J. Biol. Res. 2010 n.1 - Vol. LXXXIII

Role of Cell-Cell Bond for the Viability and the Function of Vascular Smooth Muscle Cells

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KEYWORDS: Cell Cell Bond, AngiotensinII, Fibrin Provisional Tissue, IGF1R, IGFBP3

Abstract

Vascular smooth muscle cell (VSMC) viability and homeostasis is regulated by cell-matrix and cell-cell contact: disruption of these interactions are responsible of a switch from a mature to a high proliferative phenotype. VSMCs migration, rate of growth and apoptosis, and the extent of their extracellular matrix (ECM) deposition can be also modulated by proatherogenic peptides. Among them, ATII induces the transactivation of IGF1R, which, together with the binding protein IGFBP3, represents a determinant of cell survival, growth and proliferation. Aim of our in vitro study was to verify the role of elective cell-cell bond in modulating the response to ATII. Thus, we evaluated viability, proliferation, IGF1R, IGFBP3 expression and the long term survival and production of ECM in a provisional tissue. A7r5 cell-line was used in adherent cultures or incubated in agarose-coated culture plates to inhibit cell-matrix interactions. Cells, treated or not with ATII 100 nM, were evaluated for apoptosis rate, cell cycle, IGF1R and IGFBP3 protein expression. Fibrin provisional tissue was developed polymerizing a fibrin solution containing A7r5 cells with thrombin. Histological stainings for ECM components were performed on sections of provisional tissue. An exclusive cell-cell contact resulted in the formation of floating clustered cells with a 10% higher apoptosis rate and an 8% reduction of "S" phase of the cell cycle, compared to monolayer cell cultures. ATII did not affect the cell survival in both culture conditions, but promoted a 10% decrease in "S" phase and an increases IGF1R expression only in adherent cells, while suspended cell aggregates were resistant to ATII administration; IGFBP3 was reduced both in ATII treated adherent cells and in floating clustered cells, irrespective of the treatment. VSMC conditioning in agarose-coated plates before seeding in fibrin provisional matrix reduced, but not abolished, the cell ability to colonize the clot and to produce ECM. This study demonstrates that the elective cell-cell contact induces a quiescent status in cells lacking of cell-matrix interactions and reduces the responsiveness to ATII. Therefore, preserved cell-cell contact may counteract vascular remodeling induced by proatherogenic stimuli.

Introduction

Cell-matrix and cell-cell interactions are requested for vascular smooth muscle cells (VSMCs) survival and for a preserved vessel wall function: disruption of these interactions are responsible for both a switch from a mature, contractile phenotype to a high proliferative phenotype. This is a recurrent event following percutaneous transluminal coronary angioplasty and during episodic plaque progression of early atherosclerotic lesions in arterial vessel wall. Alternatively, the loss of cell-matrix interaction can be responsible for SMCs increased apoptosis, termed anoikis, and VSMCs depletion in the vessel wall, which is displayed during the aneurysm progression and the later stages of atherosclerosis. The cell-matrix interaction is a reversible mechanism, and VSMCs migration and proliferation is noticeable both in vivo, in a biological environment as in the presence of mural thrombus, and in *in vitro* models [1]. In addition to cell-matrix bonds, another key-factors for vascular smooth muscle cells survival is represented by cell-cell interactions [2]. While it is known that cell-matrix attachment promotes cell survival, the role of cell-cell contact in cell viability has been less investigated [3]. Established cell-cell adhesion complexes are localized in caveolae, invaginations of the plasma membrane involved in cellular processes including transport and signal transduction [4]. Caveolin1 exerts a scaffolding function to organize specific lipids and lipid-modified signalling molecules within caveolae. Among these signalling molecules are included G-protein-coupled receptors, such as the receptor for Angiotensin II (ATII), receptor tyrosine kinases such as

