Apoptosis induced by elastin derived peptides and TGF-β1 in rat aortic smooth muscle cells in vitro

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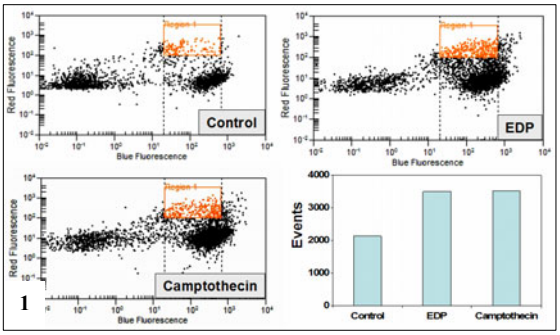
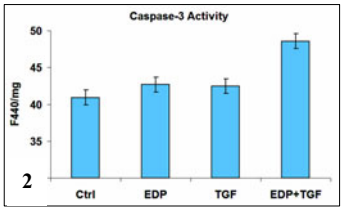
Statement of Purpose: Arterial wall calcification in the media is a prevailing aspect of diabetes, chronic kidney disease, and aging[1]; it also frequently occurs in stentless porcine aortic valves, and cryopreserved human aortic allograft valves[2]. Calcium deposition in the media is associated with proteolytic degradation of elastic fibers and the presence of TGF-β1, a major determinant in arterial injury. In response to soluble elastin degradation products (EDP) and TGF-β1 exposure for 10 days, vascular smooth muscle cells (VSMCs) secrete increased amounts of matrix metalloproteinases (MMPs) and produce typical bone proteins, such as alkaline phosphatase, osteocalcin and Cbfa-15[3]. The causal relationship between elastin degradation and calcification is not fully understood, but apoptotic cell death, a process observed in advanced atherosclerotic plaques[4], might also be related to medial calcification. The increased production of MMPs may induce apoptotic cell death by degrading the extracellular matrix from which VSMCs derive survival signals[5]. In vivo, apoptotic cells were detected at the calcification sites in a rat abdominal aorta injury model[6], and in vitro it was shown that VSMCs undergo apoptosis in multicellular nodules that calcify[7]. Apoptosis is characterized by a series of events that include phosphatidyl serine translocation within the plasma membrane (an "eat-me" signal sent by apoptotic cells), activation of caspase cascade, culminating with the activation of caspase-3, which in turn leads to DNA fragmentation. In the present study, we investigated whether EDP and TGF- β1, two factors usually present in the proteolytic milieu of injury, could induce apoptosis in VSMCs in vitro before the onset of calcification.

Methods: Primary rat aortic smooth muscle cells (RASMCs) were cultured in the presence of 100 ug/ml EDP and 10 ng/ml TGF- β1 and after 3 days the cells were analyzed for apoptosis markers. Translocation of phosphatidyl serine to the cell surface was detected by flow cytometry, measuring Annexin V binding to the cell surface, in conjunction with a calcein retention test that verifies the integrity of the cell membrane (Cell fluorescence kit, Agilent). Positive controls for this assay were obtained by inducing apoptosis with 2 uM Camptothecin. Caspase-3 activity was measured using the fluorometric caspase-3 assay kit (BD Pharmingen). Apoptotic cells with DNA breakdown were detected using the DeadEnd Colorimetric TUNEL System (Promega). DNase I-treated cells served as positive controls. DNA fragmentation was also visualized by gel electrophoresis using a lab-on-a-chip technique and the DNA 12000 assay kit (Agilent Bioanalyzer).

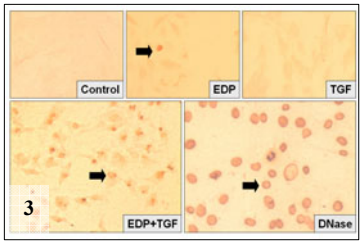
Results/Discussion: Initial studies showed that the number of EDP-treated RASMCs that translocate phosphatidyl serine from the inner to the outer lipid layer of the plasma membrane is similar to the number of camptothecin-treated cells that undergo apoptosis (Fig.1).



The activity of caspase-3 was markedly increased in cells treated with EDP and TGF-β1 together, but not in cells treated with either of the two factors separately (Fig. 2).



Nuclei of cells treated with EDP and TGF showed a positive reaction for the TUNEL assay, similar to cells treated with DNase I (Fig.3). DNA fragmentation was identified in cells treated with EDP and TGF- β1 concomitantly, but not from cells exposed to these agents separately (data not shown).



Conclusions: In a RASMCs cultured system, a simultaneous exposure to EDP and TGF-β1 for 3 days induces positive reactions for three markers of apoptosis: plasma membrane modifications, caspase-3 activation, and DNA fragmentation in a significant number of cells. These early events might be associated with the later changes noticed in the same culture system, specifically concerning the osteoblastic differentiation of vascular cells.

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