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The Role of Mechanical Stretching in the Activation and Localization of Adhesion Proteins and Related Intracellular Molecules

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ABSTRACT

The molecular complexity of the processes which lead to cell adhesion includes membrane and cytoskeletal proteins, involved in the focal adhesion formation, as well as signaling molecules tightly associated with the main intracellular regulatory cascades (Akt/PKB and MAPK/Erk). Dynamic environments, which create substrate deformations at determined frequencies and timing, have significant influences on adhesion mechanisms and in general in cellular behavior. In this work, we investigated the role of mechanical stretching (10% substrate deformation, 1 Hz frequency applied up to 60 min) on adhesion proteins (vinculin and focal adhesion kinase—FAK), related RhoGTPases (Rac1 and RhoA), and intracellular pathways (Akt/PKB and MAPK/Erk) in terms of activation and membrane recruitment in relation with cytoskeletal changes observed (membrane ruffling and filopodia formation). These changes are due to intracellular molecular rearrangements, acting with sequential concerted dynamics, able to modify the cytoskeletal conformation. The observed cellular response adds some important issues for better understanding the cellular behavior in environment which mimic as close as possible the physiological conditions. J. Cell. Biochem. 112: 1403–1409, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: MECHANICAL STRESS; FOCAL ADHESION KINASE; VINCULIN; RHOGTPASES; AKT; ERK

Several cellular functions as well as tissue development are strictly regulated by physical forces, which influence cell and tissue behavior such as bone omeostasis [Robling and Turner, 2009] or skeletal muscle differentiation and organization [Kurpinski et al., 2006; Halka et al., 2008]. The conversion of physical stimuli into biochemical signals is named mechanotransduction, and leads to dramatic changes on protein synthesis and on cell morphology and physiology. Anyway, this critical phenomenon remains not completely understood, even if focal adhesion proteins and related intracellular pathways have been assumed as major actors of the sight [Jani and Schöck, 2009; Baker and Zaman, 2010]. Focal contacts are structurally defined adhesion sites between cultured cells and the extracellular matrix (ECM) formed by more than 50 different proteins, tightly associated with actin filaments. The major transmembrane ECM receptors belong to integrin family, which show a large extracellular domain responsible for ligand binding, a single transmembrane and a cytoplasmic domains [Schwartz and DeSimone, 2008]. Integrins are associated in the cytoplasmic region with a dense and heterogeneous protein network, which create a dynamic bridge able to sense and transmit the mechanical forces to the intracellular-related proteins and the cytoskeleton, such as vinculin [Mierke, 2009] or Focal adhesion kinase (FAK) [Lehoux and Tedgui, 1998].

Vinculin comprises a globular head, a hinge domain, and a short tail [Winkler, 1996]. It can be in a close-inactive conformation by an intramolecular interaction between the head and tail domains. When phosphatidylinositol (4,5)-biphosphate is bind on the specific site on vinculin tail, this protein switch to an open-active conformation exposing binding sites for several adhesion and cytoskeletal molecules. Thus, vinculin is assumed to provide a structural link between actin filaments and the focal sites on the membrane. Anyway, vinculin appears not only a structural link between adhesion receptors and the actin cytoskeleton, but even a dynamic regulator of cell adhesion with a role in regulating cell migration through controlling membrane protrusion by localizing actin assembly to sites of newly engaged

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integrins [DeMali and Burridge, 2003].

FAK is a broadly expressed cytoplasmic tyrosine kinase that is a downstream component of an integrin-regulated signaling pathway. At the cellular level, FAK controls cell migration, proliferation, and survival through direct interactions with phosphatidyloinositol 3-kinase (PI3 kinase)/Akt and indirect activation of Erk once phosphorylated [Parsons et al., 2000]. Moreover, literature reports several works showing the effects of mechanical stress on the activation of these pathways [Albinsson and Hellstrand, 2007]. Furthermore, FAK is tightly related with members of Rho GTPase family, regulating the signal transduction from membrane receptors to a variety of cellular events related with cell morphology, motility, cytoskeletal system, and cell survival [Parsons et al., 2000].