insulin-like growth factor type I receptor (IGF1R), SRC family tyrosine kinases, protein kinases and nitric-oxide synthase [5]. Beside mechanical signals, VSMCs migration, rate of growth and apoptosis, and the extent of their extracellular matrix deposition can be modulated by vasoactive peptides such as ATII, via its receptor AT1R [6]. The downstream signalling and growth promoting activity of ATII on VSMCs activates the IGF1-IGF1R pathway, causing the transactivation of IGF1R, which is required for functioning of the cell signaling processes initiated by other growth factors and for normal cell development [7]. The IGF1R activation via IGF1 is modulated by different binding proteins, among them the IGFBP3, which regulates the bioavailability of the ligand to the receptor. Thus, the balance between IGF1R and IGFBP3 represents a critical determinant of cell survival, growth and of mitogenic response [8]. Aim of our study was to establish an in vitro model to test the role of an exclusive cell-cell bond when VSMCs undergo to hormonal and mechanical stimuli. To accomplish that, we have transiently inhibited cell-matrix contact in SMCs and we have verified whether an elective cell-cell bond is able to modulate the response to exogenous administration of ATII in terms of cell viability, proliferation and IGF1R and IGFBP3 expression. Then, to gain insight on the reversible phenotype of VSMCs, we have characterized the migratory ability and the production of extra cellular matrix of these conditioned cells, when cultured for a long term in a provisional tissue of fibrin.

Materials and methods

Cell culture. A7r5 cell line was obtained from European Collection of Cell Cultures (ECACC). Adherent, high confluent cells were used in all the experiments. The cell-matrix contact was inhibited by cell incubation in agarose-coated culture plates. A 2 mm-thick layer of gel was produced with a low melting agarose solution (1% w/v in PBS). Cells need 3 to 4 hours to form floating clusters. *ATII* treatment. Adherent and clustered cells, treated or not with ATII 100 nM, were compared after different times changing on the basis of the experimental purposes. *Flow cytometry.* Apoptosis rate: Cells were labelled with AnnexinV-FITC/Propidium Iodide (Immunostep, Salamanca, E) and apoptosis, defined as cells with Annexin V-positive and PI-negative staining, was evaluated on a minimum of $1 \cdot 10^4$ unfixed cells per sample. Cell cycle: Cells were permeabilized and nuclear DNA content was stained with a hypotonic solution (PI 50 µg/ml in Sodium Citrate/Triton X100) at room temperature. The nuclear DNA content evaluation and the cell cycle analysis were done using CellQuest and ModFit LT software (Becton Dickinson). *Immunoblot.* Cells were lysed in RIPA lysis buffer additioned of 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 µM sodium orthovanadate, and 0.1 µM phenylmethylsulfonyl fluoride. Total cellular proteins were determined using the BCA protein assay kit (Pierce Rockford, IL, USA). Proteins were separated on SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with horseradish peroxidaseconjugated secondary antibody. We analyzed the expression of IGF1R and IGFBP3, (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Antigen detection was performed with ECLPLUS substrate (Amersham Biosciences, Germany). Protein expression was quantified by densitometric analysis on a GeneGenius gel documentation system (Syngene). *Fibrin* provisional tissue. Fibrin solution (Sigma, 4 mg/ml in PBS), A7r5 cells ($6 \cdot 10^5$/ml), thrombin (Sigma, 2,5 U/ml in PBS) were added to a 24well culture plate. After polymerization, culture medium (DMEM, 10% FBS, 25 µg/ml Aprotinin) was added and renewed every 2 days for 3 weeks; cells were cultured in a humidified incubator (37°C, 5% CO2). *Histology.* On 8 µm tick sections from paraffin embedded tissues were performed the following stainings: Hematoxylin – Eosin for cell counterstaining, Masson's Trichromic for collagen, Weighert Van Gieson for elastic fibers.

Statistical analysis. Experiments were performed at least 3 times. All data are expressed as means±standard errors. Data were analyzed by Student's t tests; significant differences were taken if $p<0.05$.

