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RESEARCH REPORT

Ras activation in *Hirudo medicinalis* angiogenic process

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Abstract

In some leeches like *Hirudo medicinalis*, any kind of stimulation (surgical wound or growth factor injection) provokes the botryoidal tissue response. This peculiar tissue, localized in the loose connective tissue between gut and body wall, is formed by granular botryoidal cells and flattened endothelial-like cells. Under stimulation, the botryoidal tissue changes its shape to form new capillaries. In mammals, the molecular regulation of the angiogenic phenotype requires coordinated input from a number of signalling molecules: among them the GTPase Ras is one of the major actor. In our current study, we determine whether Ras activation alone would be sufficient to drive vessels formation from leech botryoidal tissue. Our findings indicate that assembly and disassembly of actin filaments regulated by Ras protein is involved in morphological modification of botryoidal tissue cells during leech angiogenic process.

Key Words: leech; Ras; cytoskeleton; angiogenesis

Introduction

Hirudo medicinalis shows a simple anatomical organization: a muscular-cutaneous sac, practically avascular (de Eguileor *et al*., 2001a, 2004), containing several organs embedded in a loose connective tissue. In Hirudidae between the gut and the body wall, a peculiar tissue, the botryoidal tissue is localized. This multifunctional tissue, formed by clusters of roundish granular botryoidal cells, and small, flattened, endothelial-like cells, displaying myelo/erythroid and storage functions (Sawyer, 1986), is involved in angiogenesis (de Eguileor *et al*., 2001b). We have previously demonstrated that both surgical and biochemical stimuli (i.e. injection of cytokines such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) or granulocyte Macrophage Colony-Stimulating (GM-CSF) evoke in botryoidal tissue cells, angiogenic signalling pathways analogous to those observed in vertebrates (Grimaldi *et al*. 2006; Tettamanti *et al.,* 2003a; Tettamanti *et al.,* 2003b, 2006). Immediately after stimuli, botryoidal tissue changes its shape from a solid cord of cells to a tubular, pre-vascular structure through a dehiscence process. In this contest, the remodelling is characterized by marked cellular changes: thinning, flattening and tapering of these cells allow the definition of lumen, and the increase in diameter and length of new capillary. The molecular control of the complex angiogenic phenotype requires coordinated input from a number of signalling molecules. In mammalian, one of basic regulators of the angiogenic response is Ras. This GTPase, due to its involvement in endothelial cell motility (Sosnowski *et al*., 1993; Fox *et al*., 1994), plays an important role in several systems including bFGF-mediated wound closure and pro-angiogenic response to VEGF. Its activation is also required for the proliferation, migration, and branching morphogenesis of vascular endothelial cells (Meadows *et al*., 2001, 2004). Ras protein controls signal transduction pathways (Carpenter 2000; Kranenburg and Moolenaar, 2001) leading to gene expression changes and cell motility through the MAP/ERK kinase cascade. (Ehrhardt *et al*., 2002).

The focus of the present work is to demonstrate that as in vertebrates (Joneson *et al.*, 1996; Leblanc *et al.,* 1998; Meadows *et al*., 2001; Kranenburg, 2004; Meadows *et al.,* 2004; Serban et al., 2008) Ras plays a key role in the leech angiogenic process by remodelling actin cytoskeleton even in absence of VEGF, generally considered the principal regulator of angiogenesis.