The Rho family GTPases participate in regulation of actin cytoskeleton and cell adhesion events [Lars and Hall, 1999]. The GTPases Rho, Rac1, and Cdc42 are known to regulate cell shape changes through the effects on the cytoskeleton and cell adhesion. Rho has been implicated in several cellular events such as the formation of actin stress fibers and focal adhesions [Ridley et al., 1995], cell morphology [Paterson et al., 1990], cell motility [Takaishi et al., 1994], membrane ruffling [Nishiyama et al., 1994], smooth muscle contraction [Puetz et al., 2009]. Rac1 is responsible for controlling membrane ruffling [Ridley and Hall, 1992] and the formation of lamellipodia and cell motility [Ridley et al., 1995] and actin polymerization [Machesky and Hall, 1997]. Cdc42 controls the formation of filopodia [Nobes and Hall, 1995]. Thus, in migrating cells Rac1 is generally required at the leading edge for lamellipodium extension and formation of new adhesions, regulating actin polymerization, whereas Rho controls cell contractility and tail retraction.

In this work, we applied a stretching uniaxial mechanical stress to C_2C_{12} myoblasts cells in order to evaluate the role of focal adhesion proteins and related signal molecules (Rho GTPases family, PI3 kinase/Akt, and Erk pathways) in the reorganization of the cytoskeleton. We assumed the morphological changes as a target of the intracellular translocation of the involved activated proteins from the cytoplasm to the membrane.

MATERIALS AND METHODS

MATERIALS

After sterilizing in autoclave (20 min at 121°C), silicone sheets (thick 0.1 mm; Specialty Manufacturing Inc., Saginaw, MI) coated with fibronectin (10 μg/ml, 1 h at room temperature before cell seeding; Sigma, Milano, Italy) were used as deformable substrate. Samples were cut into 3- by 2-cm samples.

CELL CULTURE

C_2C_{12} myoblast cells (ATCC CRL1772) isolated from mouse muscle were used (10×10^4 cells/cm^2). Cells were cultured in DMEM enriched with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Euroclone, Milano, Italy). Cells seeded on fibronectin pre-coated silicone sheets were maintained at 37°C in a humidified atmosphere with 5% CO_2 under static conditions for 24 h before applying mechanical stress.

MECHANICAL STRESS

The Instron 5564 testing instrument (Instron Corporation, Canton, MA) was used in the present study in order to apply and control different parameters (elongation, frequency, timing, etc.) referred to the substrate deformation. The device comprises an electronic control console, a loading frame capable of testing up to 2.5 N in tension, and a drive system that induces tension on the samples until a predefined deformation and then returns to the starting position. Samples were soaked in a vertical culture chamber (Ugo Basile, Milano, Italy), filled with culture medium and maintained at 37°C with 5% CO_2 in a closed bath. Cyclic uniaxial 10% substrate deformation with return to the starting position was applied at a frequency of 1 Hz for 10, 30, and 60 min and compared with non-stressed controls.

FLUORESCENCE

For actin filaments detection cells were fixed in formaldehyde 3.7% for 30 min at room temperature and then labeled with rhodamin-phalloidin TRITC conjugated (Sigma). Cells were observed by fluorescent microscopy (Leica Microsystems DM2500) at 40× magnification. Images are representative of all results obtained. Tests were performed in triplicate.

WESTERN BLOT ANALYSES

Subcellular fraction. Cells were lysed in subcellular fractionation buffer (250 mm Sucrose, 20 mM HEPES pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA with 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 nM PMSF added before use) for 20 min on ice. Briefly, samples were centrifuged at 720*g* for 5 min and the nuclear pellets were collected and resuspended in loading buffer (standard lysis buffer with 10% glycerol and 0.1% SDS added). The supernatants removed were centrifuged consecutively at different speed to collect the mitochondrial and cytosolic fractions and finally at 100,000*g* for 1 h to collect the membrane fraction, following resuspended in loading buffer. The only membrane franctions were used in this work, processed by SDS–PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, England). Blotted proteins were blocked with 5% non-fat dried milk on PBS, pH 7.4, for 1 h at room temperature and then incubated overnight with primary antibodies (FAK, FAK^{397Y}, Vinculin, Rac1, RhoA, pAkt, Akt, pERK1/2, and ERK1/2) at a ratio of 1:500 in PBS. After washing, membranes were incubated with secondary antibodies peroxidase conjugated (Amersham Biosciences) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (Amersham Biosciences) in a chemisensitive visualizer (VersaDoc Imaging Systems, Biorad, Milano, Italy). Tests were performed in triplicate.