Results

VSMCs incubation in agarose-coated wells for six to twenty-four hours to hamper the cell-matrix interaction promoted an exclusive cell-to-cell contact, resulting in the formation of floating clustered cells mediated by Ca^{2+}-dependent cadherins. Clustered cells and confluent adherent cells were compared in our experiments; in addition, the same cells were evaluated at different time points after having been exposed to treatment with ATII. The cell viability was evaluated using AnnexinV-FITC/Propidium Iodide double staining assay after a 24 hour inhibition of cell-matrix adhesion. The apoptotic rate in suspended clustered cells was about 10% higher compared to monolayer cell cultures ($p<0.05$). 100 nM ATII treatment did not affect levels of cell survival in both culture conditions.
A twenty-four hour inhibition of cell-matrix interaction caused a 8% reduction of "S" phase of the cell cycle in comparison to adherent cells ($p<0.05$). ATII treatment (100 nM for 24 hours) promoted about a 10% decrease in "S" phase in adherent cells ($p<0.05$) but not in cell aggregates (Fig. 1).
Once reseeded on plastic culture flask, cells showed an increase of S phase, particularly 24h after the reseeding (Fig. 2); such effect is still detectable 48 hours later (data not shown).
Since ATII induced signalling and growth promoting activity in VSMCs acts through the IGF1–IGF1R axis, we set to determine the protein expression of IGF1R and its binding protein IGFBP3. After a 16 hour incubation with ATII, adherent cells increased up to 70% the IGF1R expression, compared to untreated cells. IGF1R level in untreated cell aggregates was similar to that in adherent control cells, and ATII supplementation failed to induce significant changes in the expression of IGF1R. Once reseeded, cells kept in the absence of matrix interaction showed about a 50% increase in IGF1R expression. IGFBP3 was reduced by ATII treatment in adherent cells; in floating clustered cells IGFBP3 expression decreased compared to adherent control cells and was not affected by ATII administration.

No significant differences have been displayed by floating clustered cells after reseeding on plastic culture flasks, compared to adherent control cells (Fig. 3).
Control SMCs seeded in the fibrin provisional tissue

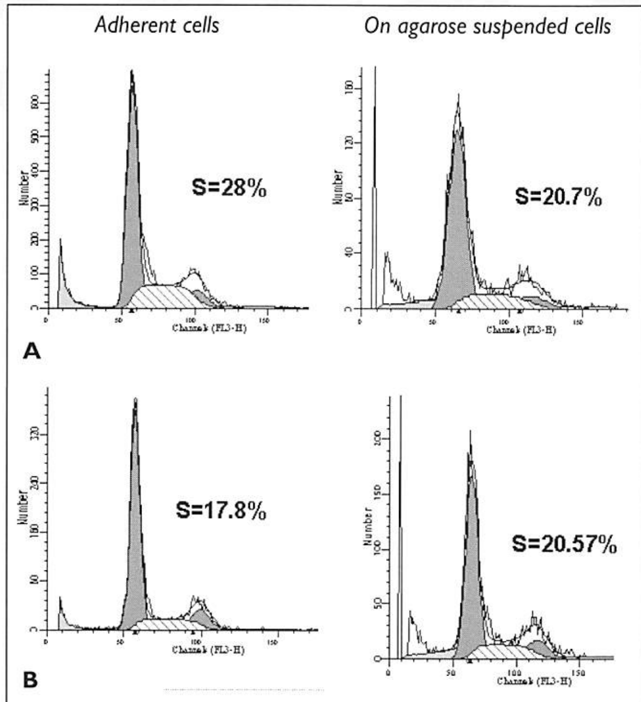

Fig. 1 - Influence on cell cycle distribution of a 24 hour treatment with ATII (100nM) on adherent cells (left panels) and on suspended clustered cells (right panels). A: untreated cells; B: ATII treated cells. "S" =Synthesis phase.