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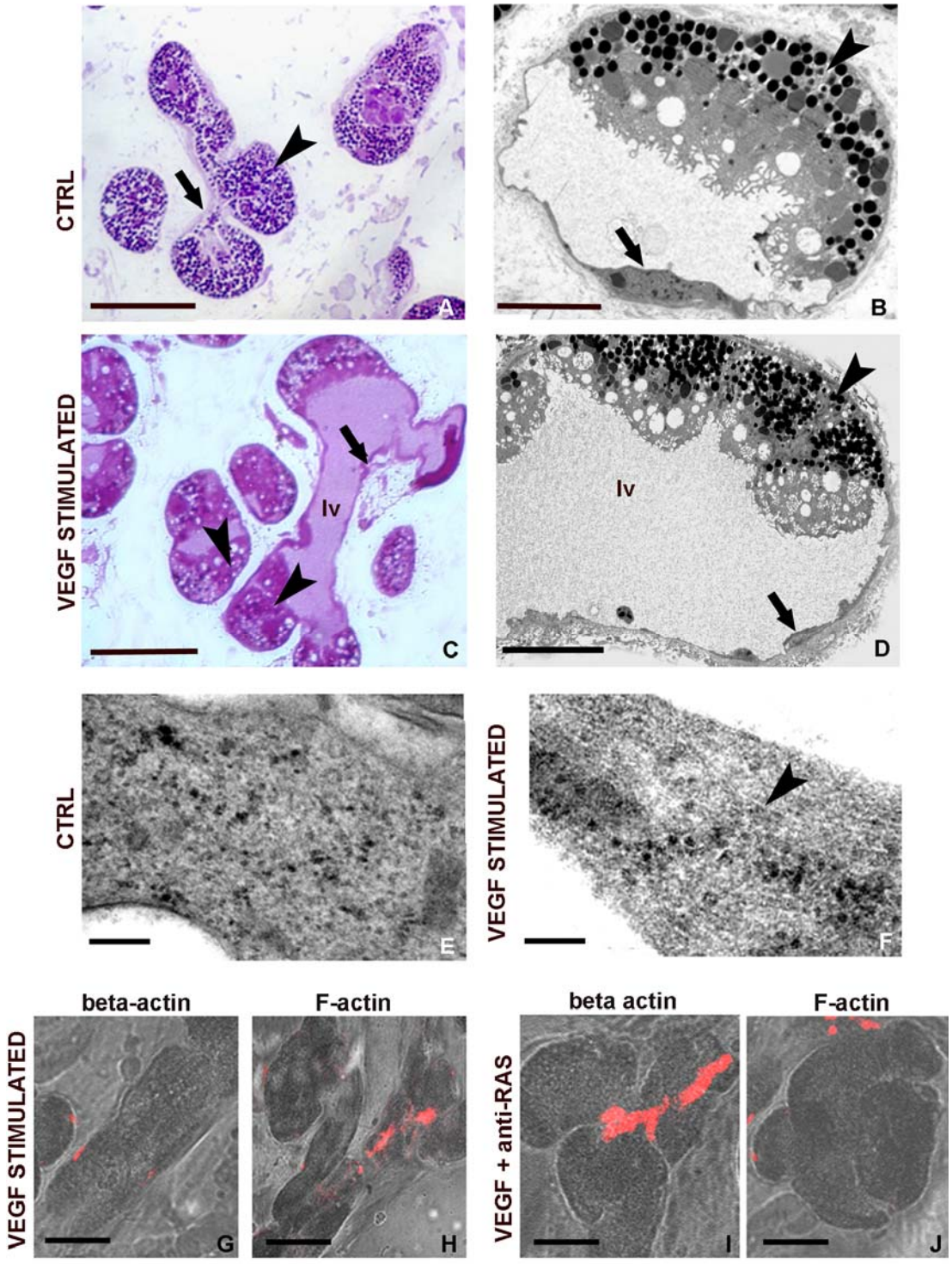

Fig. 1 A-J Morphological and immunocytochemical analysis of control and VEGF stimulated botryoidal tissue cells. Semi-thin (A, C) and thin (B, D-F) sections of control and VEGF stimulated cultured botryoidal tissue. The control tissue is formed by clusters of botryoidal cells with a granule-filled cytoplasm (arrowheads in A and B) and endothelial cells (arrows in A and B). After 6 h from VEGF administration, large cavities (lv in C and D) lined by granulated botryoidal cells (arrowheads in B and C) and by flattened endothelial-like cells (arrows in C, D) are visible. Bundles of actin filaments are visible at TEM in the cytoplasm of VEGF stimulated endothelial cells (arrowheads in F), but not in the control untreated endothelial cells (E). In both VEGF stimulated (G, H) and VEGF+anti-RAS (I, J) treated botryoidal tissue, the diffuse distribution of actin, lacking a specific organization in bundles is detected by immunofluorescence using a specific monoclonal antibody anti-beta actin (G, I), while F-actin (H, J) is stained using -conjugated phalloidin. The formation of actin filaments is highly reduced in VEGF+anti-RAS treated endothelial cells (J). Bars in A, C, G-J: 100 μm; bar in B: 4 μm; bar in D:10 μm; bars in E, F: 200 nm.

Material and Methods

Animals and Treatments

Leeches (*Hirudo medicinalis*, Annelida, Hirudinea, Hirudidae from Ricarimpex, Eysines, France) were kept in water at 19 - 20 °C in aerated tanks. Animals were fed weekly with calf blood. Before each experiment, leeches were anaesthetized with a 10 % ethanol solution.