Total lysis. Cell were lysed in RIPA buffer (25 mM Tris HCl pH8.0, 150 mM NaCl, 1% sodium deoxycholate, 1 mM Na_3VO_3, 0.1% SDS, 50 mM sodium fluoride, 1% Triton, 0.5 M EDTA, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 nM PMSF) for 30 min on ice. Protein concentration was determined with bicinchoninic acid assay (Pierce, Rockford, IL). Then, 20 μg of total proteins in loading buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.5% bromophenol blue) were used for SDS–PAGE and transferred to a nitrocellulose membrane (Amer-

sham Biosciences). Blotted proteins were blocked with 5% non-fat dried milk on PBS, pH 7.4, for 1 hour at room temperature and then incubated overnight with primary antibodies (FAK, FAK^{397Y}, Vinculin, Rac1, RhoA, pAkt, Akt, pERK1/2, and ERK1/2) at a ratio of 1:500 in PBS. After washing, membranes were incubated with secondary antibodies peroxidase conjugated (Amersham Biosciences) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (Amersham Biosciences) in a chemisensitive visualizer (VersaDoc; BioRad). Tests were performed in triplicate for each experimental condition.

AFFINITY PRECIPITATION/IMMUNOBLOT (RHO GTPASES ACTIVATION KIT ASSAY—RAC1 AND RHOA)

Cells were cultured reducing serum in culture overnight before proceeding with the experiments and fMLP (100 nM) was used to induce positive controls for Rac1 and RhoA activation (data not shown) [Chen et al., 2004]. The Rac1/RhoA Activation Assay Kit (Millipore, Milano, Italy) was used as indicated by the producer; briefly, cells were lysed in MLB buffer (MLB buffer 5×: 125 mM HEPES pH 7.5, 750 mM NaCl, 5% Igepal, 50 mM $MgCl_2$, 5 mM EDTA, 10% glycerol) on ice and transferred to microfuge tubes. The cell lysates were pre-clear with glutathione agarose and rock fpr 10 min at 4°C. The agarose beads were collected by pulsing for 5 s in the microfuge at 14,000*g*. The supernatants were discarded and Rac1/RhoA assay reagent (PAK-1 PBD agarose and RBD, respectively) were added. The reaction mixtures were rock at 4°C for 60 min. The beads were collected again by centrifuging at 14,000*g* for 5 s and resuspended in Leamli reducing sample buffer before loading the samples on SDS–PAGE gel for electrophoresis. Then, we proceeded as previously described in Western blot section.

DENSITOMETRY

A semi-quantitative examination was carried out on results obtained from Western blot analyses. Images acquired were analyzed with an image analysis software (QuantityOne; Biorad). To take into account optical density and extension of protein bands, these were evaluated as a grey scale index/mm^2 in pixels (optical density).

STATISTICAL ANALYSES

Results were expressed as mean ± SD. Statistical significance was determined by Student's *t*-test. Statistical analysis of variance (ANOVA) was used and the significance of differences between means was assessed by Bonferroni's method, taking $P \leq 0.05$ as the minimum level of significance.

RESULTS

The morphological changes observed after the mechanical stress are shown in Figure 1. Controls showed actin stress fibers well-defined among all the cell body. The cells displayed a typical fibroblast-like morphology of adherent cells (Fig. 1A). After the mechanical stretching was applied, cells presented different morphological

