibody (1:1000, Clone 1A4, Sigma, Natick, MA, USA). Labeling of  $\alpha$ -smooth-muscle actin was achieved via an H<sub>2</sub>O<sub>2</sub>/DAB reaction after 4 hours of incubation with the primary monoclonal antibody and incubation with a secondary rabbit-anti-mouse antibody conjugated to HRP.

### In vitro studies

#### Cell lines

Two muscle cell lines were used in this study, both obtained from the American Type Culture Collection (Manassas, VA): the mouse C2C12 skeletal muscle cell line and the rat H9C2(2-1) cardiomyocyte cell line (Su et al., 1999). Importantly, the latter cell line has been described to possess features of skeletal muscle differentiation, including myotube formation (Menard et al., 1999). This is in contrast to heart muscle cells in vivo that do not fuse into myotubes but become connected by intercalated disks. The cells were grown in a humidified incubator at 5% CO<sub>2</sub> and 37°C in growth medium (GM) consisting of DMEM (ICN Biomedicals BV, Zoetermeer, The Netherlands) supplemented with 2 mM L-glutamine (Serva, Heidelberg, Germany), 10% FCS (Gibco, Paisly, UK) and 0.05 mg/ml gentamycin (AUV, Cuijk, The Netherlands). At 70-80% confluency, cells were trypsinized (0.125% trypsin (Gibco), 0.02% EDTA and 0.02% glucose in PBS) for 1-3 minutes and split at a 1:5-1:10 ratio. Myotube formation was induced by replacing GM with differentiation medium (DM) (Van der Loop et al., 1996). The only difference between GM and DM is that the latter contains 2% normal horse serum (Gibco) instead of 10% FCS. To limit autofluorescence, all the experiments were performed with cells maintained in GM or DM deficient in neutral red (ICN Biomedicals BV).

As a positive control for myoblast differentiation-dependent annexin V binding, the BHK-21/C13 cell line (Flow Laboratories, Irvine, UK) was used, which has been reported to exhibit myoblast like characteristics, including the formation of multinucleated

myotubes (Van der Loop et al., 1996). As negative controls, the myeloid cell line U937 (American Type Culture Collection) and the non-small-cell lung cancer cell line MR65 (Gropp, Philips Universitäts Klinik, Marburg, Germany) were used.

### Reagents

In this study, several variants of human recombinant annexin V were used: (1) human recombinant annexin V conjugated to Oregon Green (annexin V-fluo) at a final concentration of 250 ng/ml (annexin V-Oregon Green, NeXins Research BV); (2) unlabeled recombinant human annexin V (AnxV; 1-100  $\mu$ g/ml); (3) its null mutant (M1234; 100  $\mu$ g/ml), which has mutations in all four Ca<sup>2+</sup> binding sites resulting in a loss of PS binding capacity (Mira et al., 1997); and (4) M1234 conjugated to Oregon Green (1  $\mu$ g/ml).

To test cell viability and apoptosis, the following reagents were used: propidium iodide (PI, 5  $\mu$ g/ml; Molecular Probes, Eugene, OR), CMXRos (Mitotracker® Red, 500 ng/ml; Molecular Probes); Hoechst 33258 (10  $\mu$ g/ml; Molecular Probes), and zVAD(OMe)-fmk (100  $\mu$ M, diluted in DMSO; Alexis Biochemicals, Leiden, The Netherlands).

For immunofluorescence studies, rabbit-derived antibodies were used against active caspase 3 (polyclonal antibody CM1, 1:40, Idun Pharmaceuticals Inc., La Jolla, CA), and against annexin V (1:100). Furthermore, mouse derived mAbs were used directed against the sarcomeric protein titin (9D10, 1:10; Developmental Studies Hybridoma Bank). As secondary antibodies, Texas-Red-conjugated swine anti-rabbit Ig, or rabbit anti-mouse Ig were used as appropriate (DAKO, A/S, Glostrup, DK). As negative controls, the primary antibody was omitted; all negative control samples showed an absence of immunoreactivity.

### Immunocytochemistry

Cells cultured in the presence of annexin V-fluo were rinsed with ice cold annexin V binding buffer and thereafter fixed with 4% paraformaldehyde in annexin V binding buffer at 4°C, pH 7.4 for 10 minutes. Then the cells were rinsed twice with PBS, permeabilized for 10 minutes with 0.005% SDS in PBS at room temperature, rinsed with PBS containing 1% BSA and incubated at 4°C with CM1 antibody overnight, or one of the other antibodies for 2 hours. Subsequently, samples were rinsed with the same buffer and incubated for 2 hours with the appropriate fluorochrome-conjugated secondary antibody. After incubation with the secondary antibody, the samples were rinsed again and mounted with glycerol containing DAPI (Sigma Chemicals, St Louis, MO).

### Annexin V binding assays

To test for cell surface exposure of PS, annexin V-fluo was added to the medium. The cells were maintained in culture for a period ranging from 15 minutes up to several days in a humidified 5% CO<sub>2</sub> incubator at 37°C. For the longer culture periods, medium including annexin V-fluo was renewed every 2 days. Cells were studied upon binding of annexin V-fluo using an inverted fluorescence microscope with appropriate excitation and emission filters (Zeiss, Oberkochen, Germany; Leica Microsystems BV, Rijswijk, The Netherlands). Image acquisition was achieved using Ikaros (2.3) (MetaSystems, Heidelberg, Germany) or Openlab (Improvision, Lexington, MA) software. Images and composite figures were prepared using Adobe PhotoShop (5.0.2) and Illustrator (8.0) software, respectively (Adobe Systems Inc., San Jose, CA). To some images, deconvolution software was applied to remove out-of-focus information using the Openlab package (Improvision).

### Myotube fusion-inhibition assay

To test whether recombinant human annexin V could inhibit myotube formation, C2C12 and H9C2 cells were grown in 96 well plates ( $\mu$ Clear™ black tissue culture microplates, Greiner Laborotechnik, Frickenhausen, Germany). When the C2C12 cultures had reached 25% or 50% confluency and the H9C2 cultures had reached 95%

confluency, myoblast differentiation and myotube formation was induced by replacing GM with DM containing AnxV (1-100  $\mu\text{g/ml}$ ) immediately after the medium switch. Medium with the same amount of annexin V was refreshed twice a day for the lower doses (1-40  $\mu\text{g/ml}$ ) and every third day for the highest dose (100  $\mu\text{g/ml}$ ). To ascertain that annexin V interacted with the myoblasts in a PS-dependent manner, we used the non-PS-binding annexin V mutant M1234 at a concentration of 100  $\mu\text{g/ml}$ . As a positive control, cells were incubated with DM without AnxV or M1234.

On DMd0 and at the end of the culture period (i.e. DMd5 for C2C12 and DMd11 for H9C2), dual interference contrast-microscopy images were captured of each well. After culturing, cells were fixed in 4% paraformaldehyde in annexin V binding buffer and stained for the sarcomeric protein titin as described above. Subsequently, the complete bottom of the wells with cells attached were cut out with a scalpel and mounted on a coverslide using glycerol/DAPI. In each sample, all multinuclear cells were identified by titin staining and their nuclei were counted on a Zeiss microscope using the 40 $\times$  1.2 NA oil objective. For each well, the total number of multinucleated cells and nuclei therein, and the ratio between both were calculated. Averages $\pm$ s.e.m. were calculated for the data in each group (control, M1234 and AnxV), using MS Excel 98 (Microsoft, Redmond, WA). To test for significance, the Mann-Whitney (non-parametric) test was applied using SPSS (10.07a) for Macintosh (MacKiev, Cupertino, CA).