Cell culture

Leeches were longitudinally dissected on the dorsal side using sterilized razor blades and botryoidal tissue cells, localized between the muscular body wall and the gut, were harvested from the animals using sterilized forceps. Cells were then transferred to a single well of 24 well plates in 400 ml of tissue culture medium containing DMEM medium (Celbio, Milan, Italy) modified by dilution (1:4) to reach iso-osmolality and supplemented with 1% glutamine,10 % fetal bovine serum and 1 % antibiotic antimycotic solution (Sigma, St. Louis, MO) as already described (Grimaldi *et al.*, 2008, 2009). Cells were maintained at 20 °C for 24 h before to be differently processed.

Cell culture and VEGF administration

To evaluate the effect of VEGF165 on botryoidal tissue cells shape modification cells were plated in 24-multiwell plates in medium alone or in medium supplemented with 100 ng/ml of VEGF165. All cultures were performed in quadruplicate and scored at 6 *in vitro* using an inverted Olympus microscope (Olympus, Tokyo, Japan). Data were recorded with a DS-5M-L1 digital camera system (Nikon, Tokyo, Japan).

Cell culture and transformation assays

Plasmids (kindly gifted by Renata Zippel, University of Milano, Italy) utilized for botryoidal cells transfection were: pc3mycRas61, coding for a constitutively activated form of Ras (Glu61 substituted with Leu) and pEGFP-CI (coding for a green fluorescence protein). DNA transfection of botryoidal tissue cells were performed using calcium phosphate precipitation (calcium phosphate kit, Sigma). Cells were kept in a 1xHEPES-buffered saline solution pH 7.05 (Sigma) containing 8.4 μl $CaCl_2$ (2,5M), 2,5 μg of pEGFP-CI and 4.5 μg of pc3mycRas61. Control experiments were performed using a 1xHEPES-buffered saline solution pH 7.05 containing only 8.4 μl $CaCl_2$ (2,5M) and 7 μg of pEGFP-CI. After 20 h the medium containing DNA were removed and cells were treated for 2 minutes with a 50 % glycerol in 1xHEPES-buffered saline solution pH 7.05 to increase efficiency of transfection. Cells were then plated in culture medium and analyzed after 3 days. Transfection efficiency was visualized by using a fluorescence microscope Olympus (excitation/emission filters 490/525 nm for fluorescein isothiocyanate-FITC).

Inhibition of Ras signalling in Ras transformed cells

An antibody anti-Ras and the synthetic inhibitor of the MAP kinase pathway PD 098059, (2′-amino-3′methoxyflavone, Alexis, Bingham, U.K.) were used to block Ras activity (Alessi *et al*., 1995). For antibody-mediated neutralization experiments, 1 μg of the polyclonal antibody anti-rabbit Ras (StressGen Biotechnologies Corporation, Victoria, BC, Canada), was added to the medium in which GFP/Ras61 co-transfected cells or VEGF stimulated cells were cultured. PD 098059 was dissolved in DMSO to give a final concentration of 37 μM and stored at -80 °C. GFP/Ras61 transformed cells were then cultured in a medium containing PD 098059 to a final concentration of 100 μM. Cells plated in both modified culture medium were analyzed after 3 days.

Inhibition of actin bundles formation

Cytochalasin D (Goddette and Frieden, 1987), that has the ability to bind actin filaments, was use to block polymerization and the elongation of actin. GFP or GFP/Ras61 transformed cells were incubated with culture medium containing 5 μg/ml of cytochalasin D (Sigma) and analyzed after 3 days.

Optical and Electron microscopy

Botryoidal tissue cells were fixed for 2 h in 0.1 M cacodylate buffer pH 7.2, containing 2 % glutaraldehyde. Specimens were then washed in the same buffer and postfixed for 2 hrs with 1 % osmic acid in cacodylate buffer, pH 7.2. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin) according to Moore *et al.* (1960), and subsequently observed under a light microscope (Olympus). Data were recorded with a DS-5M-L1 digital camera system (Nikon). Images were combined with Adobe Photoshop (Adobe Systems, Inc.). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan). Data were recorded with a MORADA digital camera system (Olympus).

