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A novel time resolved fluorometric assay of anoikis using Europium-labelled Annexin V in cultured adherent cells

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Background: Adherent cells undergo apoptosis when detached from their home ground, a process called anoikis (homelessness).

***Methods*: We developed a new and sensitive method to analyse apoptosis and anoikis of adherent cell types using a time resolved fluorometric assay with Europium-labelled Annexin V. Anoikis was induced with tumor necrosis factor-α/cycloheximide and three cell fractions of the cell cultures were prepared and analysed. Fraction 1 consisted of adherent cells, analysed while growing on their support (without detachment by trypsinisation). Fraction 2 contained detached cells due to anoikis (floating cells) and fraction 3 contained apoptotic bodies. Both fractions 2 and 3 were present in the culture medium and were isolated by differential centrifugation.**

***Results*: TNF-α treatment of three different types of adherent cell cultures induced a significant increase of the amount of floating cells (anoikis) and apoptotic bodies compared to control cell cultures. Also in the adherent cell fractions a small amount of apoptosis was observed.**

***Conclusions*: The novel time resolved assay provides the ability to analyse the cell death cascade in adherent cell cultures of the same sample at the same time in a sensitive and reproducible way.**

Keywords: adherent cells; Annexin V; anoikis; apoptosis; Europium; time resolved fluorometric assay.

Introduction

Adherent cells are dependent for survival on continuous engagement of cellular integrins to the extra cellular matrix.[1,2] Detachment of adherent cells from the extra cellular matrix induces almost immediately apoptosis, a phenomenon designated 'anoikis' or homelessness.[3,4] Anoikis is of relevance in the physiological development of tissues.[5,6] Without anoikis, detached cells could possibly reattach to distantly localised matrices, resume growth and contribute to the metastatic growth of cancers.[5,7,8] Apoptosis occurring in detached cells would abrogate this mechanism and in this way provide a stringent control of appropriate cell number and tissue organisation. Therefore anoikis is a form of apoptosis with pertinent relevance to tissue homeostasis. On the other hand activation of death receptors in adherent cells will cause detachment of those cells from their support inducing anoikis.[5] To measure anoikis in adherent cells, it is therefore of utmost importance that the viable cells are not disrupted from their location and cells detached from their support should be taken into consideration.[9,10] Several techniques can be used to identify different stages of the apoptotic pathway of cells *in vitro*.[9,11] At present analyses of a wide choice of parameters are used ranging from simple cell sizing to measuring of cell membrane properties, cytoplasmic constituents, cell organelles, DNA content and nuclear chromatin.[11] Most of the techniques for reliable quantitative measurement of apoptosis are excellent for the analysis of apoptosis of cells in suspension but are not suitable for adherent cells because of the occurrence of anoikis due to detachment of the cells.[12] Annexin V binding is extensively used to analyse apoptosis.[13] This is based on the high, Ca^{2+}-dependent affinity of Annexin V to phosphatidylserine (PS). Cells under viable conditions maintain phospholipid asymmetry over the two leaflets of the plasma membrane with PS mainly located at the inner leaflet. During apoptosis (or necrosis) PS is translocated to the outer leaflet of the plasma membrane and can then be recognised by Annexin V binding. Fluorescently labelled Annexin V has been used to analyse apoptosis by means of flow cytometry or microscopy.[11] For performing flow cytometry on adherent cells, cells should first be detached from the support. This procedure of detaching the cells induces apoptosis itself.[10] Therefore, flow cytometry can