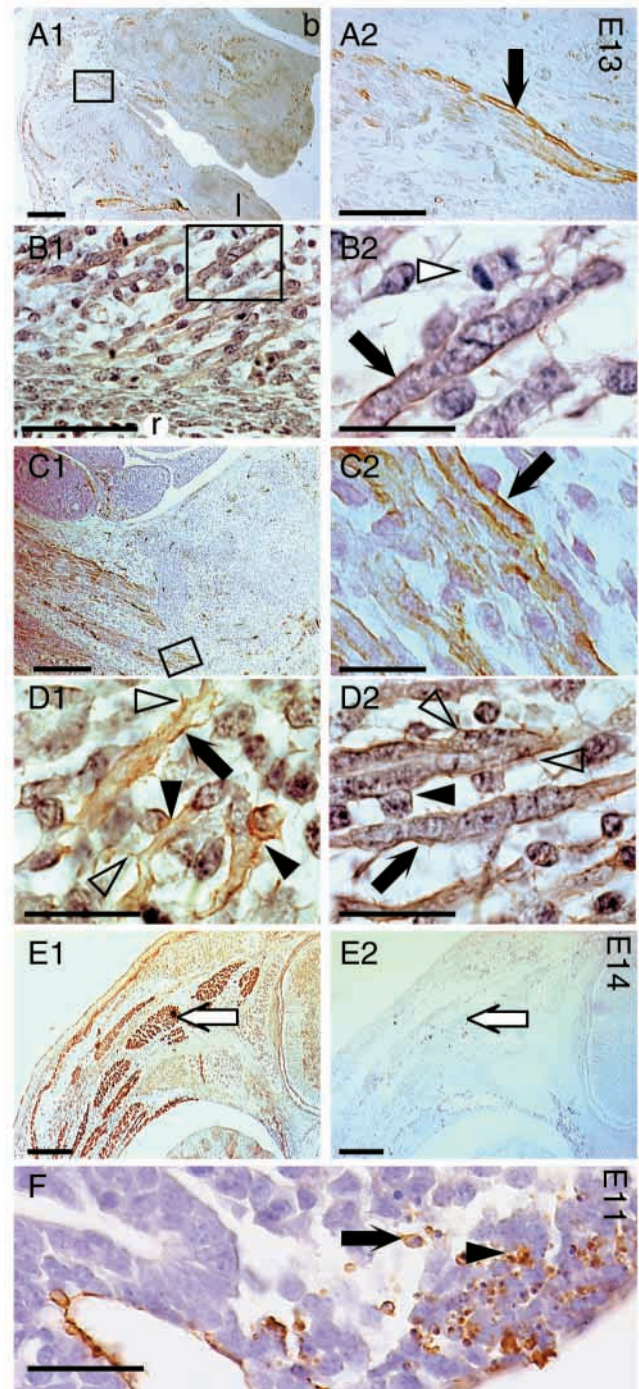
#### Control experiments

To determine whether fluorescence observed in differentiating H9C2 and C2C12 myoblasts was due to the presence of annexin V-fluo and not of unconjugated fluorochrome or autofluorescence, annexin V was immunocytochemically visualized. Using the same immunocytochemical procedure, it was verified whether unlabeled human recombinant annexin V had bound to differentiating myoblasts and the level of endogenous annexin V expression in C2C12 and H9C2 cells was assessed. Expression of annexin V was not detected in C2C12 cells, whereas, in apoptotic H9C2 cells, some endogenous annexin V expression was observed. To quantify the amount of annexin V in this cell line, an ELISA was performed, according to the manufacturer's instructions (Zymutest, Hyphen Biomed, Andressy, France). Cell lysates were obtained by removing the medium, adding 100  $\mu\text{l}$  of lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and collecting the cells with a rubber policeman. Subsequently, the suspension was subjected to three freeze-thaw cycles, and stored at  $-70^{\circ}\text{C}$  until simultaneous analysis. The levels of endogenous annexin V-protein measured in this cell line were, however, very low, with values ranging from 0.0028-0.0008% per total protein content.

## RESULTS

### PS exposure during muscle cell development in mouse embryos

In areas of skeletal muscle differentiation, labeling with annexin V was observed in E13 mouse embryos, where most of the myotubes in the trunk region were positive for cell surface exposure of PS (Fig. 1). Fig. 1A1-C2 show examples of annexin V-positive primary myotubes in the cervical, thoracic and lumbar regions. On the surface of these annexin V-positive myotubes, annexin V-positive protrusions were found (Fig. 1D1,D2, open arrowheads). In-between the



**Fig. 1.** Transient PS exposure by differentiating myoblasts in mouse embryos. Primary myotubes expose PS transiently at E13 (black arrows) in the cervical area (boxed area A1,A2), in-between the developing ribs (boxed area B1,B2) and the lumbar region (boxed area C1,C2). Frequently, annexin V-positive rounded cells were found attached to myotubes (D1,D2, arrowheads). Also in these sections, indications were found that annexin V-positive extensions arise from these developing myotubes (D1,D2, open arrowheads). Mitotic cells were negative for annexin V (B2, white arrowhead). At E14, annexin V staining of myotubes is virtually absent: compare the labeling for muscle using an anti- $\alpha$ -smooth muscle actin antibody (E1) with the labeling of surface-exposed PS (E2) in the same muscle (white arrows) in an adjacent section. For comparison, F shows annexin V-labeled apoptotic cells in the fusing E11 branchial arches, both in the mesodermal compartment (arrow) and in the ectoderm (arrowhead). Bars, 25  $\mu\text{m}$  (C2,D1,D2,F); 40  $\mu\text{m}$  (B2); 200  $\mu\text{m}$  (A2,B1,C1); 500  $\mu\text{m}$  (A1,E1,E2). Abbreviations: b, brain; l, limb; r, rib.

myotubes, a mixed population of mononucleate myoblasts, and fibroblasts, were observed (Ontell and Kozeka, 1984). Many of these cells were negative for annexin V-biotin, but a subpopulation of these cells, in particular the more rounded cells with little cytoplasm were observed to be annexin V-positive and attached to myotubes (Fig. 1D1,D2, black arrowheads). The labeled primary myotubes were characterized by their large diameter, and non-condensed cytoplasm and nucleus (Ashby et al., 1993b; Stockdale, 1992). Hence, these cells could be clearly discriminated from condensed apoptotic cells. Whereas only a few of such apoptotic cells were found in the developing muscle, many were observed elsewhere in the embryo at specific sites where cell death is known to occur (Fig. 1F). Only at these sites was profound phagocytic activity was observed. At E14, when most primary myotubes had formed, these  $\alpha$ -smooth muscle actin-positive cells (Fig. 1E1) were mostly negative for annexin V-biotin (Fig. 1E2).

