Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 19, issue 1, 2009, pp. 7-13

FLOW CYTOMETRIC ANALYSIS OF CELL DEATH AND VIABILITY OF OSTEOARTHRITIC CHONDROCYTES CULTURED IN PRESENCE OF APOPTOSIS INHIBITORS FOR TISSUE ENGINEERING

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ABSTRACT. In autologous cell implantation (ACI), the autologous chondrocytes recovered from the patient are amplified in tissue culture prior to re-implantation. Recently, we demonstrated that a dual mechanism, apoptosis and replicative senescence, could be responsible for pathogenesis of osteoarthritis diseases. In some systems, inhibition of caspases can prevent apoptosis and may therefore have important therapeutic implications. To confirm this view, chondrocyte apoptosis was induced in vitro with Ca^{2+}, a potential inducer of apoptosis in vivo, either in the presence or absence of the caspase inhibitor, Ac-DEVD-fmk or of leupeptin specific inhibitor of calpains. Cell viability and death were analyzed by flow cytometry. Our results provide that the progression of cartilage lesions is related to an apoptosis phenomenon and suggest the potential efficacy of caspase inhibitors. DEVD-cmk, however, was less efficient to prevent cell death as compared to leupeptin and the mixture of both had complementary effect and prevented all hallmark of apoptosis

Keywords: osteoarthritis, chondrocytes, apoptosis, flow-cytometry, autologous cell implantation, tissue engineering

INTRODUCTION

Tissue engineering has emerged throut a combination of many developments in biology, medicine, material science, engineering and manufacturing and their strategy is the use biologically based mechanisms to achieve the repair and healing of damaged and diseased tissues. Articular cartilage provides its own particular challenges for tissue engineering because it is frequently damaged as a result of trauma and degenerative joint diseases. Cartilage has no blood vessels, is not innervated and normal mechanisms of tissue repair, involving the recruitment of cells to the site of damage, do not occur. The challenge is to produce cartilage tissue with suitable structure and properties ex vivo, which can be implanted into joints to provide a natural repair. In autologous cell implantation (ACI), a currently practiced cell-based therapy to repair cartilage defects, the autologous chondrocytes recovered from the patient are amplified in tissue culture prior to re-implantation. The technique now withwell over a decade of clinical experience has shown encouraging clinical results [Harrison P.E., 2000]. The ability to manipulate and reconstitute tissue structure and function in vitro has tremendous clinical implications and is likely to have a key role in cell therapies in coming years.

Destruction of articular cartilage is an irreversible consequence of arthritis. The causes of osteoarthritis (OA) are multifactorial, and although aging is the most strongly associated risk, mechanical, hormonal, and genetic factors all contribute to varying degrees. OA emerges as a clinical syndrome when these determinants result in sufficient joint damage to cause impairment of function and the appearance of symptoms.

Chondrocyte is an unique cell type in articular cartilage tissue and is essential for cartilage formation and functionality.

Since articular cartilage lacks blood supply and possibly a source of stem cells, chondrocyte viability may be one of the critical limiting factors in the repair response. A number of recent findings have suggested a strong association between apoptosis, age-related diseases and aging [Warner, 1999; Zhang, 2003]. With reference to the relation between apoptosis and cartilage degeneration in osteoarthritis, a number of recent studies have shown that apoptotic cell death takes place at an increased rate in osteoarthritic cartilage and correlate reduced cell density in human OA cartilage with apoptosis [D'Lima et al., 2001; Aigner & Kim, 2002; Aigner et al., 2004; Sharif et al., 2004]. In cartilage there are no macrophages and the fate of apoptotic bodies is uncertain. In those conditions, secondary necrosis would inevitably result with the disadvantage of uncontrolled release of lysosomal enzymes from apoptotic bodies causing serious damages to the extracellular matrix, the products of cell death such as pyrophosphate and precipitated calcium may contribute to pathologic cartilage degradation including calcification [Lotz et al., 1999; Sandell &Aigner, 2000], unless the cell had developed alternative mechanisms of destruction [Roach et al., 2004]. Recently, we demonstrated that a dual mechanism, apoptosis and replicative senescence,

