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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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Introduction

Adherent cells are dependent for survival from continuous engagement of cellular integrins to the extra cellular matrix. Detachment of adherent cells from the matrix induces almost immediately apoptosis, a phenomenon designated as 'anoikis' or homelessness (1). Activation of death receptors in adherent cells will cause detachment of those cells from their support inducing anoikis. Anoikis is of pertinent relevance to tissue homeostasis (2). Annexin V binding is extensively used to analyse apoptosis. During apoptosis (or necrosis) phosphatidyl serine is translocated from the inner to the outer leaflet of the plasma membrane and is recognised by Annexin V binding (3).

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®, Wallac Oy, Turku, Finland) (4). By using Europium-labelled Annexin V, the occurrence of apoptosis was measured in three different cell fractions derived from adherent cell cultures. 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.

Methods

Three different human adherent cell types microvascular endothelial cells (HMEC), umbilical vein endothelial cells (HUVEC) and umbilical vein smooth muscle cells (SMC) were cultured 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 to induce apoptosis/anoikis.

For this assay, three fractions of the adherent cell cultures were prepared and analysed. Fraction 1 consisted of viable adherent cells and these cells were analysed while growing on their support (without detachment by trypsinisation). Culture media of the adherent cell cultures were collected and two other fractions were isolated by differential centrifugation. Fraction 2 contained detached cells due to anoikis (floating cells) and fraction 3 contained apoptotic bodies. To analyse apoptosis, the three fractions of the cell cultures were separately washed and incubated with Europium-labelled Annexin V. Time resolved fluorescence was measured in each sample. Fluorescence was normalised against control cell cultures. Adherent and floating cells were stained with May-Grünwald-Giemsa and examined for their morphology by microscopy.

Results

In control cell cultures of HMEC, HUVEC and SMC, without apoptotic inducers, high fluorescence intensity was found in the adherent cell fractions and no increase was found after treatment with TNF-α/CHX of the three cell cultures (figure 1A, B and C respectively). However, in this figure, the fluorescence intensity was not correlated to the amount of cells measured. After TNF-α/CHX treatment, a decrease of confluency was observed (figure 1E and F). 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 1D). TNF-α/CHX treatment induced a significant increase of the amount of floating cells and apoptotic bodies compared to control cultures.

Discussion

A new method to analyse apoptosis/anoikis in adherent cell cultures using Europium-labelled Annexin V in a DELFIA" system is described. 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. 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 methods using flowcytometry. An additional advantage is that by analysing the three different cell fractions derived from adherent cell cultures as described in this paper, necrosis is excluded from the analyses.