Immunofluorescence staining

Cultured cells were fixed with 4 % paraformaldehyde in phosphate-buffer (PBS) for 30 min at 4 °C. After washing in PBS cells were pre-incubated for 30 min in a blocking solution (PBS, 2 % bovine serum albumin (BSA), tween 0.1 %). Cells were then incubated for 1 h at 37 °C with the primary antibodies mouse anti-beta actin (Sigma), to visualize G-actin, and rabbit anti-Ras (StressGen Biotechnologies Corp.), diluted 1:100 in blocking solution. The washed specimens were incubated for 1 h at room temperature with the appropriate secondary antibody Cy3 conjugated (1:200) (Jackson, Immuno Research Laboratories, West Grove, PA, USA). Samples were directly examined with a fluorescence microscope Olympus or embedded in Polyfreeze tissue freezing medium (Polysciences, Eppelheim, Germany), immediately frozen in liquid nitrogen. and cut with a Leica CM 1850. The staining was visualized using excitation/emission filters 550/580 nm for Cy3. Data were recorded with a DS-5M-L1 digital camera system (Nikon). Images were combined with Adobe Photoshop (Adobe Systems, Inc.). In control samples, primary antibodies were omitted and cells,

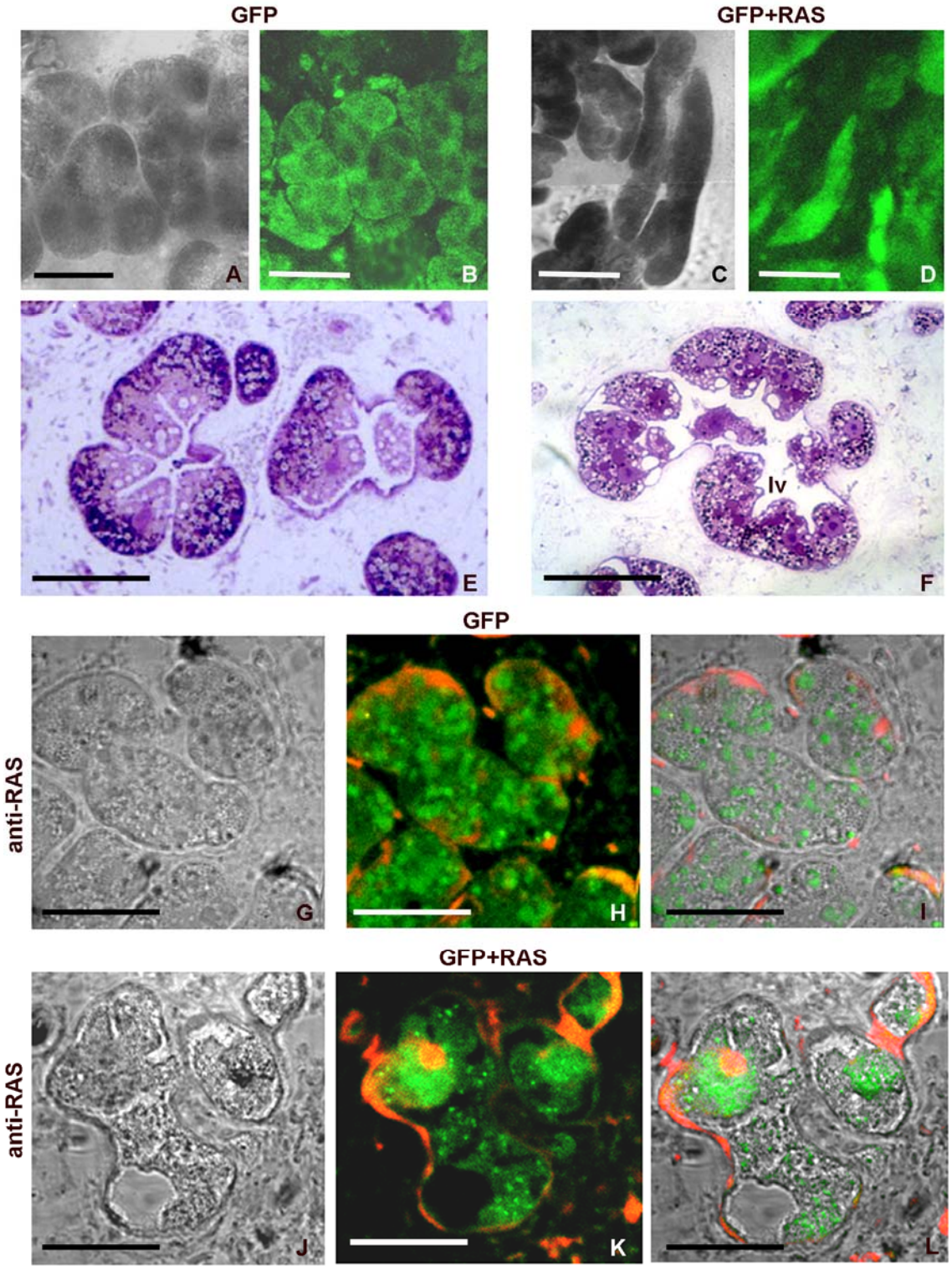

Fig. 2 A-L Ectopic expression of Ras61 enhances a dehiscent process in botroyidal tissue cells. The morphology of GFP transformed botryoidal cells in growth medium (A, B) and sectioned (E) is similar to that observed in control untreated cells. Botryoidal tissue cells maintain a rope shape. In cultured (C, D) and sectioned (F) GFP/RAS61 transformed cells morphological changes are observed. Botryoidal tissue cells show an elongated phenotype (C, D) and undergo a dehiscence process lining a new cavity (lv in F). The expression of RAS was detected by immunofluorescence in both GFP (G-I) and GFP/RAS61 (J-L) transformed cells. After RAS61 transfection, the expression of RAS protein (in red in H, I, K, L) is mainly localized in the elongated and flattened endothelial cells (J-L). The GFP marker indicates infected cells (green in B, D, H, I. K, L). Bars in A-L: 25 μm.

pre-incubated for 30 min with PBS/BSA, were incubated only with the secondary antibodies. To visualize F-actin filaments, cells were treated for 10 min at 4 °C with a permeabilizing solution (HEPES 20 mM, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl2, 0.5 % Triton X-100), then washed with PBS buffer and finally incubated 1 h at 37 °C with tetramethylrhodamine (TRITC)-labeled phalloidin (Sigma) (diluted 50 µg/ml) in PBS buffer.

Results

Morphological analysis of cultured botryoidal tissue cells