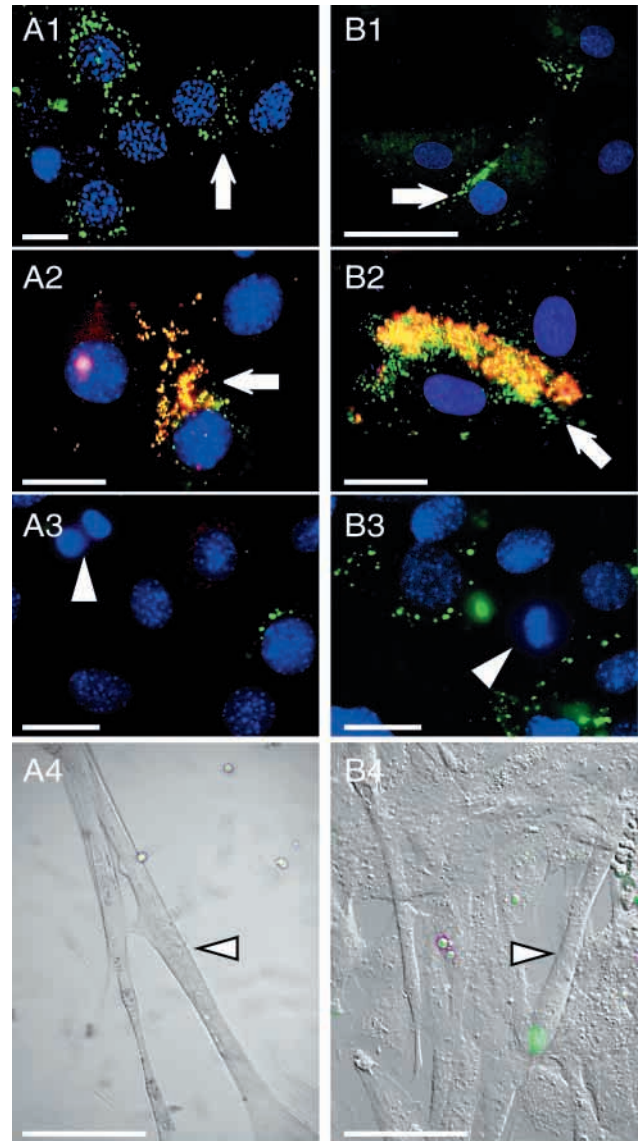
In summary, our data from developing myotubes indicate that these viable muscle cells transiently expose PS in a developmentally regulated manner. No accumulation of phagocytes was observed in areas of myoblast differentiation.

#### PS exposure in differentiating myoblast cultures

C2C12 and H9C2 muscle cell lines undergo differentiation after serum deprivation, as indicated by the process of cellular elongation, fusion into di- and trinuclear elongated cells and formation of extremely elongated multinucleated myotubes.

These C2C12 and H9C2 cells were able to bind annexin V-fluo transiently (Fig. 2). C2C12 muscle cells were found to bind annexin V within 8 hours after switching from GM to DM (Fig. 2A1,A2). After 2 days, binding was maximal with approximately 60% of the cells positive for annexin V. By contrast, proliferating C2C12 muscle cells (Fig. 2A3) and myotubes (Fig. 2A4) after 8 days in DM were not labeled with annexin V-fluo. H9C2 cells behave similarly (Fig. 2B1-4), except that the first binding of annexin V-fluo was observed after 2.5 days in DM (Fig. 2B1,B2), was maximal after 8 days (on average 40% of the cells) and absent again after 12 days. Mitotic cells (Fig. 2B3) and myotubes (Fig. 2B4) were negative for annexin V-fluo. In annexin V-positive cells the distribution pattern of this marker changed time dependently. Between 15 minutes and 2 hours of incubation the annexin V-fluo labeling was seen at the cell surface, in-between cells. After longer incubation periods the annexin V-fluo became gradually internalized, as could be demonstrated by rinsing with  $\text{Ca}^{2+}$ -depleted medium (which dissociates annexin V from cell surface-exposed PS), resulting in only a partial loss of the annexin-fluo labeling.

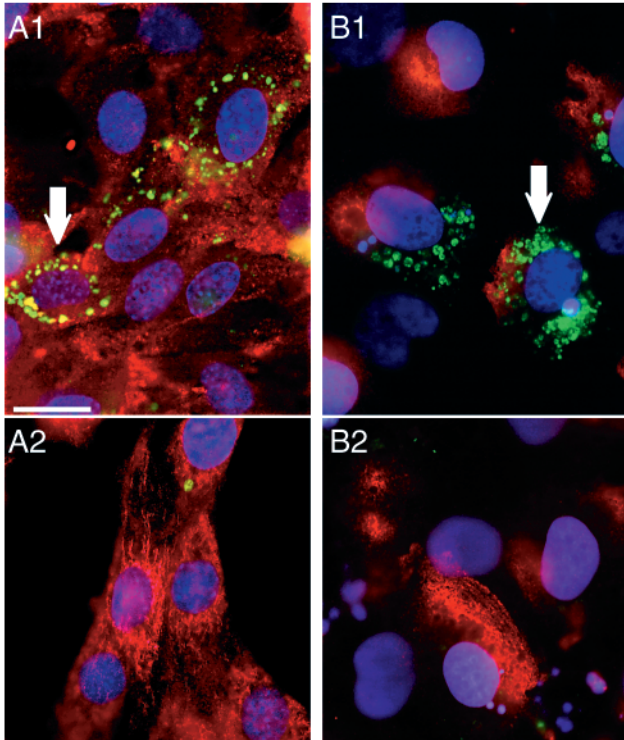
Once negative for annexin V-fluo, cells could not be relabeled with freshly added annexin V-fluo, even at quadruple doses (1  $\mu\text{g}/\text{ml}$ ), indicating that the loss of the annexin V-fluo signal is not due to depletion of this marker, and stressing the transient nature of PS exposure. In Fig. 2A2,B2, cells are shown that were incubated with annexin V-fluo in culture and stained with an anti-human annexin V antibody after fixation. The co-localization of both markers indicates that the green annexin V-fluo signal reflects interaction of intact annexin V conjugates with the myoblasts, even after prolonged incubation periods. In addition, analogous experiments showed that unlabeled human



**Fig. 2.** Double labeling with annexin V-fluo (green) and for DNA (DAPI, blue) of C2C12 (A) and H9C2 (B) muscle cells shows that differentiating myoblasts are labeled with annexin V at cell-cell contact areas (A1,A2,B1,B2, arrows), while mitotic myoblasts (A3,B3, arrowheads) and multinucleated myotubes (A4,B4, arrowheads) are negative for annexin V. Immunocytochemistry with an anti-human annexin V antibody (red) shows co-localization of these two markers resulting in yellow areas in the overlay (A2,B2). Bars, 20  $\mu\text{m}$  (A1-3,B1-3); 60  $\mu\text{m}$  (A4,B4).

recombinant annexin V, which was used in the fusion inhibition studies (see below), also specifically bound differentiating myoblasts since the pattern of immunostaining of this unlabeled annexin V accurately mirrored the annexin V-fluo signal.