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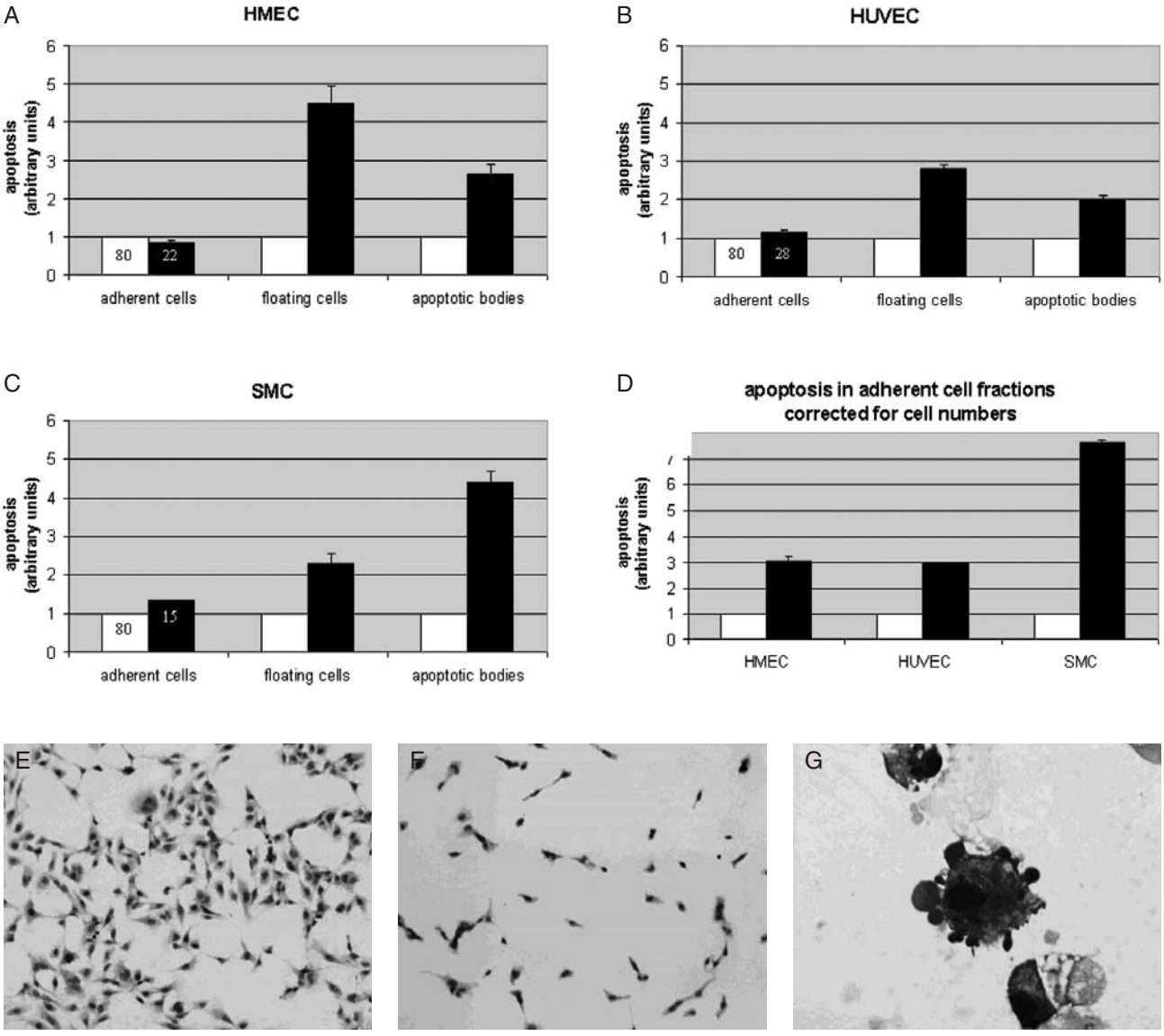

Figure 1. Cell death measured in three different adherent cell cultures. Cells were cultured and treated with TNF-α/CHX in culture medium. Apoptosis was then analysed by Europium-labelled Annexin V as described in Methods. Apoptosis induced by TNF-α/CHX (closed bars) was normalised against apoptosis found in control cell cultures (open bars). Three fractions of the cell cultures were analysed (adherent cells, floating cells and apoptotic bodies). Fluorescence of these three fractions of (A) HMEC (B) HUVEC and (C) SMC cultures were separately analysed. Numbers in the diagrams show the percentages of confluency of the adherent cell fractions determined by microscopy. Fluorescence intensity of Europium-labelled Annexin V of the adherent cell fractions were corrected for the cell numbers measured (D). SEM values are indicated by bars. Cell morphology was analysed by May-Grünwald-Giemsa staining. Shown are adherent HMEC (control cells: E and TNF-α/CHX treated cells: F, magnification: 100X), and floating HMEC presenting apoptotic body formation (G, magnification: 1000X).

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. The fluorescence intensity of Europium-labelled Annexin V of TNF-a/CHX treated cells compared to control cells was increased when correlated to the number of cells analysed. Based on this observation we can speculate that we also showed TNF-a/CHX induced apoptosis in the adherent cell fractions. According to our knowledge this is the first direct quantitative technique to measure anoikis in adherent cell cultures.

References

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