Cultured botryoidal tissue, extracted from untreated control leech, is formed by clustered cells mostly packed in cord or lining practically virtual spaces (Figs 1A, B). These cells (de Eguileor *et al*., 2001) belong to different categories due to their size and to the presence of large amount of granules in the cytoplasm: large roundish granular botryoidal and small flattened endothelial-like cells are easily recognizable (Figs 1A, B).

Effects of VEGF in vitro

The VEGF administration (Figs 1C, D) is responsible of botryoidal tissue cells changes. A dehiscence process led the compact cell cords to gradually line new cavities. During early phases of angiogenesis (*i.e.*, during the formation of prevascular lacunae), botryoidal cells acquired a semilunar shape (Figs 1C, D) while small, flattened and elongated endothelial-like cells become visible interposed among botryoidal ones (Figs 1C, D). Unlike control, VEGF stimulated endothelial-like cells showed bundles of cytoskeletal filaments (Figs 1E, F).

Characterization of cultured botryoidal tissue cells cytoskeleton

After VEGF administration, botryoidal tissue cells, showing an elongated phenotype, displayed a weak expression of anti-beta actin that localize diffuse G-actin, and a strong TRITC-conjugated phalloidin staining F-actin (*i.e.,* detecting filament bundles) (Figs 1G, H). In contrast VEGF/anti-Ras treated cells showed a high positivity for the beta-alpha actin antibody while a low signal was detectable for F-actin staining (Figs 1I, J).

Effect of Ras61 on botryoidal tissue cells

Botryoidal tissue cells have been transformed both with GFP and Ras61 (constitutively activated RAS protein) in order to: a) define the effects of Ras activation on botryoidal tissue phenotypes; b) to determine whether Ras activation was sufficient to cause actin filaments polymerization, driving angiogenic responses without the synergistic VEGF induction. Botryoidal tissue cells co-expressing Ras61 and GFP showed transformed phenotype (Figs 2A-F). Cells expressing only GFP maintained a cord shape (Figs 2 A, B, E) while the ectopic expression of Ras61 promoted *in vitro* the elongation and the formation of new vessels, (Figs 2 C, D, F). The ectopic expression of Ras61 in both GFP and GFP/Ras61 co-transfected cells was evaluated by using an antibody anti-Ras. In the control GFP transfected cells, Ras was low expressed at the periphery of the botryoidal tissue cells organized in clusters (Figs 2G-I). In contrast, in RAS61 transformed cells, Ras signalling was strong and it was mainly localized in the flattened endothelial cells lining the new lumen of vessel (Figs 2J-L).