Since myoblast differentiation is a highly organized process, we wondered whether the temporal window of annexin V labeling is related to a particular phase of myoblast differentiation, as for example indicated by the molecular organization at the sarcomeric level. To this end, cells were double-stained for annexin V and titin, which has been shown

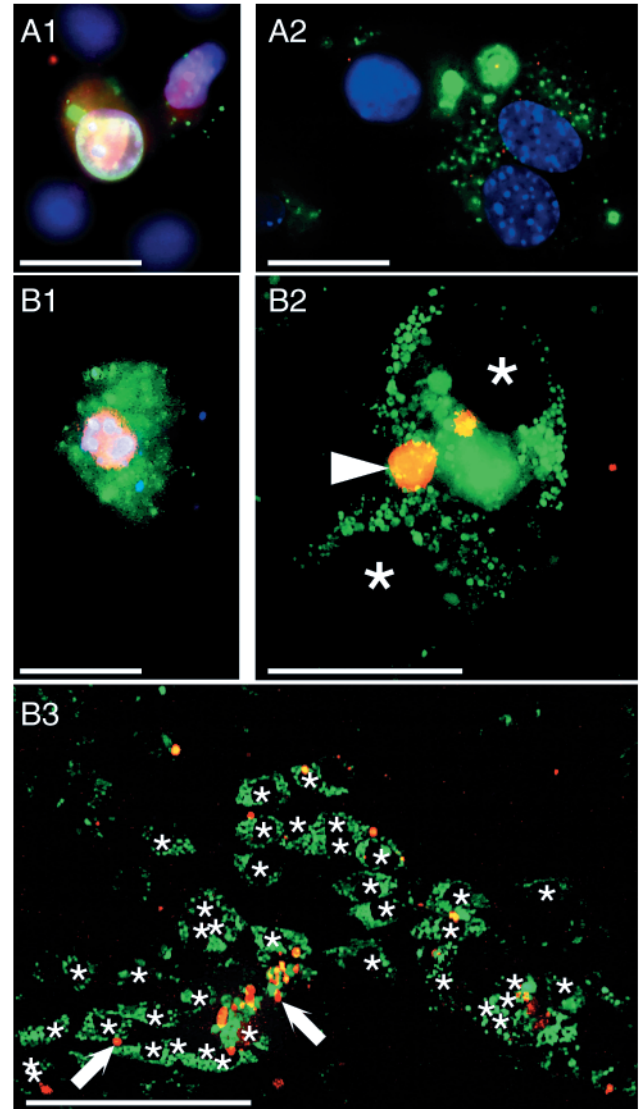


**Fig. 3.** Triple labeling with annexin V-fluo (green), for titin (red) and DNA (DAPI, blue) of C2C12 (A1,A2) and H9C2 (B1,B2) myoblasts shows that annexin V labels mainly early differentiating muscle cells with a punctate titin expression pattern (A1,B1, arrows). At later stages of differentiation, when the titin-expression pattern becomes filamentous, myoblasts were almost completely negative for annexin V-fluo (A2,B2). Bar, 20  $\mu$ m.

to be one of the earliest proteins to become expressed and organized in the developing sarcomere (van der Ven et al., 1993). Essentially, the labeling patterns in C2C12 and H9C2 cells were similar (Fig. 3). Double labeling for both markers was mainly observed at a phase when titin was expressed in dot-like aggregates (Fig. 3A1,B1). By contrast, virtually all cells exhibiting a filamentous titin organization were negative for annexin V-fluo (Fig. 3A2,B2).

To confirm that the annexin V-fluo interaction with muscle cells observed in our culture system is a PS-specific event, we performed negative control experiments using M1234 conjugated to Oregon Green. In none of the experiments with C2C12 or H9C2 cells was any labeling observed, even after incubation periods of multiple days or doses up to 1  $\mu$ g/ml (data not shown). Double labeling experiments showed that in differentiating C2C12 and H9C2 cell cultures the annexin V-fluo-positive cells had a non-disrupted plasma membrane as concluded from PI exclusion (Fig. 4A2,B2,B3), which indicates that annexin V stained the cells via an interaction with PS present at the outer leaflet of the plasma membrane. Only sporadically, cells were positive for both annexin V-fluo and PI (Fig. 4A1,B1,B3, arrows), indicating a possible intracellular annexin V-fluo labeling, which was accompanied by a postapoptotic/necrotic morphology. Mainly, annexin V-fluo was observed to label clusters of cells amidst unlabeled cells (Fig. 4B3).

Finally, testing annexin V-fluo in cultures of other cell lines



**Fig. 4.** Differentiating annexin V-binding C2C12 (A1,A2) and H9C2 (B1-3) myoblasts have an intact plasma membrane as detected by PI exclusion. In all cultures low numbers of solitary annexin V (green) and PI (red) labeled cells were found (A1,B1,B3, arrows). These postapoptotic/necrotic cells can clearly be discriminated from annexin V-positive differentiating myoblasts where annexin V labeling is preferentially located at cell-cell contact areas (A2, nuclei stained with DAPI; B2, asterices indicate location of nuclei), and which are PI impermeable (A2,B2,B3, asterisks indicate location of nuclei). In-between the cells shown in B2, an annexin V- and PI-positive cell fragment, most probably resulting from apoptotic cell membrane blebbing, is present (arrowhead). Bars, 25  $\mu$ m (A1,A2,B1,B2); 200  $\mu$ m (B3).

showed that normally only viable differentiating muscle cells bind annexin V. In line with our hypothesis, differentiating BHK cells bound annexin V-fluo similar to the C2C12 and H9C2 muscle cell lines after growth factor deprivation. In BHK cells, annexin V-fluo was detected at contact areas of cells with a non-apoptotic morphology as determined with DAPI staining and dual interference contrast microscopy. By contrast, U937 and MR65 cultures were only annexin V-positive when necrotic or apoptotic (data not shown).

### Annexin V fusion-inhibition studies

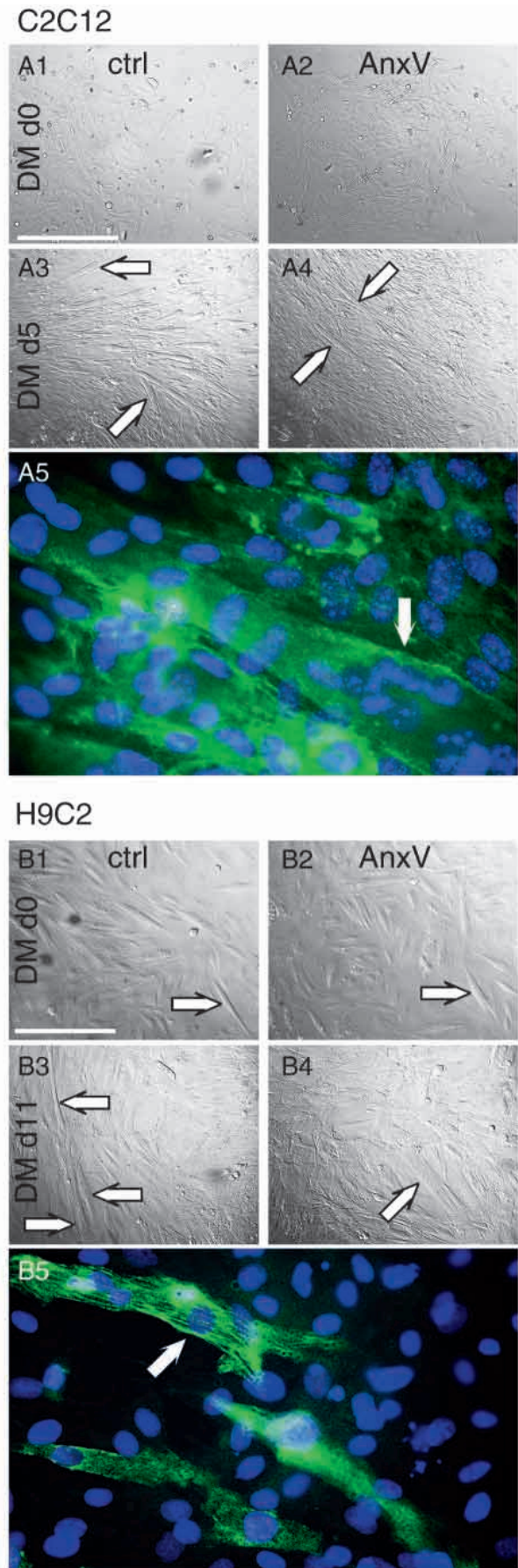
To investigate a possible causal relationship between PS exposure and myoblast fusion, we analyzed whether annexin V can inhibit the formation of myotubes in differentiating C2C12 and H9C2 cells. C2C12 cells grown to 25% or 50% confluency (Fig. 5A1,A2) and H9C2 cells grown to 95% confluency (Fig. 5B1,B2) were induced to differentiate by medium switch. We also induced C2C12 myoblast differentiation in cells grown to 80-100% confluency. However, in these cultures the numbers of myotubes that had formed at DMd5 were too high to permit accurate counting of myotube numbers and nuclei.