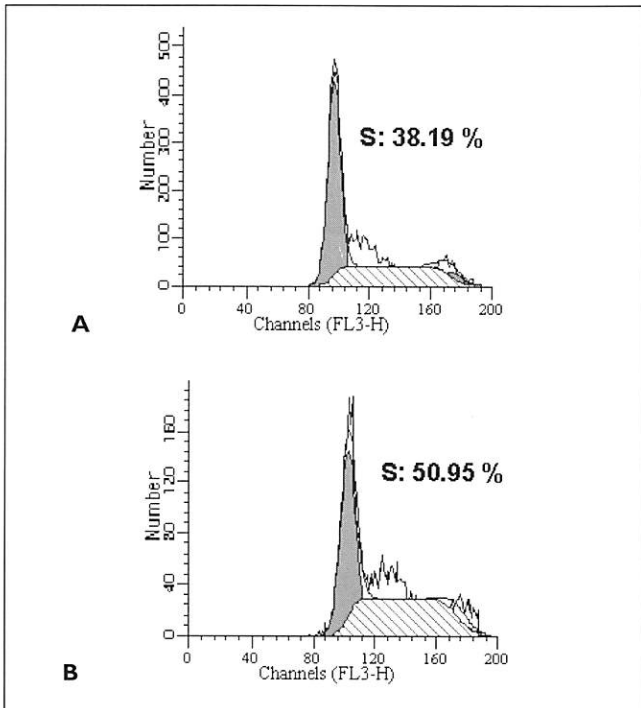

Fig. 2. Influence of an overnight incubation in agarose coated plates onagarose on cell cycle distribution evaluated 24 hours after reseeding in adherence. A: cells incubated in adherence before detachment and reseeding, used as a parallel control; B: cells incubated overnight on agarose and reseeded in plastic wells; "S" =Synthesis phase.

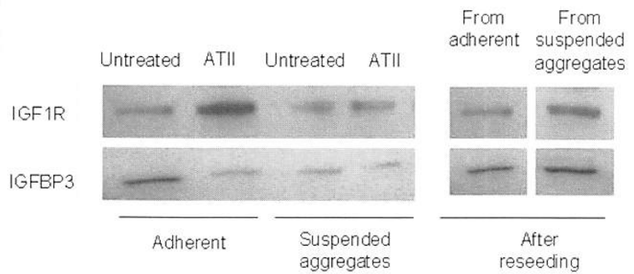

Fig. 3 - Immunoblot quantification of IGF1R and IGFBP3 expression in A7r5 cells: blots on the left, cells untreated or after treatment with ATII on adherent and on agarosesuspended cell cultures; blots on the right, cells incubated overnight both in adherent condition or on agarose, and reseeded in plastic wells.

showed a robust deposition of extra cellular matrix, primarily collagen, over time (3 weeks). Overnight SMC conditioning in agarose-coated plates before seeding in this provisional matrix reduced, but not abolished, cell ability to colonize the clot and to produce extra cellular matrix (Fig. 4).