Paralleling, cells expressing only GFP showed a strong peripheral localization of G actin (Fig. 3A), and a weak rhodamine-phalloidin positivity indicating that there were very few actin filaments (Fig. 3B) as also ultrastructurally validated (Fig. 3C). In GFP/Ras61 co-transfected cells (Figs 3D-F) the presence of F-actin (confirmed by staining with TRITC-phalloidin), mainly located at level of endothelial like cells, was preponderant in respect to G-actin (confirmed by staining with anti-beta actin). The presence of filament bundles was verified by ultrastructural analysis (Figs 3F).

Therefore, we assessed the role of Ras signalling in endothelial cells cytoskeleton reorganization by inhibition of Ras signalling in Ras transformed cells. The effect of Ras was abolished when an antibody anti-Ras or the selective MAP kinase kinase 1 (MEK1) inhibitor, PD 098059, were administrated in vitro (Figs 4 A, B). A similar result was obtained adding to the culture medium the inhibitor of actin polymerization Cytochalasin D (Fig. 4C). All three different treatments inhibited the formation of F-actin, as shown by TRITC-phalloidin staining (Figs 4 D-F) blocking the consequent modification of the botryoidal tissue shape.

Discussion

Reorganization of the actin cytoskeleton, composed of actin filaments and many specialized actin-binding proteins (Stossel, 1993; Small, 1994; Zigmond, 1996; Takai *et al*., 2001), has a key role in many cellular functions such as cytokinesis, change of cell shape, cell motility and cell adhesion. The modulations of actin scaffold is due to the tightly regulated polymerization and depolymerisation of actin filaments and numerous studies have identified in GTPase Ras intervention the *clou* event leading to remodelling of the actin cytoskeleton (Joneson *et al.* 1996; Serban *et al*., 2008). In vertebrates Ras activation is sufficient to induce many of the requisite phenotypes for angiogenesis by inducing profound morphological changes of the vascular endothelium (Xu, *et al.,* 1998), however discordant opinions are about the importance in assigning the different factors involvement. In fact it is known that Ras signalling, even if its mode of action is unclear, is capable of driving an angiogenic switch in the phenotype of primary endothelial cells in the absence of angiogenic factors (Meadows *et al*., 2004). In addition some evidences have accumulated that both the classical extracellular activated kinase ERK1/2 mitogen activated protein (MAP) kinase pathway and the phosphoinositide 3-kinase (PI 3-kinase) pathway can contribute to alterations in the actin cytoskeleton (Castro Barros and Marshall, 2005).

To assess the role of Ras/MAP/ERK signalling on cells shape modification we used, as an *in vitro* approach, the botryoidal tissue of *H. medicinalis.* This tissue (made of endothelial and botryoidal cells)