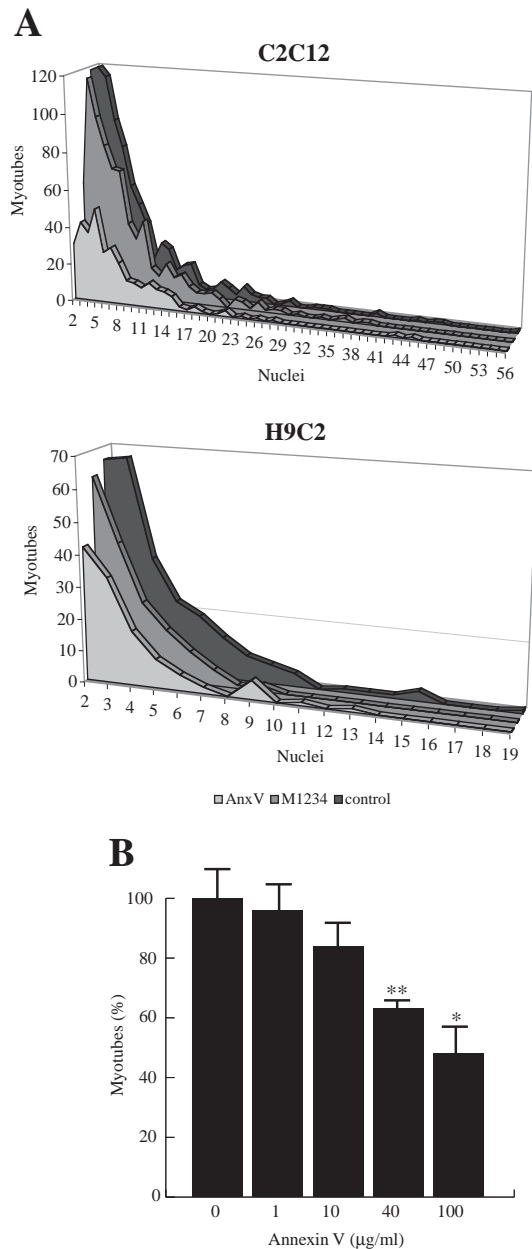
In almost completely confluent cultures, first signs of 'spontaneous' myoblast differentiation and myotube formation were observed before the medium switch (Fig. 5B1,B2, arrows). In samples cultured in DM without AnxV (Fig. 5A3,B3) and in samples cultured in DM containing M1234 (data not shown), cells became elongated upon medium switch, aligned and formed giant multinucleated cells. Maximum myotube formation was observed on DMd5 and DMd11 for C2C12 and H9C2 cells, respectively. When cultured in DM containing AnxV, only a few C2C12 myotubes had formed (Fig. 5A4). H9C2 cultures responded similarly to incubation with DM with AnxV, although the decrease in myotube numbers as compared with control incubations was less extreme (Fig. 5B4).

For the quantitative and statistical analysis of differences in myotube formation between myoblasts cultured with or without AnxV, we counted all myotubes and nuclei therein in 5-17 wells per group. To this end C2C12 cells at DMd5 (Fig. 5A5) and H9C2 cells at DMd11 (Fig. 5B5) were labeled for titin to identify differentiated multinucleated cells, and DAPI to count the nuclei. Fig. 6A illustrates the differences in myotube numbers containing a given number of nuclei for C2C12 cells that were induced to differentiate when grown to 50% confluency and H9C2 cells grown to 95% confluency before switching to DM. It is evident from this figure that for C2C12, AnxV significantly reduces the number of myotubes compared with control and M1234 incubations, whereas for H9C2 this inhibitory effect is less pronounced.

C2C12 cultures that were induced to differentiate at 25%

**Fig. 5.** Recombinant human annexin V (AnxV) inhibits myotube formation in differentiating C2C12 and H9C2 muscle cell cultures. C2C12 cells were induced to differentiate in DM when the cultures were at 25% (not shown) and 50% confluency (A1,A2), and H9C2 cells at 95% confluency (B1,B2). In samples cultured in the presence of M1234 (not shown) or in the absence of annexin V (ctrl) myotube formation could clearly be observed in C2C12 cells at day 5 (DM d5, A3, arrows) and H9C2 cells at day 11 (DM d11, B3, arrows), and to a lesser extent in cells cultured in DM containing 100 µg/ml of recombinant human annexin V (A4,B4, arrows). Note that cells cultured in GM differentiate spontaneously and form myotubes when reaching 100% confluency (e.g. B1,B2, arrows). To assess the effect of annexin V on myotube formation quantitatively, multinucleated cells and their nuclei were counted in C2C12 cultures at DM d5 (A5) and H9C2 cultures at DM d11 (B5), after staining of DNA with DAPI (blue), and with titin (green) to detect differentiating cells. The quantitative results are shown in Fig. 6 and Table 1. Bar, 100 µm (A1-4,B1-4); 25 µm (A5,B5).





**Fig. 6.** (A) Annexin V inhibits myotube formation in differentiating C2C12 and H9C2 myoblast cultures via an interaction with PS. C2C12 and H9C2 cells were induced to differentiate in DM (control), DM with PS binding-deficient M1234, or DM with 100 µg/ml annexin V (AnxV). The graphs depict myotube distribution as a function of the number of nuclei they contained on DMd5 in cultures of C2C12 cells that were induced to differentiate at 50% confluency, and on DMd11 in cultures of H9C2 cells that were induced to differentiate at 95% confluency. Data from 6 wells per group were combined. Statistical analysis of this data is shown in Table 1. (B) Annexin V inhibits myotube formation dose dependently. C2C12 cells were induced to differentiate in DM with indicated concentrations of annexin V and the number of myotubes were counted in each sample ( $n=6$  per dose). The graph depicts the average number of myotubes per well as a percentage of control on DMd5 in myoblast cultures that were induced to differentiate at 50% confluency. A significant decrease in the number of myotubes formed was observed at a concentration of 40 µg/ml (\*\* $p=0.001$ ) and 100 µg/ml (\* $p=0.002$ ).