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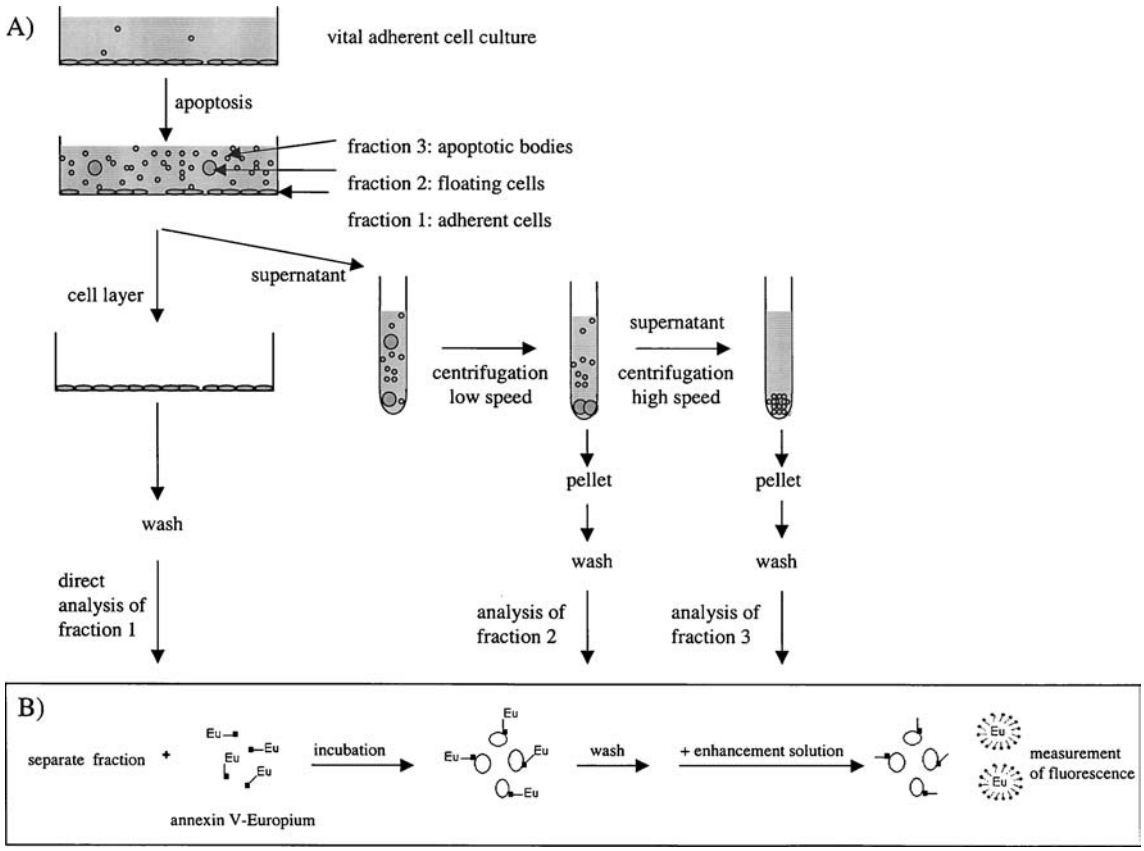
Figure 1. Work flow diagram of the different steps involved in the Europium-labelled Annexin V time resolved fluorometric assay to analyse the cell death cascade. (A) Three fractions of the adherent cell cultures were prepared. Fraction 1 consisted of adherent cells, analysed while growing on their support (without detachment by trypsinisation). Fraction 2 contained detached cells due to anoikis (floating cells) and fraction 3 contained apoptotic bodies. Both fractions 2 and 3 were present in the culture medium and were isolated by differential centrifugation. (B) Apoptosis was then analysed in the three separate fractions by using the DELFIA® principle (Wallac Oy, Turku, Finland). DELFIA® assays utilise a lanthanide (Europium) chelate label which becomes highly fluorescent after forming a new lanthanide chelate within a protective micelle in specially developed enhancement solution (Wallac Oy, Turku, Finland).



never be an optimal quantitative technique to measure apoptosis in adherent cells.[9,10]

We developed a new very sensitive method to analyse anoikis in adherent cell cultures using the principles of the Dissociation Enhanced Lanthanide Fluoro Immuno Assay (DELFIA®,[14] Wallac Oy, Turku, Finland). DELFIA® assays utilise a lanthanide metal (Europium) chelate label which is practically non-fluorescent. However, after binding of Europium-labelled Annexin V to PS, Europium is efficiently dissociated from the labelled compound by the low pH of the commercial available enhancement solution (Wallac Oy, Turku, Finland). The free Europium ion then rapidly forms a new, highly fluorescent and stable chelate with the components of this enhancement solution. The principles of the DELFIA® method are represented schematically in Figure 1b.[15–17] By using this novel time resolved fluorometric assay using Europium-labelled Annexin V, the occurrence of apoptosis was measured in three different cell fractions derived from adherent cell cultures as represented schematically in Figure 1a. The occurrence of apoptosis was analysed in cells which were still attached to the culture surface, anoikis was measured in detached cells (floating cells) and the final stage of the apoptotic pathway was investigated by analysing apoptotic bodies.[18] Because by differential centrifugation we are able to separate detached cells and apoptotic bodies from the cell debris which is present in the culture medium due to necrotic cell death, necrosis is excluded from the analyses.

Results of our new test were compared with results of the standard DNA fragmentation assay according to Nicoletti[19] and with results of flow cytometric measurements of apoptotic bodies.[20] With the very sensitive time resolved fluorometric Annexin V assay we could analyse the processes of apoptosis and anoikis in more detail compared to existing assays.