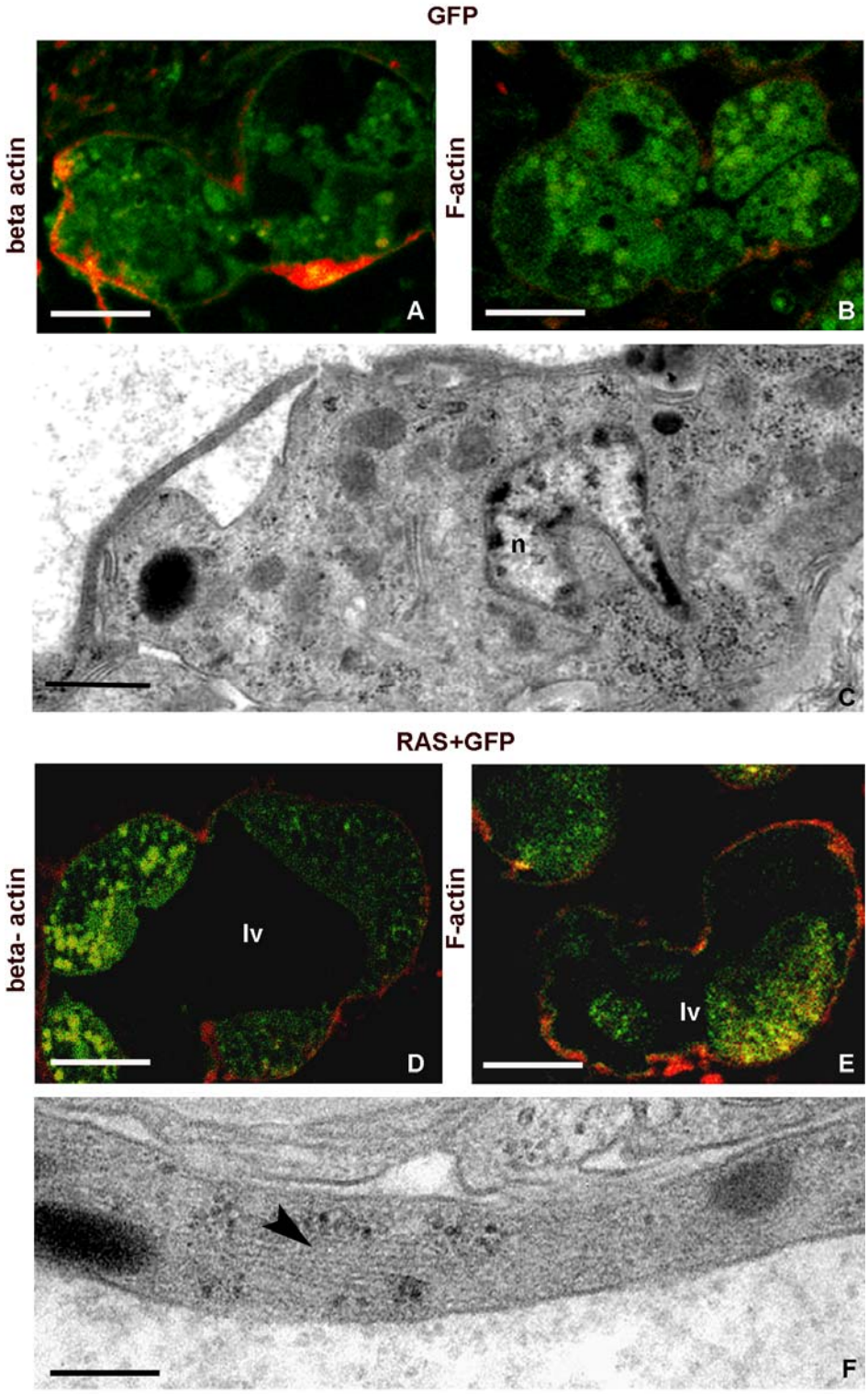

Fig. 3 A-F Analysis of cytoskeleton in sectioned GFP and GFP/RAS61 transformed cells. Distribution of diffuse and polymerized actin in transformed cells is assessed respectively with an anti-beta actin antibody (A, B) and by staining with -phalloidin (D, E). GFP transformed cells contain more diffuse actin (red in A) than polymerized F-actin (red in B), as also showed by electron microscopy image (C). In GFP/RAS61 tranformed cells a down-regulation of G-actin isoform (red in D) and a strong signal for F-actin (red in E) is observed. Bundles of actin filaments are visible at TEM (arrowhead in F). The GFP marker indicates infected cells (green in A, B, D, E). lv: lacunae vessel; n: nucleus. Bars in A, B, D, E: 10 μm; bar in C: 400 nm; bar in F: 200 nm.

can be considered an useful tool to evaluate Ras effects on cell shape modification since it, during angiogenesis in response to VEGF administration, undergoes to a radical remodelling leading to prevascular/vascular lumen formation characterized by marked cellular changes (*i.e.*, by flattening, lengthening and stretching especially of endothelial-like cells (de Eguileor *et al*., 2001b).

An interesting outcome of our investigation showed that constitutively activated protein Ras61 expression in stably transfected cells causes modification of actin cytoskeleton with vessels cavities formation also in total absence of the angiogenic factors VEGF, whereas cells expressing only GFP show the typical solid cord structure of un-stimulated tissue. Moreover the angiogenic phenotype in both GFP/Ras61 transformed cells and VEGF stimulated cells was inhibited by using an antibody anti-Ras.

Immunodetection and ultrastructural analysis of cytoskeleton alterations of both VEGF stimulated and Ras61-transformed botryoidal tissue cells show the presence of oriented bundles of polymerized filaments in all botryoidal tissue cells. These cells with an elongated and flattened shape are characterized by actin filament bundles and in their cytoplasm the presence of polymerized F-actin (rhodamine-phalloidin positivity) is predominant in respect to G actin (anti-beta globular actin positivity). These data are validated by treatment of GFP/RAS61 co-transfected botryoidal tissue cells with Cytochalasin D, an inhibitor of F-actin polymerization (Goddette and Frieden, 1987), leading to disappearance of actin bundles (decrease in fluorescence intensity of phalloidin). On the contrary, the diffuse distribution of G-actin, challenges the hypothesis of the relationship between down-regulation of actin polymerization and morphological changes of botryoidal tissue cells.