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Article received: october 2008; published: march 2009

could be responsible for pathogenesis of osteoarthritis diseases. Apoptotic phenomenon appears to be consistent and relevant and could contribute to cartilage degeneration [Buia et al., 2008]. The apoptotic pathway has been shown to include the activation of a cascade of cysteine dependent aspartate specific proteases (caspases) which lead to characteristic changes in DNA and other cellular constituents. In some systems, inhibition of caspases can prevent apoptosis, enhance cell survival in vitro and in vivo and may therefore have important therapeutic implications [Rudell, 1999, Bratosin et al., 2001]. Pharmacological inhibitors of these enzymes have been explored as potential therapeutic agents in experimental models of diseases that are associated with increased cell death. In vitro studies of chondrocytes showed that caspase inhibitors can prevent cell death and maintain chondrocytes function (Nutall et al., 2000; D'Lima et al., 2001].

To confirm this view, chondrocyte apoptosis was induced in vitro with Ca2+, a potential inducer of apoptosis in vivo, either in the presence or absence of the caspase inhibitor, Ac-DEVD-fmk, or of leupeptin specific inhibitor of calpain. Cell viability and death were analyzed by flow cytometry, a method ideally adapted for the study of cell death and for rapid and individual analysis of a large number of cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Cambrex Bio Science (Verviers, Belgium), fetal calf serum, penicillin, streptomycin, amphotericin B and L-glutamine were from Gibco (Carlsbad, USA). Hyaluronidase, trypsin, collagenase from *Clostridium histoliticum* and calcein-AM were from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein-conjugated Annexin-V (Annexin-V-FITC), propidium iodide and HEPES buffer were purchased from Pharmingen (San Diego, CA, USA). Ac-Leu-Leu-Arg-CHO (leupeptin) and Ac-DEVD-cmk were from Bachem France (Voisins-le-Bretonneux, France).The flow cytometer was a Becton-Dickinson FACScan apparatus (San Jose, CA, USA) with CellQuest Pro software for acquisition and analysis.

Isolation of chondrocytes

Articular chondrocytes were isolated as described elsewhere [Green W.T., 1971; Kuettner *et al.*, 1982] with a few modifications from intact unaffected zones of the tissue from patients with osteoarthritis undergoing arthroplasty under sterile techniques (CFR 2 Hospital, Bucharest, Romania). All enzymatic solutions were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with a mixture of antibiotics and antimycotics (penicillin 10 U/ml, streptomycin 10 mg/ml, amphotericin B 0.025 mg/ml), with L-glutamine 0.002M and 10% of fetal calf serum. The pieces of cartilage were minced into small pieces and incubated with 0.1% of sheep teste hyaluronidase in DMEM medium for 20 min at 37°C. They were then incubated with 0.1% hyaluronidase in DMEM medium for 20 min at 37°C and maintained in a trypsin solution (0.25 g/100 ml PBS buffer pH 7.4) for 60 min at 37° C. Then the pieces were incubated (overnight, 37°C and 5% CO_2) in 0.2 % collagenase in DMEM medium with 10% fetal calf serum (FCS). The dissociated chondrocytes were centrifuged, rinsed twice and plated at a density of 2–3 x 10^6 cells per 60-mm culture dish in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine 0.002M and containing 10% FCS.

Induction of cell death

Chondrocyte apoptosis was induced by resuspending the chondrocytes in 10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4, in absence or in presence of Ac-DEVD-cmk (200 µM), Ac-Leu-Leu-Arg-CHO (leupeptin) (200 µM) or a mixture of cysteine protease inhibitors Ac-DEVD-cmk and leupeptin (200 µM each) and containing 10% FCS. The chondrocytes were evaluated by flow cytometry after 24 h incubation endpoints.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACScan cytometer using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Morphological changes assessment of chondrocytes by light scattered measurements

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides informations about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the lightwere associated with cell shrinkage.