Materials

Dulbecco's Vogt Modified Eagle's minimal essential Medium (DMEM) was purchased from Gibco BRL (Breda, The Netherlands). Penicillin, streptomycin, fetal bovine serum, trypsin/ethylene diamine tetra-acetic acid (EDTA) and RPMI-1640 medium were purchased from Biowhittaker (Verviers, Belgium). Camptothecin (CPT), tumour necrosis factor (TNF)-α, gelatin type B from bovine skin, cycloheximide (CHX), and propidium iodide (PI) were from Sigma (St. Louis, Missouri, USA). N-[2-hydroxyethyl] piperazine-N′-[2-ethane sulfonic acid] (HEPES) was from Brunschwig (Amsterdam, The Netherlands). Medium 199 (M199) (with 20 mM HEPES) and L-Glutamine were from Life Technologies (Grand Island, New York, USA). Fetal bovine serum was heat-inactivated (30 min, 56°C) before use. Human serum was acquired by overnight incubation of whole blood (collected from 16 healthy volunteers) at 4°C. Serum was obtained by centrifugation and subsequently pooled and stored at −80°C. Before use, serum was heat inactivated (30 min, 56°C) and filter-sterilised. Europium-label-reagent and enhancement solution were from Amersham Pharmacia Biotech (Woerden, The Netherlands). FlowCount™ Fluorospheres were from Beckman-Coulter (Mijdrecht, The Netherlands). Other buffer components and salts (NaCl, KCl, $MgCl_2$, $CaCl_2 2H_2O$, $NaHCO_3$) and solutions of May-Grünwald and Giemsa were obtained from Merck (Darmstadt, Germany). Annexin V and fluorescein isothiocyanate-(FITC)-labelled Annexin V were provided by Dr. Reutelingsperger (University of Maastricht, The Netherlands). HMEC cultures were donated by Dr. P. Koolwijk of the Netherlands Organisation for Applied Scientific Research (TNO-PG, Leiden, The Netherlands) in co-operation with E. W. Ades of Centers for Disease Control and Prevention (CDC, Atlanta, USA) and T. J. Lawley of Emory University (Atlanta, USA).

Methods

Culturing of human micro-vascular endothelial cells (HMECs)

HMECs (gift from Dr. P. Koolwijk, TNO, Leiden, The Netherlands) were cultured in 1% (w/v) gelatin-coated culture flasks at a seeding density of 40,000 cells/cm^2. Culture medium consisted of M199 medium supplemented with 10% (v/v) pooled human serum, 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Medium was refreshed every 2–3 days. Confluent cultures were subcultured after detachment using a trypsin solution (0.05% trypsin/0.02% EDTA in PBS).

Culturing of human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated from human umbilical veins, according to the method of Jaffe *et al*.[21] Umbilical cords were obtained from women terminating normal pregnancies. The procedure followed was in accordance with the policies of the Institutional ethical review board of the Hospital Group (ECOM). Cells were cultured in 1% (w/v) gelatin-coated culture flasks at a seeding density of 40,000 cells/cm^2. Culture medium consisted of 50% M199 medium and 50% RPMI-1640 medium, supplemented with 20% (v/v) pooled human serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. Medium was refreshed every 2-3 days. Confluent cultures were subcultured (to a maximum of 5 passages) after detachment using a trypsin solution (0.05% trypsin/0.02% EDTA in PBS).

Culturing of human umbilical vein smooth muscle cells (SMCs)

SMCs were isolated from human umbilical veins according to the method of Heimli *et al*.[22] with some minor modifications as described previously.[23] Cells were cultured in 1% (w/v) gelatin-coated culture flasks at a seeding density of 40,000 cells/cm^2. Culture medium consisted of DMEM supplemented with 10% (v/v) pooled human serum, 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.[22] Medium was refreshed every 2–3 days. Confluent cultures were subcultured (to a maximum of 12 passages) after detachment using a trypsin solution (0.125% trypsin/0.05% EDTA in PBS).[24,25]

Modulation of apoptosis/anoikis

All cell cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. Cells were grown to 80% confluence and subsequently either cultured in their standard culture medium (control cells) or treated for 16 h with 3 nM TNF-α and 50 μM CHX in standard culture medium (TNF-α/CHX) to induce apoptosis/anoikis.[26]

Labelling Annexin V with Europium

Annexin V was first transferred in carbonate buffer (50 mM $NaHCO_3$/150 mM NaCl, pH 8.5) by gel filtration using a Sephadex® G-25 m PD-10 Column (Amersham Pharmacia Biotech, Woerden, The Netherlands). Europium-label-reagens (2 mg/mL) was used to label Annexin V with Europium during 20 h at RT. The obtained Eu^{3+}-labelled Annexin V was

purified by gel filtration using a Sephadex® G-25 m PD-10 Column in carbonate buffer (50 mM $NaHCO_3$/150 mM NaCl, pH 8.5). The final fraction was diluted with glycerol (1:1) to a final concentration of 0.4 g/L Europium-labelled Annexin V.