We demonstrate also that MAP kinase activity is essential to the maintenance of the Ras-induced phenotype, utilizing a potent inhibitor, the PD 098059 that reduces the basal MAP kinase activity in Ras-transformed botryoidal cells that are unable to form vessels.

Summarizing: i) RAS is the most involved factor in actin cytoskeleton reorganization of botryoidal tissue cells; ii) the cytoskeletal reorganization of botryoidal tissue cells lead to new vessel formation; iii) Ras/MAP/ERK signalling on actin filaments polymerization and their spatial arrangements are directly involved in the morphological modification of botryoidal tissue cells.

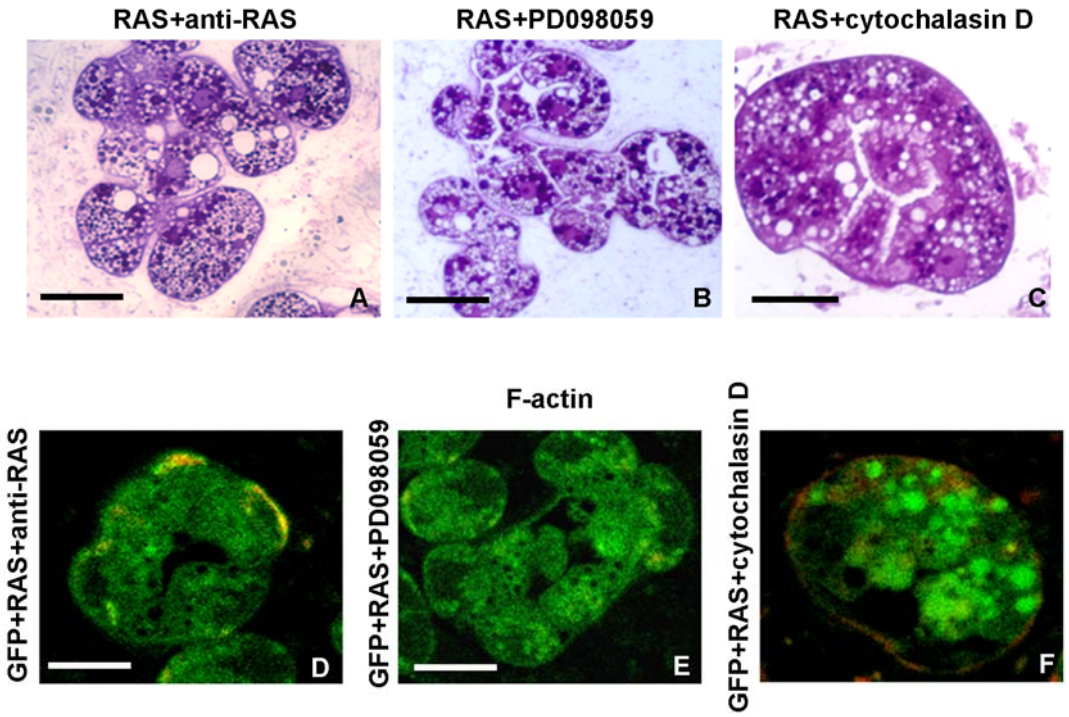

Fig. 4 A-F Effect of anti-RAS; PD 098059 and Cytochalasin D on RAS61 activated cells. A-C: semithin sections of RAS-61 transformed botryoidal tissue cells. The anti-Ras antibody (A), the MAP kinase kinase 1 (MEK1) inhibitor PD09805 (B) and the inhibitor of actin polymerization Cytochalasin D (C) inhibit Ras induced vessel formation. D-F: cryosections of GFP/RAS-61 co-transformed botryoidal tissue cells treated with anti-Ras antibody (D), the MAP kinase kinase 1 (MEK1) inhibitor PD09805 (E) and the inhibitor of actin polymerization Cytochalasin D (F). A low signal for F-actin is detected with TRITC-conjugated phalloidin (red in D-F). Bars in A, B, D, E: 10 μm; bars in C, F: 5 μm.

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