Cell death assays

Cell death was determined using an annexin-V-FITC/propidium iodide apoptosis kit. Annexin-V is a Ca^{2+}- dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. Cells that stain positive for annexin-V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both annexin-V-FITC and PI are either in the end stage of

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 19, issue 1, 2009, pp. 7-13

apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both annexin-V-FITC and PI are alive and not undergoing measurable apoptosis.

Chondrocytes were washed with PBS buffer pH 7.4 and the cells ($2x10^5$) were resuspended in 100 µl of 1x binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). 10 µl propidium iodide and 5 µl annexin-V-FITC were added and incubated for 30 min at room temperature in the dark. After adding 400 µl of 1x binding buffer, the suspension was analysed in the flow cytometer and gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (PI fluorescence). The light scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. All studies were performed at least three times with three replicates each time.

Cell viability assay using calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin *et al.* [Bratosin *et al.*, 2005]. The membrane-permeable dye calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 µM in PBS buffer pH 7.4. Chondrocytes ($4 x10^5$ in 200 µl PBS buffer) were incubated with 10 µl calcein-AM working solution (final concentration in calcein-AM: 5 µM) for 45 min at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

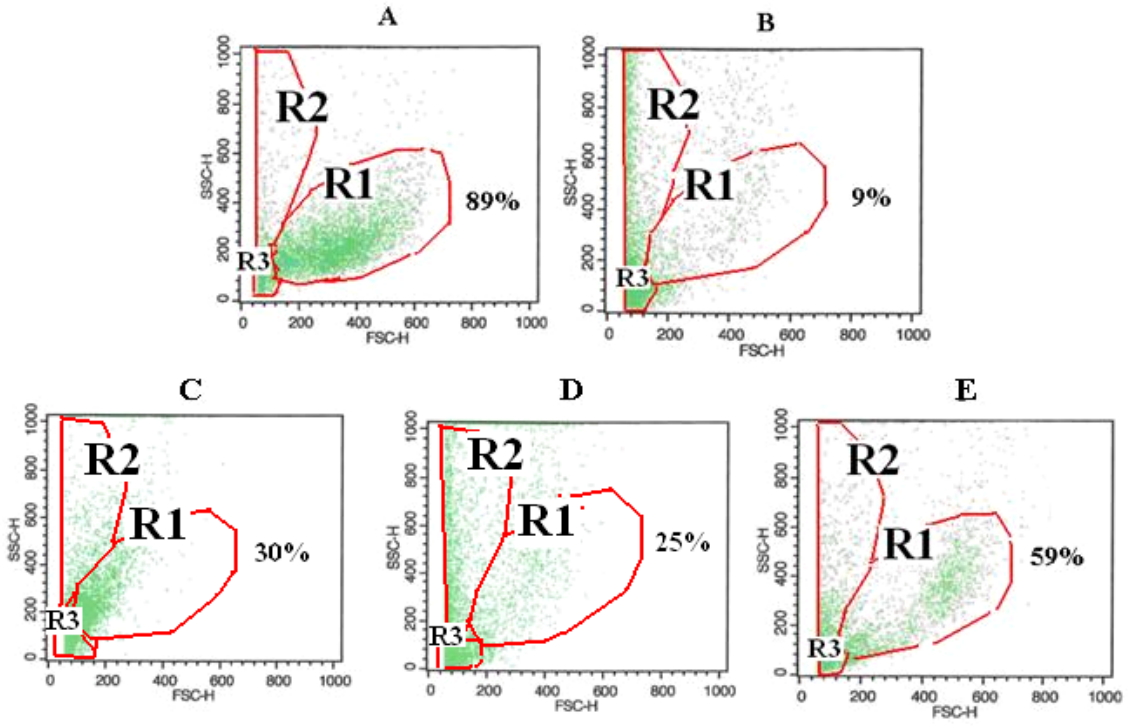

Figure 1. Comparative dot-plot analysis FSC/SSC of morphological changes of human non-osteoarthritic chondrocytes. **(A)** cultured in the presence of Ca^{2+} (2.5 mM) for apoptosis induction, in absence **(B)** or in presence of Ac-DEVD-cmk (200 µM) **(C),** Ac-Leu-Leu-Arg-CHO (leupeptin) (200 µM) **(D)** or a mixture of cysteine protease inhibitors Ac-DEVD-cmk and leupeptin (200 µM each). Dot-plot analysis FSC/SSC of chondrocyte shape changes. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness). R1: viable chondrocytes; R2: apoptotic chondrocytes; R3: cellular remainders of chondrocytes. Number of counted cells: 5,000. Results presented are from one representative experiment of three performed.

RESULTS AND DISCUSSION

Light scattering properties of chondrocytes in osteoarthritis

The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the

forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents [Darzynkiewicz et al., 1997].

Figure 1 shows that the morphological changes of human apoptotic chondrocytes were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of characteristic features of apoptosis. The percentage of cells in region R1 (increased FSC and decreased SSC) of apoptotic chondrocytes (Fig.1B) decreased drastically from 89 % for "normal" chondrocytes (Fig. 1A) to 9 %. Concomitantly, the proportion of the apoptotic chondrocytes (region R2: decreased FSC and increased SSC) increased proportionally. Region R3 is constituted of cell fragments.