**Table 1. Annexin V inhibits myotube formation in differentiating C2C12 and H9C2 cell cultures**

A C2C12 (25% confluency)		
	Myotubes/well	Nuclei/myotube
Control ( $n=17$ )	56±5	6.3±0.4
M1234 ( $n=5$ )	45±8	8.1±0.4*
AnxV ( $n=6$ )	23±4‡,§	7.1±0.7
B C2C12 (50% confluency)		
	Myotubes/well	Nuclei/myotube
Control ( $n=6$ )	141±17	7.1±0.2
M1234 ( $n=6$ )	123±14	7.3±0.4
AnxV ( $n=6$ )	67±13‡,¶	7.2±0.2
C H9C2 (95% confluency)		
	Myotubes/well	Nuclei/myotube
Control ( $n=6$ )	42±7	4.2±0.2
M1234 ( $n=6$ )	27±4*	3.4±0.3‡
AnxV ( $n=6$ )	19±4*	3.5±0.2

Myotube formation by differentiating C2C12 and H9C2 cells. Myoblast differentiation and myotube formation was induced in cultures of C2C12 cells grown to 25% (A) or 50% (B) confluency, and H9C2 cells grown to 95% confluency (C) by switching from GM to DM, which contained no annexin V (control), 100 µg/ml of mutant annexin V (M1234), or 100 µg/ml of annexin V (AnxV). Number of myotubes and nuclei therein were determined in each sample at DMd5 and DMd11 in C2C12 and H9C2 cultures, respectively. The average values±s.e.m. are shown for each group. The Mann-Whitney test (non-parametric) was applied to determine significance of differences between control versus M1234 or AnxV (\* $P<0.05$ ; ‡ $P<0.005$ ) and between M1234 versus AnxV (§ $P<0.05$ ; ¶ $P<0.005$ ).

confluency (Table 1A), showed no significant differences between myoblast differentiation in DM or in DM plus M1234 with respect to myotubes per well, whereas myoblast differentiation in DM with AnxV resulted in significantly fewer myotubes compared with control ( $p=0.001$ ) and M1234 ( $p=0.014$ ). The average number of nuclei per myotube varied between 6.3 and 8.1. Inducing differentiation of C2C12 cells at 50% confluency (Table 1B) resulted in a profound increase in the number of myotubes formed compared with numbers in the 25% confluence culture, whereas the average number of nuclei per myotube was in the same order of magnitude. Statistically significant differences were observed for the number of myotubes formed in the presence of AnxV, compared with myotube formation in the presence of M1234 ( $p=0.005$ ) or DM only ( $p=0.002$ ). In H9C2 cells (Table 1C), a trend was visible for the number of myotubes with highest values for control cultures, intermediate values for cells differentiating in medium with M1234, and lowest values for cells differentiating in medium with AnxV. However, statistically significant differences were only observed between control samples and M1234 ( $p<0.033$ ) and AnxV ( $p<0.019$ ) incubated samples. In this cell line the average number of nuclei per myotube varied between 4.2 and 4.2.

A dosage-dependent effect of annexin V on myotube formation is obvious from Fig. 6B, which shows a trend of an inhibitory effect already at the lowest dose of 1 µg/ml, and a significant inhibitory effect at a dose of 40 µg/ml ( $p=0.001$ ) and above.

Generally these observations strongly indicate that recombinant human annexin V inhibits myotube formation by homotypic interaction between muscle cells via surface-exposed PS.



### PS-exposing myoblasts: differentiation or apoptosis?

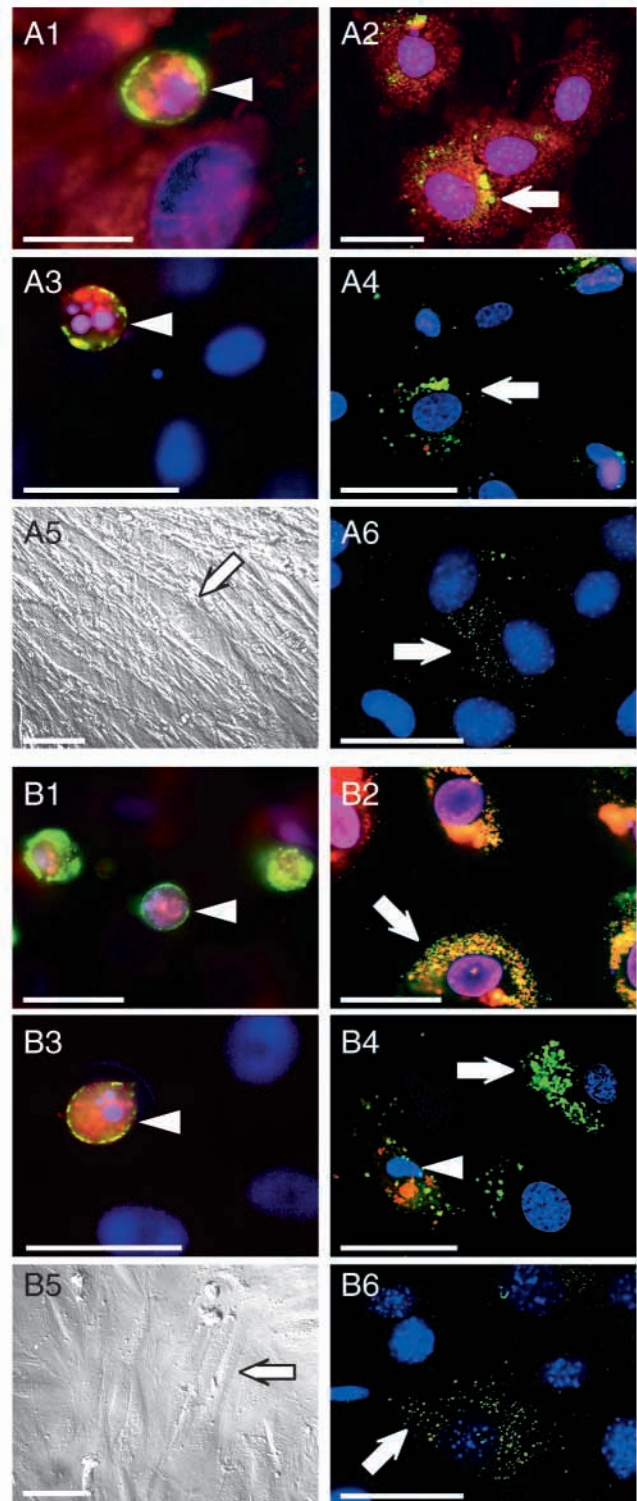
PS exposure in apoptotic cells is suggested to be a downstream effect of mitochondrial changes and activation of the caspase cascade in the apoptosis signaling pathway. To establish whether a comparable molecular mechanism underlies PS exposure in fusing myoblasts, these events were investigated during *in vitro* myoblast differentiation (Fig. 7).

Detection of PS exposure with annexin V-fluo, in combination with CMXRos labeling to indicate the status of the mitochondrial membrane potential, and DAPI to show nuclear morphology, demonstrated clear differences between apoptotic and differentiating C2C12 and H9C2 cells. Apoptotic myoblasts are stained with annexin V-fluo at the cell's circumference, the CMXRos labeling is diffuse, while the nucleus is pyknotic (Fig. 7A1,B1). By contrast, differentiating myoblasts have a granular annexin V-fluo distribution pattern, a granular CMXRos labeling indicating an intact mitochondrial membrane potential, and uncondensed nuclei (Fig. 7A2,B2). Activated caspase 3 was present in apoptotic, annexin V-fluo-positive C2C12 and H9C2 myoblasts (Fig. 7A3,B3,B4, arrowhead). By contrast, differentiating annexin V-fluo-positive myoblasts were negative for active caspase (Fig. 7A4,B4, arrow). Further confirmation that annexin V binding to differentiating myoblasts is unrelated to caspase activity was obtained from experiments in which C2C12 and H9C2 cells were cultured in DM supplemented with the cell-permeable, general caspase inhibitor zVAD(OMe)-fmk. Up to 5 days after switching to differentiation medium, the cell cultures with zVAD(OMe)-fmk showed similar numbers of myotubes (Fig. 7A5,B5) and comparable distribution of annexin V-fluo (Fig. 7A6,B6) as the cultures without this caspase inhibitor. In these cases, it is again evident that after extended incubation periods annexin V is internalized.