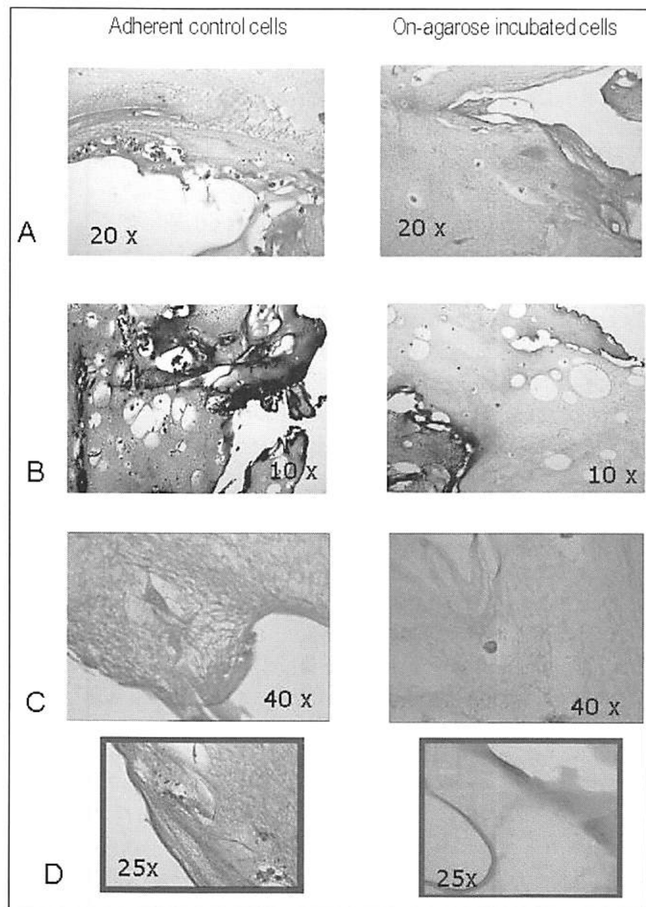

Fig. 4. Histology from long-term (3 weeks) cultures of A7r5 cells embedded in a fibrin provisional tissue. Left side: samples containing cells from adherent control cultures seeded in fibrin gel. Right side: samples containing cells seeded in fibrin gel after an overnight on-agarose incubation. A: H&E stain; B: Masson's Trichromic stain; C and D: WeighertVan Gieson stain.

Discussion

This study demonstrates that the elective cell-cell contact is able to counteract apoptosis in VSMCs, to maintain a quiescent status in cells lacking of cell-matrix interaction and to reduce the response to ATII.
Previous studies demonstrated that the antiapoptotic contribution of cadherin-mediated cell-cell contact is equally effective in maintaining cell survival as cell-matrix contact in VSMCs of human saphenous vein, and that anoikis of primary human CECs can be inhibited, despite loss of cellmatrix adhesion, when cell-cell adhesion is preserved through β-catenin-, Src-, and PI3-K/Akt-dependent pathway.
According to these statements, we found that the formation of cell aggregates induced in A7r5 cells by a 24 hours lack of matrix interaction resulted in a modest increase (10%) of apoptotic rate compared to adherent culture cells; moreover, we observed that clustered cells were maintained in a steady state, mimicking tissue homeostasis, with a reduction in the synthesis phase of the cell cycle [3, 9].
Then, we highlighted some differences in VSMCs response to the proliferative stimulation by vasoactive peptide ATII with regard to the possibility of substrate adhesion or of cell-cell clustering. It has been demonstrated that IGF1R transactivation is important for the downstream signalling of ATII [10]. In our study, monolayer cell cultures exposed to ATII exhibited a time-dependent increase of IGF1R expression, as expected. Conversely, suspended cell aggregates were resistant to ATII administration, maintaining similar IGF1R total levels as adherent untreated ones; IGFBP3, which besides its function as mediator of IGF1 bio-availability, acts as a proapoptotic agent through a secretion and re-uptake mechanism caveolae and clathrin-mediated [11], was reduced both in ATII treated adherent cells, and in agarose induced cell aggregates, irrespective of the treatment. Thus, a promoted cell-cell contact provokes a shift toward a prosurvival IGF1R and IGFBP3 ratio.
We further have demonstrated that the quiescent status induced by the cell-cell bond is a reversible phenomenon, as shown by the ability of on-agarose, clustered VSMCs to increase the synthesis phase, to proliferate after re-seeding, and, on long term, to migrate and produce extracellular matrix components when embedded in a fibrin clot, to form a provisional tissue. In conclusion this work provides novel links about the effect of preserved cell-cell contact in counteracting vascular remodelling induced by proatherogenic stimuli and implicates options for prevention and therapy of atherosclerotic disease, with respect to impaired extracellular matrix deposition and cell depletion.

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