Europium-labelled Annexin V time resolved fluorometric assay to measure apoptosis

For this time resolved fluorometric assay, three fractions of the adherent cell cultures grown in 96 wells plates (Corning, Badhoevedorp, The Netherlands) were prepared and analysed as represented schematically in Figure 1. Fraction 1 consisted of viable adherent cells and these cells were analysed while growing on their support (without detachment by trypsinisation). Supernatants of the adherent cell cultures were collected and two other fractions were isolated: floating cells and apoptotic bodies. After centrifugation of the supernatants (low speed, 1,000 g, 3 min, room temperature (RT)), apoptotic cells detached from their support were obtained from the pellets (fraction 2, floating cells). Supernatants of these centrifuged samples were collected and centrifuged (high speed, 3,500 g, 15 min, RT). Apoptotic bodies derived from the apoptotic cells as a result of a final stage of apoptosis were then obtained from the pellets (fraction 3). To analyse apoptosis, the three fractions of the cell cultures (adherent cells, floating cells and apoptotic bodies) were separately washed with a solution of 10 mM HEPES, supplemented with 137 mM NaCl, 2.68 mM KCl, 1.7 mM $MgCl_2$, 25 mM glucose and 2.5 mM $CaCl_2 \cdot 2H_2O$ and pH 7.4 (HEPES buffer). Adherent cells (fraction 1) were then incubated with Europium-labelled Annexin V (final concentration of 0.4 mg/l in HEPES buffer for 30 min at RT). Pellets of floating cells (fraction 2) and apoptotic bodies (fraction 3) were resuspended in Europium-labelled Annexin V (final concentration of 0.4 mg/l) in HEPES buffer for 30 min at RT. After washing with HEPES buffer, the three fractions were incubated with (commercial available) enhancement solution.[15] for 5 min at RT to convert the Europium label into the highly fluorescent chelate. Time resolved fluorescence was measured in each sample on a Viktor fluorescence analyser (PerkinElmer Life Sciences, Turku, Finland). Excitation and emission wavelengths were 340 and 615 nm respectively. Fluorescence was normalised against control cell cultures.

DNA fragmentation assay to measure apoptosis

The occurrence of apoptosis in the adherent and floating cell fractions together was measured by flow cytometry using PI according to Nicoletti *et al*.[19] After culturing, cells already present in the culture medium (floating cells) were collected and subsequently adherent cells were detached from the support using a 0.125% trypsin/0.05% EDTA solution. The two fractions were combined, washed with PBS, fixed in 70% ethanol and after another wash stained with 15 μM PI for 30 min at 37°C.[11] PI fluorescence of individual cells was measured with a Coulter Epics XL flow cytometer, using System II TM software with the XL-2 or DOS configuration. Excitation was elicited at 488 nm with the Argon laser and emission was measured using the long pass (>570 nm) filters. In each sample 10,000 events were measured and data were analysed with Coulter program Expo II. Percentages of PI positive cells were compared to the total amount of cells and normalised against control cells which reflects the amount of apoptosis in the two cell fractions (adherent and floating cell fraction) together.

Flow cytometric measurements of apoptotic bodies

After culturing, 200 μL aliquots of culture medium/supernatant were centrifuged (1,000 g, 3 min, RT). 100 μL aliquots of supernatants were stained for 30 min (in the dark) with FITC-labelled Annexin V (final concentration of 12.8 μg/mL) in HEPES buffer. 40,000 Flow-Count^{TM} Fluorospheres (with a nominal diameter of 10 μm) were then added as internal standard and the amount of apoptotic bodies were analysed on a Coulter Epics XL flow cytometer, using System II TM software with the XL-2 or DOS configuration. Excitation was elicited at 488 nm with the Argon laser and emission was measured using the standard band pass (530 $\pm$ 20 nm) filters. In each sample a fixed number of 2,000 Flow-Count^{TM} Fluorospheres were measured and the ratio of the amount of Annexin V positive apoptotic bodies was calculated based on the number of added Flow-Count^{TM} Fluorospheres.[20] Analyses were done with Coulter program Expo II. Ratios were normalised against control cells.

Morphology

After cells were cultured to 80% of confluence, anoikis was induced by TNF-α/CHX. Adherent cells still present on their culturing support and apoptotic cells detached from their support (floating cells) were analysed. An aliquot of the culture medium containing floating cells was centrifuged on glass slides at 700 rpm for 10 minutes with low acceleration using a Shandron cyto centrifuge (Shandron, Pittsburg, USA). Adherent cells still present on their culturing support and floating cells collected on the glass slides were fixed with methanol, stained with May-Grünwald-Giemsa and examined for their

morphology. Confluency was quantified by counting cells based on light microscopy.

Statistical analyses

All data represent mean ± standard error of the mean (SEM) of several experiments performed in duplicate. Data were analysed using Student's T-test and considered significantly different at p-values <0.05.