### DISCUSSION

At present only limited data are available on surface molecules that regulate signaling between myoblasts during muscle development. In this study we show that differentiating myoblasts transiently express PS at an early phase of myotube formation, a process that seems not to involve loss of mitochondrial metabolic activity and activation

of the caspase cascade, as observed for PS exposure during apoptosis. This translocation of PS from the inner to the outer plasma membrane leaflet is predominantly found at cell-cell contact areas. The formation of multinucleated myotubes from individual differentiating myoblasts can specifically be blocked with PS-binding annexin V, indicating that cell surface exposure of PS is essential to the execution of this process.



**Fig. 7.** Annexin V-binding by differentiating C2C12 (A) and H9C2 (B) myoblasts does not require loss of mitochondrial inner membrane potential or caspase activity. Apoptotic myoblasts are positive for annexin V-fluo (green) and show a diffuse labeling for CMXRos (A1,B1, arrowhead, red) and immunoreactivity for the anti-active caspase 3 antibody (A3,B3,B4, arrowhead, red). By contrast, differentiating C2C12 and H9C2 cells bind annexin V-fluo while having a granular labeling for CMXRos (A2,B2, arrow) and show no active caspase 3 protein (A4,B4, arrow). In line with the latter results, culturing cells in DM containing the caspase inhibitor zVAD-(OMe)-fmk at a dose of 100  $\mu$ M blocks neither myotube formation (A5,B5, arrow) nor annexin V-fluo binding (A6,B6, arrow). These two figures, in which out-of-focus information has been removed by image restoration using deconvolution software, further illustrate the intracellular granular labeling of myoblasts with annexin V-fluo after longer incubation periods. Bars, 25  $\mu$ m.

### Muscle cells transiently expose PS during differentiation

In this study we present data showing that, during embryogenesis, morphologically viable myoblasts transiently bind annexin V, and thus express PS at particular stages of development. These cells were, however, not in contact with phagocytes, nor were any phagocytes detected in their vicinity, as would be expected when these cells were apoptotic (Abood and Jones, 1991; van den Hoff et al., 2000). In the underlying study, we have focussed on skeletal muscle development. In this tissue, binding of annexin V appeared to be a synchronous process that was present at E13 without the accumulation of pyknotic cells. Annexin V was not observed anymore at E14 or later, when most primary myotubes have formed, and was preceding the phase of massive death of primary myotubes that occurs after E15 (Ashby et al., 1993a).

Since, in the organism, cell surface exposure of PS is a trigger for phagocytic removal of apoptotic cells (Fadok et al., 1992b; van den Eijnde et al., 1999), one may wonder how PS-exposing, differentiating myoblasts remain unnoticed by phagocytes. Macrophages show tissue specificity with respect to the receptors used for clearance of apoptotic cells. In E13 mouse embryos, shielding of PS specifically on apoptotic neurons using annexin V was found to inhibit phagocytosis, whereas apoptotic mesenchymal cells in the limbs were ingested by phagocytes (van den Eijnde et al., 1999). This finding corroborates studies by Fadok and co-workers, who showed that depending on their activation status, macrophages may recognize apoptotic cells in a PS-dependent or -independent manner via integrins (Fadok et al., 1992a). Another mechanism may relate to the observations that apoptotic cells can attract phagocytes actively by secreting chemotactic factors (Knies et al., 1998). PS-exposing viable muscle cells may, by lacking such factors, avoid the attention of scavenging cells despite the fact that they bear a putative signal for cell removal (Knies et al., 1998; Wilson et al., 1996).

Similar to the *in vivo* situation, two populations of annexin V binding cells could be discriminated in C2C12 and H9C2 cell cultures, that is, apoptotic and differentiating muscle cells. In contrast to apoptotic cells, differentiating cells showed intracellular labeling when incubated with annexin V-fluo for longer time periods. Endocytosis of annexin V-fluo through pinocytosis can largely be excluded since co-labeling of annexin V-fluo with DiI (labeling of the membrane of pinocytic vesicles) showed hardly any overlap (S.M.v.d.E. et al., unpublished). The nature of the annexin V-fluo vesicles in differentiating muscle cells is not yet known. However, based upon their cellular localization they resemble: (1) the cytoplasmic vesicles found in the gap junction-like pre-fusion complex that forms at an early stage of myotube formation in *Drosophila*, directly following the initial phase of cell recognition and adhesion; and (2) the plasma membrane vesicles found during the phase of plasma membrane breakdown, which is believed to effect cytoplasmic continuity and actual fusion of myoblasts by removing excess plasma membrane in-between the fusing cells (Doberstein et al., 1997; Paululat et al., 1999).

In our *in vitro* experiments, annexin V was found to almost exclusively label mononucleated cells in contact with other mononucleated cells or small myotubes containing a few nuclei. Large, multinucleated myotubes were unlabeled.

Labeling for the sarcomeric protein titin revealed further detail about the time-point of PS exposure in myoblast differentiation. Titin is the first sarcomeric protein expressed in muscle cells (Van der Loop et al., 1996; van der Ven et al., 1993); in differentiating muscle cells titin organization changes from diffuse to punctate, thereafter becomes filamentous and finally incorporates into sarcomeres, where it exhibits the typically striated staining pattern. Annexin V-positive myoblasts were largely characterized by a punctate titin distribution pattern, while subsequent stages of titin patterns were rarely observed in these labeled cells. In view of previous immunocytochemical studies of C2C12 and H9C2 cells (Van der Loop et al., 1996) or in human skeletal muscle cell cultures and BHK cells (van der Ven and Furst, 1998; van der Ven et al., 1993), our results indicate that, in particular, early postmitotic myoblasts expose PS. Thus, these *in vitro* data corroborate our *in vivo* observations that annexin V binding is mainly restricted to myoblasts in the process of myotube formation, and that PS is therefore transiently exposed and internalized before the fusion process is actually completed. The localization of PS in the inner plasma membrane leaflet in most cells is maintained by an aminophospholipid translocase (Diaz and Schroit, 1996; Zwaal and Schroit, 1997). During apoptosis and platelet activation, PS becomes cell surface exposed by a simultaneous translocase inhibition and scramblase activation (Higgins, 1994; Verhoven et al., 1995). It remains to be resolved, however, whether the same or similar transporters regulate loss of PS asymmetry of the plasma membrane in developing myotubes.

### PS exposure during myogenesis is not related to the molecular cascade of apoptosis

A possible link between the apoptotic pathway and PS exposure during muscle cell differentiation was investigated and could be excluded at the level of loss of mitochondrial inner cell membrane potential, caspase 3 activation and caspase activity. In contrast to apoptotic H9C2 and C2C12 cells, annexin V-positive differentiating cells showed none of these molecular characteristics. In addition, we were unable to inhibit myotube formation and differentiation-dependent PS exposure with the broad spectrum caspase inhibitor zVAD(OMe)-fmk, suggesting that caspases are probably not directly involved in the loss of plasma membrane PS asymmetry. Nonetheless, we cannot rule out that PS exposure by myoblasts is triggered by a short and transient loss of mitochondrial membrane potential (Minamikawa et al., 1999). We consider it likely that PS exposure during muscle development is regulated via factors upstream of the mitochondrion. This hypothesis is substantiated by several recent studies, indicating that PS exposure does not always require caspase activity, and that this plasma membrane alteration can be reversed (Hammill et al., 1999; Williamson et al., 1995).