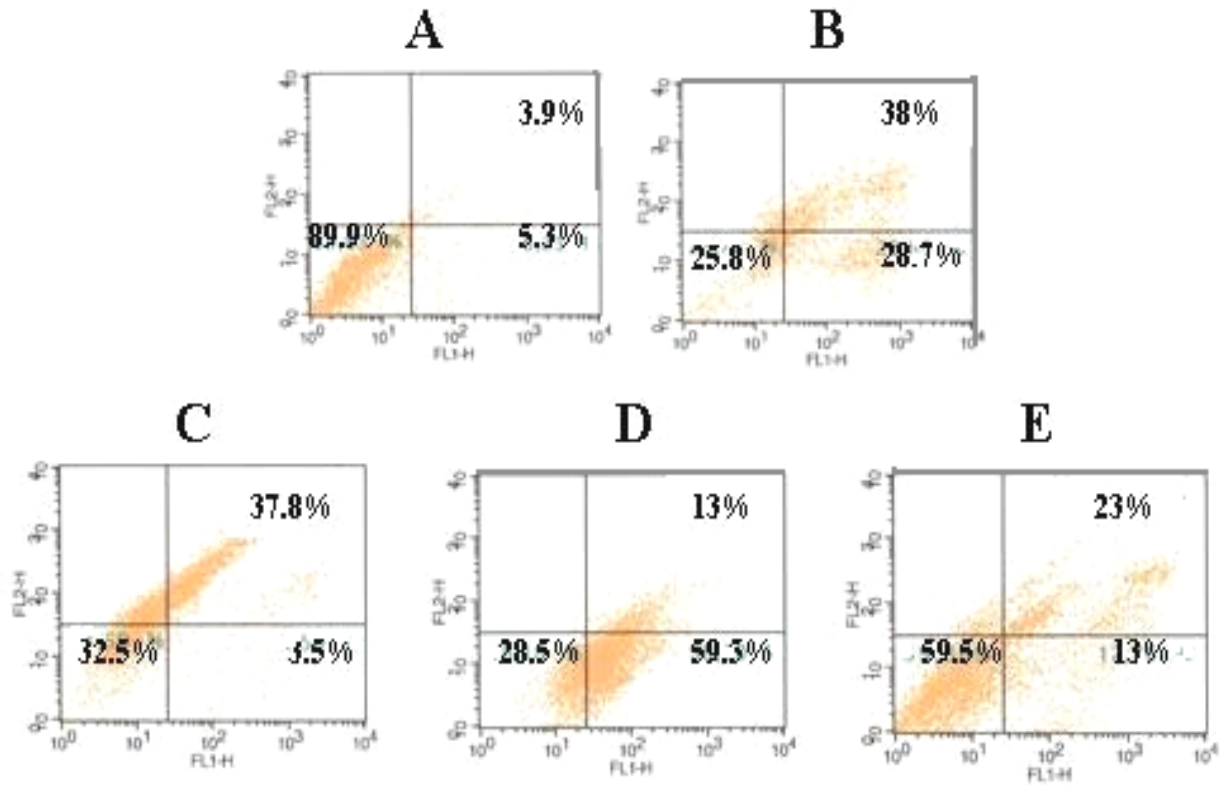

Figure 2. Comparative flow cytometric quadrant analysis of annexin-V-FITC (FL1) and propidium iodide (FL2) double-stained to assess chondrocyte phosphatidylserine exposure and cell membrane integrity, respectively. Human non-osteoarthritic chondrocytes **(A),** chondrocytes cultured in the presence of Ca^{2+} (2.5 mM) for apoptosis induction, in absence **(B)** or in presence of Ac-DEVD-cmk (200 µM) **(C),** Ac-Leu-Leu-Arg-CHO (leupeptin) (200 µM) **(D)** or a mixture of cysteine protease inhibitors Ac-DEVD-cmk and leupeptin (200 µM each). Lower left quadrant: viable cells (annexin V and propidium iodide negative cells); Lower right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); UR quadrant: dead cells (annexin-V and propidium iodide positive cells). Abscissae: annexin-V-FITC fluorescence; ordinates: propidium iodide fluorescence. Number of counted cells: 5,000. Results presented are from one representative experiment of three performed.

The morphological characteristics of chondrocytes cultured in the presence of Ca^{2+} (2.5 mM) for apoptosis induction but in presence of Ac-DEVD-cmk (200 µM) (Fig 1C), Ac-Leu-Leu-Arg-CHO (leupeptin) (200 µM) (Fig. 1D) or a mixture of cysteine protease inhibitors Ac-DEVD-cmk and leupeptin (200 µM each) in the Fig.1E show that inhibitors of cysteine proteinases (caspases and calpain inhibitors) are capable to prevent chondrocytes apoptosis. Here, we found that the addition of cysteine-protease inhibitors, Ac-DEVD-cmk and leupeptin, delayed the death of chondrocytes at the concentration of 200 µM, but with different rates of inhibition. The mixture of both inhibitors (200 µM each) almost strongly prevented the erythrocyte cell death (Fig.1E) and 59% of chondrocytes are in the cytogram, practically the same distribution (R1) to the control sample (Fig. 1A).

Study of death by annexin-V-FITC/ propidium iodide double – labelling

Chondrocytes cultured in absence or in the presence of Ca^{2+} (2.5 mM) for apoptosis induction, or incubated in presence of apoptosis inhibitors were analyzed by flow cytometry for phosphatidylserine (PS) exposure (annexin-V labelling) and membrane permeabilization (propidium iodide labelling). Phosphatidylserine residues are exposed in the external leaflet of cell membrane early during the process of apoptosis whereas the uptake of propidium iodide indicates a disrupted cellular membrane integrity generally

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 19, issue 1, 2009, pp. 7-13

observed during late apoptosis and cell necrosis. Figure 2 shows comparative flow cytometric analyses of annexin-V-FITC / propidium iodide double-stained of chondrocytes.