Results

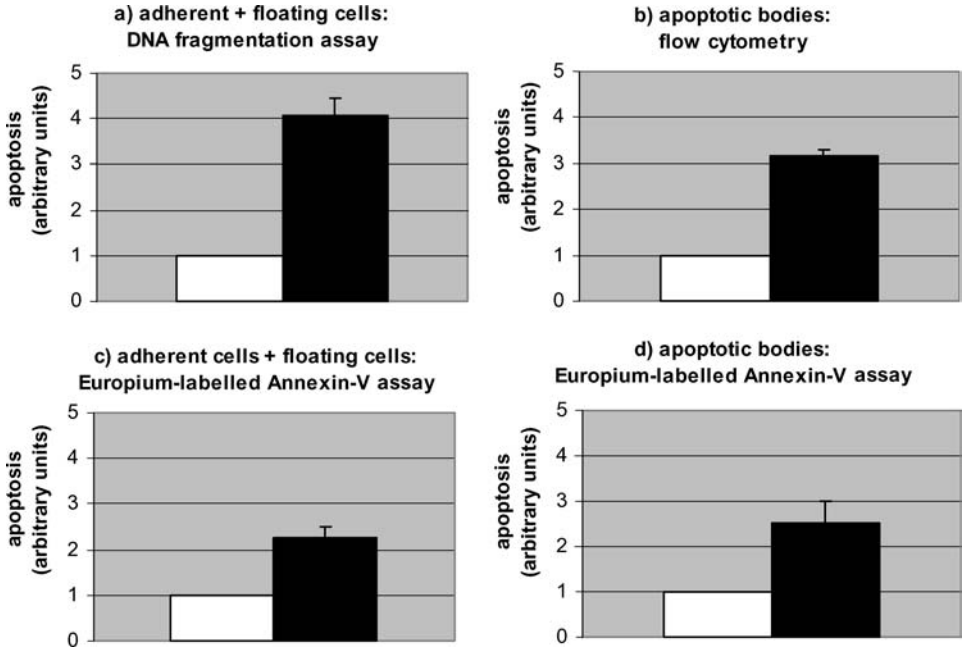
Optimisation and validation of the technique

The variables of the technique as described under Methods were first optimised. Optimal parameters for time of centrifugation, time of labelling with Europium-labelled Annexin V and concentration of the Europium-labelled Annexin V were 15 min, 30 min, and 0.4 mg/L respectively. To evaluate the reproducibility of the technique, coefficients of variation (CVs) for intra- and inter-assay precisions were determined for viable and apoptotic HMEC-cultures ($n = 10$). The CVs for intra- as well as inter-assay precisions for both viable and apoptotic cell-cultures were less than 10%. Linearity and sensitivity of the technique were evaluated by measuring PS exposure in serial dilutions of floating cells derived from control HMEC cultures. The sensitivity limit of the method was ten thousand cells per measurement and the method showed a linear response between 10,000 and at least 75,000 cells per measurement with a coefficient of correlation of 0.95.

Apoptosis of HMEC cultures determined by three different techniques

HMECs were cultured in control medium or cultured in medium supplemented with TNF-α/CHX to induce apoptosis/anoikis. Two standard methods to analyse apoptosis were used as reference tests. The traditional DNA fragmentation assay for measurement of apoptosis (Figure 2a) does not allow separately testing of apoptosis in floating cells present in the supernatant and in cells detached by trypsinisation. With use of the here described Europium-labelled Annexin V fluorometric assay it is

Figure 2. Apoptosis/anoikis of adherent HMEC cultures determined by three different techniques. HMECs were cultured and treated with TNF-α/CHX in culture medium. Apoptosis was then analysed by three different techniques as described in the methods. Apoptosis induced by TNF-α/CHX (closed bars) was normalised against apoptosis found in control cell cultures (open bars). (A) Apoptosis in HMEC cultures determined by a DNA fragmentation assay. Floating cells in the supernatant of the cell cultures and cells detached from the support by trypsinisation were analysed together. (B) Amount of apoptotic bodies present in culture medium of HMEC cultures analysed by FITC-fluorescence with use of flow cytometry. (C) Time-resolved fluorescence of Europium-labelled Annexin V of the adherent cell fraction of HMEC cultures (fraction 1) + floating cells derived from the HMEC cultures (fraction 2) determined by the time resolved fluorometric assay. Floating cells in the supernatant of the cell cultures and cells still attached to the support were analysed together to compare the results with the DNA fragmentation assay. (D) Time-resolved fluorescence of Europium-labelled Annexin V of apoptotic bodies derived from the HMEC cultures (fraction 3) determined by the time resolved fluorometric assay. SEM values are indicated by bars.



possible to analyse these fractions separately (fraction 1 and 2). However, to be able to compare the DNA fragmentation test with the time resolved fluorometric assay, floating cells in the supernatant of the cell cultures and cells still attached to the support were analysed together in this experiment (Figure 2c). With use of flow cytometry based on FITC-labelled Annexin V binding as well as particle size the amount of apoptotic bodies derived from the cell cultures was analysed (Figure 2b) and results of the Europium-labelled Annexin V fluorometric assay analysing apoptotic bodies (Figure 2d) are compared with results of this assay. As can be seen from Figure 2, results of the three different methods show all that TNF-α/CHX induces apoptosis in HMEC cultures compared to control. An advantage of our new time resolved fluorometric assay is the possibility to analyse adherent and floating cells as well as apoptotic bodies in one single assay.