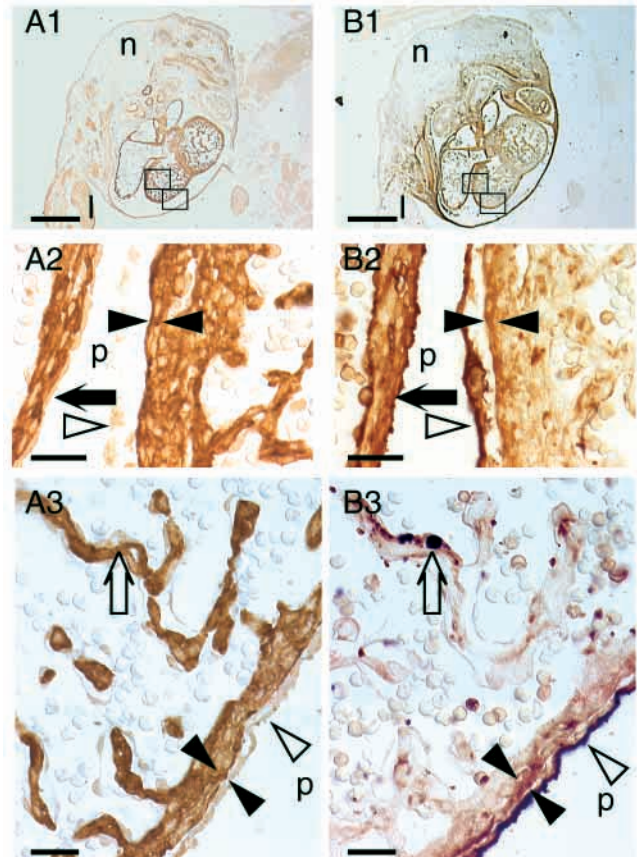
### Physiological function of PS exposure in myoblast interactions

Using the merocyanin 540 dye for labeling of loose lipid packing of plasma membranes, Sessions and Horwitz, as early as 1981, suggested that in primary cultures of chick and quail myoblasts PS was exposed at the cell surface when these were expected to fuse (Sessions and Horwitz, 1981). From these

results, they hypothesized that an increase in the percentage of the PS in the outer plasma membrane leaflet may give rise to an increase in membrane fluidity. In turn, this increased membrane fluidity could create a biochemical microenvironment facilitating the fusion process. Data presented in this study also indicate that cell surface-exposed PS is required for differentiating muscle cells to fuse into multinucleated myotubes. A significant decrease was observed in the numbers of myotubes formed in myoblast cultures containing 40 and 100  $\mu\text{g/ml}$  of recombinant human annexin V. Generally, C2C12 cells showed more prominent features of differentiation than H9C2 cells. This may relate to the fact that C2C12 cells are of skeletal myoblast origin, whereas H9C2 cells derive from cardiac myoblasts. Cells from this lineage normally become connected via intercalated disks and do not form myotubes, neither in vivo nor in primary cultures (Kostin et al., 1999). It may be expected, therefore, that H9C2 cells are at least partially deficient in the expression of molecules regulating myoblast fusion and are less efficient in executing this process. In all experiments, a limited effect of the annexin V mutant M1234 on myotube formation was observed, which was borderline-significant in H9C2 cell cultures. This finding suggests that this annexin V mutant, which does not bind PS that is present in artificial bilayers of phosphatidylcholine (C.P.M.R., unpublished), may have a residual interaction with PS in biological membranes. Alternatively, it may suggest a PS-independent interaction of M1234 with myoblasts. At present, the nature of these interactions remains to be resolved.

Data from the fusion inhibition experiments indicate that PS is of physiological importance to myotube formation. A well documented function of PS is phagocyte recognition of apoptotic cells (Fadok et al., 2000; Hamon et al., 2000; Schroit et al., 1985; Verhoven et al., 1995), involving PS exposure at the plasma membrane of both scavenger and prey (Marguet et al., 1999). Cell-cell recognition is considered the initial event in myotube formation (Doberstein et al., 1997; Paululat et al., 1999), and we therefore consider it likely that PS has an analogous function in phagocytosis and myotube formation. In this respect, the phenomenon of partial inhibition of myotube formation by high doses of annexin V may relate to the timing of interference with the fusion process, at which a fraction of the cells may have already completed their recognition phase. Interestingly, a similar partial inhibitory effect of has been observed in studies on the role of PS in phagocytic removal of apoptotic cells. In several phagocyte lineages the decrease in phagocytic activity also maximized at approximately 50%, irrespective of the inhibitory agent used (i.e. annexin V at a dose 18-180  $\mu\text{g/ml}$  (Bennett et al., 1995), PS-containing liposomes, or antibodies against a putative PS receptor (Fadok et al., 2000)). This partial inhibition of phagocytosis is explained by the existence of multiple pathways in which PS is a membrane component amongst others orchestrating the engulfment.

A function of PS in homotypic cell-cell interactions would fit our observations that PS is initially exposed at the plasma membrane at myoblast cell-cell contact areas by early differentiating muscle cells. In addition, it could explain our observation of a transient exposure of PS in the developing heart in mouse embryos at E12 (Fig. 8). In heart development, homotypic cell recognition is possibly of importance to the formation of intercalated disks. PS exposure was detected, in



**Fig. 8.** PS is transiently exposed by differentiating cardiomyocytes of E12 mouse embryos. Adjacent transverse sections were stained for  $\alpha$ -smooth muscle actin to detect cardiac muscle cells (A) and annexin V to label surface-exposed PS (B). Boxed areas in A1 and B1 are depicted at a higher magnification in A2 and B2 (upper box), and A3 and B3 (lower box). A strong annexin V-biotin labeling was observed in the smooth-walled atrial myocardium (A2,B2, arrow), the subepicardial ventricular myocardium (A2,A3,B2,B3, in-between arrowheads) and the epicardium (A2,A3,B2,B3, open arrowhead). Also, at this stage of development, some pyknotic cardiomyocytes were observed in the trabecular myocardium (A3,B3, open arrows). Bars, 25  $\mu\text{m}$  (A2,B2); 50  $\mu\text{m}$  (A3,B3); 500  $\mu\text{m}$  (A1,B1). Abbreviations: n, neural tube; l, limb; p, pericardial cavity.

particular, in sub-epicardial ventricular cardiomyocytes and the smooth-walled atrial myocardium in a spatiotemporal distribution, which did not match early descriptions of cell death foci in the developing heart (van den Hoff et al., 2000).

PS may be implicated in homotypic recognition between muscle cells by interacting with a receptor. A putative receptor for PS, which is highly expressed in developing heart and skeletal muscle, has recently been identified and cloned by Fadok and co-workers (Fadok et al., 2000). Another candidate receptor is thrombospondin, which has been reported to bind PS (Tooney et al., 1998) and to be expressed in mouse embryonic heart and skeletal muscle (Manodori et al., 2000) in a pattern that is mirrored by the pattern of in vivo PS exposure described here. Alternatively, PS exposure can mediate cell signaling. This may be achieved in a direct manner to the cytosol via lipid rafts, as has been shown to occur in differentiating B cells (Dillon et al., 2000). In addition, PS may

give rise to cell signaling in an indirect manner via catalyzing the conversion of prothrombin into thrombin. In myoblasts this could result in a signal for cell survival after the interaction of thrombin with its receptor, which is present at the myoblast surface prior to fusion (Chini et al., 1999; Suidan et al., 1996).

Taken together, our studies in combination with those of others strongly indicate a physiological role of cell surface exposure of PS in myoblast differentiation.

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