Comparative analyses of quadrant cytograms of chondrocytes death show that for chondrocytes cultured in the presence of Ca^{2+} (2.5 mM) the number of living cells (annexin$^-$/PI$^-$) decreased drastically from 89.9% (Fig. 2A, normal chondrocytes) to 25.8% respectively (Fig.2B). The proportions of Annexin-V positive and PI negative cells (Annexin$^+$/PI$^-$) were significantly increased (28.7%) for chondrocytes treated contrary to 5.3 % for non-osteoarthritic chondrocytes. The proportions of annexin-V positive and PI positive cells (Annexin$^+$/PI$^+$) indicating late apoptosis or dead cells was approximately 3.9% for "normal" chondrocytes and 38% for Ca^{2+} treated cells. The number of living cells in the samples cultured in the presence of apoptosis inhibitors, is approximately the same for Ac-DEVD-cmk (32.5%) and leupeptin (28.5%)(Fig. 2C and D), and double (59.5%) if the chondrocytes were cultured with a mixture of inhibitors Ac-DEVD-cmk and leupeptin. The number of apoptotic cells or necrotic cells in all samples is respectively reduced.

Cell viability calcein-AM assay of chondrocytes

We recently devised a new flow cytometric assay for the measurement of cell viability using calcein-AM [Bratosin *et al.*, 2005]. The assay is based on the use of acetoxymethyl ester of calcein (calcein-AM), a fluorescein derivative and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes.