Apoptosis of three different adherent cell cultures determined by the Europium-labelled Annexin V time resolved fluorometric assay

In control cell cultures of HMEC, HUVEC and SMC, without apoptotic inducers, high fluorescence intensity was found in the adherent cell fractions and no (significant) increase was found after treatment with TNF-α/CHX of the three cell cultures (Figure 3A–C respectively). However, in this figure, the fluorescence intensity was not correlated to the amount of cells measured. When the fluorescence intensity was correlated to the number of cells measured, TNF-α/CHX induced a significant increase of apoptotic cells in the adherent cell fraction of all cell types analysed (Figure 4). TNF-α/CHX treatment of these three different adherent cell cultures induced a significant increase of the amount of floating cells and apoptotic bodies compared to control cultures (Figure 3). The same responses were observed when 0.15 μM camptothecin supplemented to the culture medium or culture medium without serum (serum starvation) were used to induce anoikis (results are not shown).

Morphology

From morphological analyses it can be seen that TNF-α/CHX induces anoikis of HMECs, HUVECs and SMCs. Control cell cultures were approximately 80% confluent (for the HMEC culture see Figure 5A) whereas after treatment with TNF-α/CHX, confluencies of approximately 22% of HMECs (Figure 5B), 28% of HUVECs, and 15% of SMCs were observed. No morphological abnormalities were observed in the attached cells of both the control cell cultures and the TNF-α/CHX treated cell cultures (Figure 5C). When floating cells present in the culture medium were analysed, apoptotic cells were observed (Figure 5D). These apoptotic cells showed apoptotic body formation. Free apoptotic bodies could not be detected by light microscopy.

Figure 3. Cell death measured in three different adherent cell cultures by the Europium-labelled Annexin V time resolved fluorometric assay. Cells were cultured and treated with TNF-α/CHX in culture medium. Apoptosis was then analysed by analysing the phosphatidyl serine exposure at the outer plasma membrane with Europium-labelled Annexin V as described in the methods. Apoptosis induced by TNF-α/CHX (closed bars) was normalised against apoptosis found in control cell cultures (open bars). Three fractions of the cell cultures were analysed (adherent cells, floating cells and apoptotic bodies). Fluorescence of Europium-labelled Annexin V of these three fractions of (A) HMEC cultures (B) HUVEC cultures and (C) SMC cultures were separately analysed. Numbers in the diagrams show the percentages of confluency of the adherent cell fractions determined by microscopy and SEM values are indicated by bars.

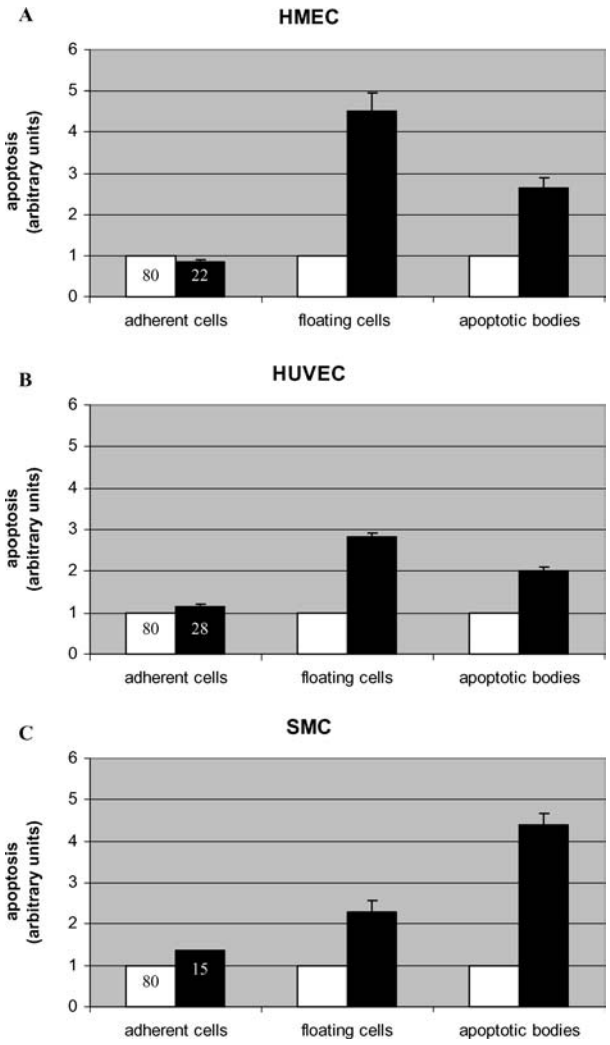
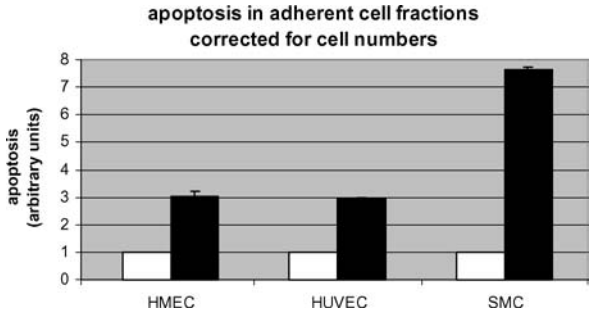
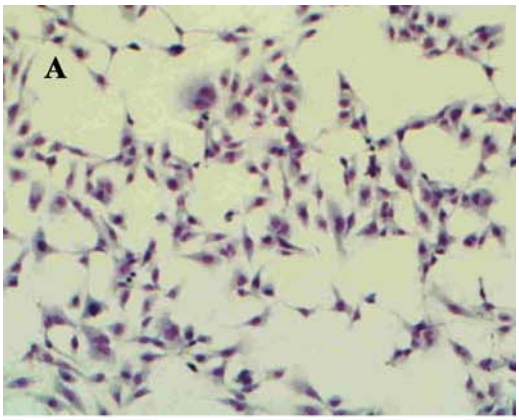


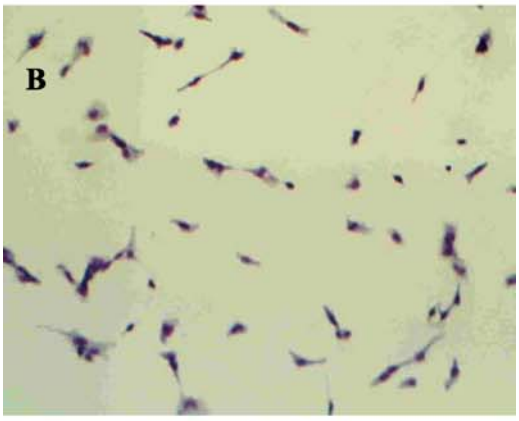
Figure 4. Fluorescence intensity of Europium-labelled Annexin V of the adherent cell fractions of HMECs, HUVECs and SMCs corrected for the cell numbers measured. Cells were cultured and treated with TNF-α/CHX in culture medium. Apoptosis was then analysed by analysing the phosphatidyl serine exposure at the outer plasma membrane with Europium-labelled Annexin V as described in the methods. Apoptosis induced by TNF-α/CHX (closed bars) was normalised against apoptosis found in control cell cultures (open bars). Fluorescence of Europium-labelled Annexin V of the adherent cell fractions of HMEC cultures, HUVEC cultures and SMC cultures was corrected for the cell numbers measured. SEM values are indicated by bars.

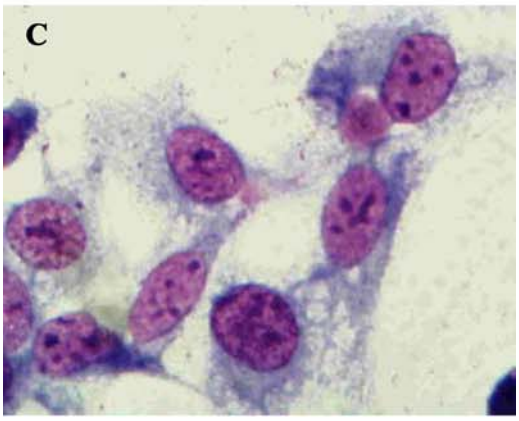


Discussion

DELFIA® assays have been employed in routine laboratory techniques[14] but have never been used to measure apoptosis or anoikis. This technique conveys three major benefits: 1st) the Stokes shift is large (almost 300 nm), 2nd) the emission peak is very sharp and 3rd) the Europium chelate fluorescence has a very long decay time compared to that of conventional fluorochromes. This significantly reduces the background signal and gives greater resolution and sensitivity. We described here a new method to analyse apoptosis/anoikis in adherent cell cultures using Europium-labelled Annexin V in a DELFIA® system. By analysing apoptosis in three different cell fractions, adherent cells, floating cells and apoptotic bodies, the occurrence of apoptosis/anoikis could be measured directly without manipulation of the cell cultures like trypsinisation. Based on the CVs for intra- and inter-assay precisions this assay proved to be reproducible and sensitive to study apoptotic cell death in different adherent cell