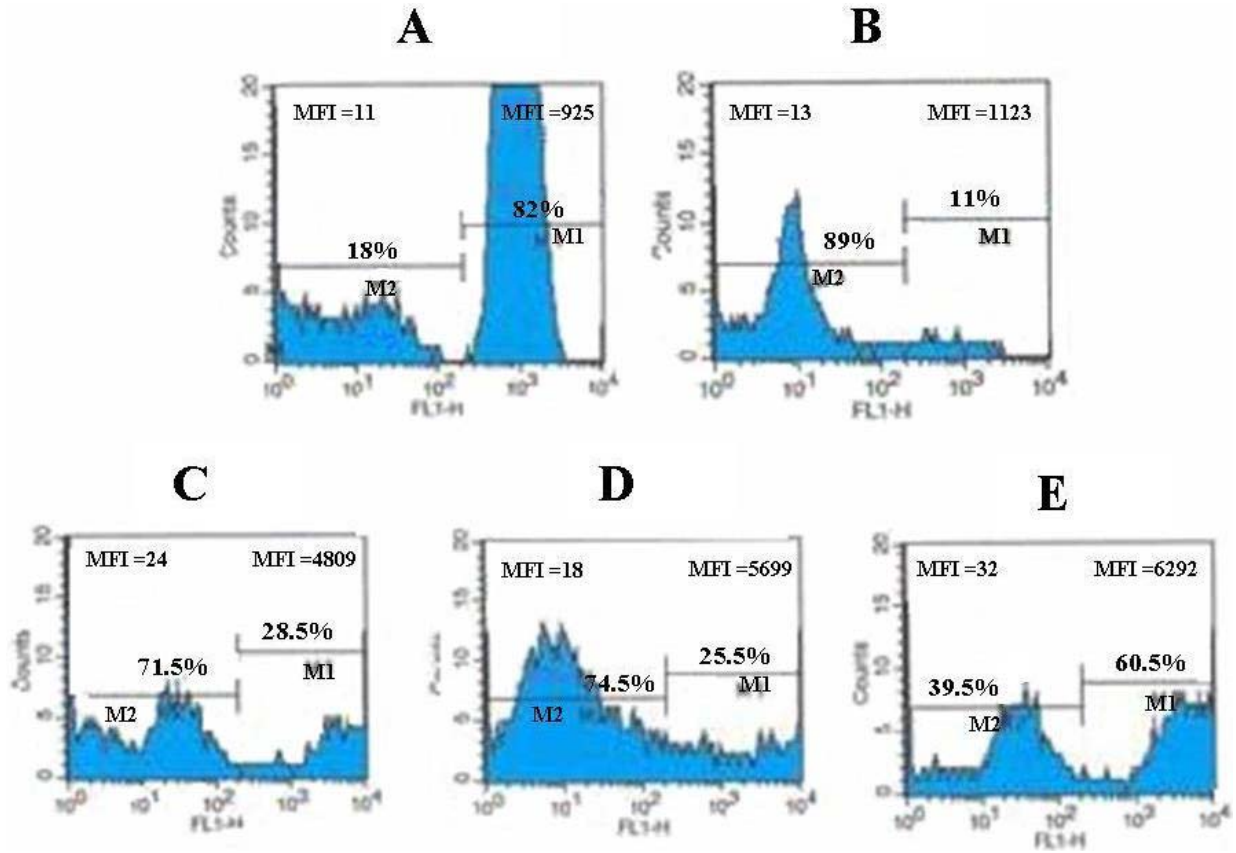

Figure 3. Comparative flow cytometric histogram analysis of chondrocytes viability by cell esterase activity measurement using calcein-AM. Human non-osteoarthritic chondrocytes **(A),** chondrocytes cultured in the presence of Ca^{2+} (2.5 mM) for apoptosis induction, in absence **(B)** or in presence of Ac-DEVD-cmk (200 µM) **(C),** Ac-Leu-Leu-Arg-CHO (leupeptin) (200 µM) **(D)** or a mixture of cysteine protease inhibitors Ac-DEVD-cmk and leupeptin (200 µM each).Abscissae: log scale green fluorescence intensity of calcein (FL1). Ordinates: relative cell number. M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Numbering refers to the cell percentage of each population. Number of counted cells: 5,000. Results presented are from one representative experiment of three performed.

Application of this assay for analysing chondrocytes cultured in absence (Fig. 3A) or in presence of Ca^{2+} (Fig. 3B) showed that two regions could be clearly and unambiguously defined: the region of fluorescent cells with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells (region M1) 82% for non- osteoarthritic chondrocytes (Fig.3A) compared to 11% for chondrocytes cultured in the Ca^{2+} presence (Fig. 3B) and the region M2 of

nonfluorescent dead cells with damaged cell membranes (18% versus 89% respectively). In this regard, it is important to mention that we have previously demonstrated that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure [Bratosin *et al.*, 2005].

The histograms of Figures 3 C, D and E show that calcein fluorescence of chondrocytes cultured in presence of Ac-DEVD-cmk (Fig. 3C), leupeptin (Fig. 3D) or a mixture of cysteine protease inhibitors (Fig.3E) a good correlation with inhibitors activity. The number of viable cells improve to 28.5% and 25.5% for chondrocytes cultured with Ac-DEVD-cmk or leupeptin only, and to 60.5% for the cells cultured in presence of a mixture of inhibitors.