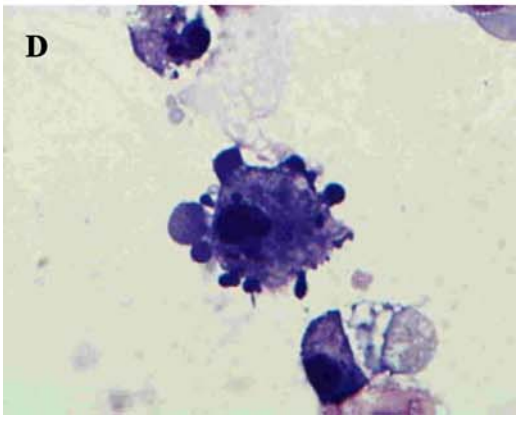


Figure 5. Morphology of HMEC cultures. Cells were cultured to 80% of confluence and treated with TNF-α/CHX in culture medium. May-Grünwald-Giemsa staining of the cells was performed to analyse morphology of the isolated three fractions of the cell cultures (adherent cells, floating cells and apoptotic bodies). (A) Control HMECs cultured in standard culture medium on gelatin-coated culture flasks (magnification: 100×). (B) HMECs of a culture treated with TNF-α/CHX, still present on the surface of gelatin-coated culture flasks (magnification: 100×). (C) HMECs of a culture treated with TNF-α/CHX, still present on the surface of gelatin-coated culture flasks (magnification: 1000×). (D) Apoptotic HMECs which were detached from their support as a result of an early stage of apoptosis (fraction 2) and centrifuged on glass slides (magnification: 1000×).

types. The assay showed a linear response between 10,000 and at least 75,000 cells per measurement. The assay can easily be performed, even in 96 wells culture dishes, in one run eliminating the inter assay variation. The most important advantage is that different stages of the apoptotic cascade can be analysed in one single assay. This is in contrast with standard flow cytometry analysing DNA fragmentation and measuring the amount of apoptotic bodies. The DNA fragmentation assay is not sensitive enough to distinguish between adherent cells and floating cells. On the other hand when the amount of apoptotic bodies is analysed by flow cytometry one cannot distinguish floating cells and apoptotic bodies. Results of both methods are comparable to results of our new Europium-labelled Annexin V time resolved fluorometric assay (Figure 2). However apoptosis-induced increments in both fractions are different due to the fact that different parameters are analysed with use of different techniques. DNA fragmentation and forming of apoptotic bodies are both late events in the apoptotic cascade compared to membrane changes measured by Annexin V binding.[11] With use of this novel time resolved fluorometric assay, double labelling using propidium iodide[11] to exclude necrosis from the analyses, can't be performed. This may be considered as a disadvantage. However, by analysing the three different cell fractions derived from adherent cell cultures as described in this paper, necrosis is excluded from the analyses.

Our study demonstrates that apoptotic stimuli significantly increase the number of floating cells and apoptotic bodies present in the culture medium, which is a direct proof for the anoikis principle of adherent cells.[2] (Figure 3). We did not find an increase of Europium fluorescence of the adherent cell fraction, which not necessarily implies that apoptosis is not induced in this fraction by TNF-α/CHX. We found a remarkable decrease of confluency in all three cell types induced by TNF-α/CHX (a decrease of 58% for HMECs, 52% for HUVECs and 65% for SMCs). Accordingly, the fluorescence intensity of Europium-labelled Annexin V of TNF-α/CHX treated cells compared to control cells was increased with a factor of 3 for HMECs and HUVECs and with a factor of 7.5 for SMCs when correlated to the number of cells analysed. Based on this observation we can speculate that we also showed TNF-α/CHX induced apoptosis in the adherent cell fractions (Figures 4 and 5). The fact that the adherent cell fraction can be studied directly in the culture dish without detachment using e.g. trypsin eliminates the possibility of inducing apoptosis by artefacts.[9,10] The relative increases of the amount of apoptotic adherent cells, floating cells and apoptotic bodies induced by TNF-α/CHX (Figures 3 and 4), indicating anoikis, were not the same in the three different adherent cell types used. These findings confirm our previous observations that cell death kinetics depend on the type of the cell.[27] Further study is needed in order to describe these differences into detail and to reveal the exact mechanism.

Conclusion

According to our knowledge this is the first direct quantitative technique to measure anoikis in adherent cell cultures. Our assay provides the ability to analyse anoikis and further steps of the apoptotic cascade of the same sample at the same time. Apoptotic stimuli increase the number of apoptotic floating cells and apoptotic bodies which is a direct proof for the anoikis principle of adherent cells.[2]

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