CONCLUSIONS

We found that chondrocyte apoptosis mediated by calcium influx shares typical features of apoptosis including cell shrinkage, phosphatidylserine exposure, and loss of cell viability. Our findings also indicated that this process of cell death was prevented by inhibitors of cysteine proteinases (caspase and calpain inhibitors). However, Ac-DEVD-cmk, was less efficient to prevent cell death as compared to leupeptin and the mixture of both had an additive effect and prevented all hallmarks of apoptosis. All together these results led us to use the mixture of both caspase and calpain inhibitors in our further experiments.

Although the caspases are key components in apoptosis, numerous results suggest the presence of other cell death pathways. Those included Ca^{2+}-dependent serine proteases, DNAse I, DNAse II and DNAse γ [Peitsch et al., 1993; Barry and Eastmann, 1993; Shiokawa and Tanuma,1998; Solary *et al.*, 1998; Shiokawa and Tanuma, 2001]. Our observations may be crucial for understanding how apoptosis regulates chondrocytes survival and participate in pathogenesis of osteoarthritis.

ACKNOWLEDGMENTS

This work was supported by the Romanian Ministry of Education and Research (National Plan of Research-Development and Innovation, Programme BIOTECH, contract 4625/2004). Authors are grateful with many thanks to Dr. Francis Goudaliez, Director of MacoPharma C°, Tourcoing, France, for providing reagents and FACScan flow cytometer. We express our gratitude to Victoria Andrei for skilful assistance.

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