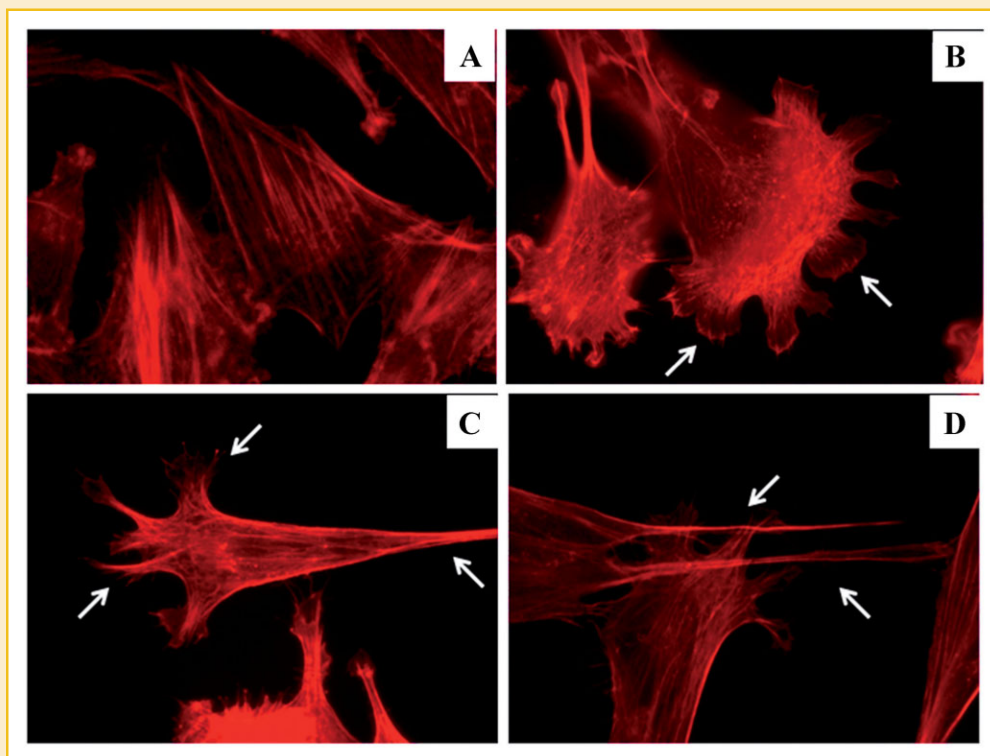

Fig. 1. Cells morphology obtained after actin filaments staining with rhodamine-phalloidin. Pictures represent static control (A) and cells subjected to dynamic conditions (10% substrate deformation, 1 Hz frequency) for 10 (B), 30 (C), and 60 (D) min. Pictures are representative of all the results obtained in the different experimental conditions.

characteristics, starting from a distinct membrane ruffling after 10 min of 10% substrate deformation at 1 Hz frequency, as indicated by arrows in Figure 1B. The actin stress fibers disaggregated and evident actin acrs were formed (Fig. 1B). An extended mechanical stimulation (30 min) showed the formation of short filopodia (arrows) together with a maintained, even if less represented, membrane ruffling (Fig. 1C). Significant changes appeared after 60 min, when a considerable presence of long filopodia was detected together with trailing tails, characteristics of migrating cells (arrows Fig. 1D). Membrane ruffles were not extensively identified. Following the morphological observation, the presence, localization and, eventually, the activation of adhesion proteins were studied. Figure 2 showed the behavior of FAK and vinculin in presence of mechanical stress as indicated by Western blot analyses (Fig. 2A,C) of the subcellular fraction and the total lysis, together with the relative densitometric analyses (Fig. 2B,D). The membrane fraction evidenced an increased presence of FAK after 10 min of mechanical stretching at the same dynamic conditions used for the morphological analyses. This increase was constantly maintained until 60 min of stimulation was reached. These data were in agreement with the increasing of the activated fraction (FAK^{Y387}) which raised up after 10 min and following gradually decreased, even if the activation remained higher when compared to static controls. The mechanical stimulation had no influence on FAK expression when the total lysates were considered. The membrane fractions showed an increased amount of vinculin after 30 min of substrate deformation, while again the broad expression was constantly maintained (Fig. 2C). Data were confirmed by the densitometrical analyses (Fig. 2D). The involvement of RhoGTPases family in cytoskeletal modifications as well as in cell migration is already proved in literature. Thus, we proceeded by investigating the role of Rac1 and RhoA in the morphological changes caused by the dynamic environment (Fig. 3). Rac1 membrane recruiting resulted after 10 min and massively after 30 min, while the level was significantly decreased after 60 min. Rac1 was transiently activated after 10 min while the total expression resulted not modified during all the experimental timing (Fig. 3A). Densitometry performed in triplicate in all the experimental conditions confirmed the visual data reported (Fig. 3B). RhoA behaved similarly, showing a massive membrane recruitment after 30 min following the transient activation at 10 min of substrate deformation. Again, the total amount of RhoA resulted not altered by the presence of the mechanical stress (Fig. 3C). The densitometric analyses are shown in Figure 3D. As RhoGTPases are tightly associated with Akt/PKB and MAPK/Erk pathways we considered the localization and activation of these two proteins in presence of mechanical stress (Fig. 4). The Akt membrane fraction presented an increased presence of the protein after 10 and 30 min, after which the level decreased. Instead, the activated fraction raised up at 30 and more at 60 min while the total expression of the protein was constant among time (Fig. 4A). Once more the visual data were accompanied by the densitometry, summarizing the results obtained in three different experiments (Fig. 4B).

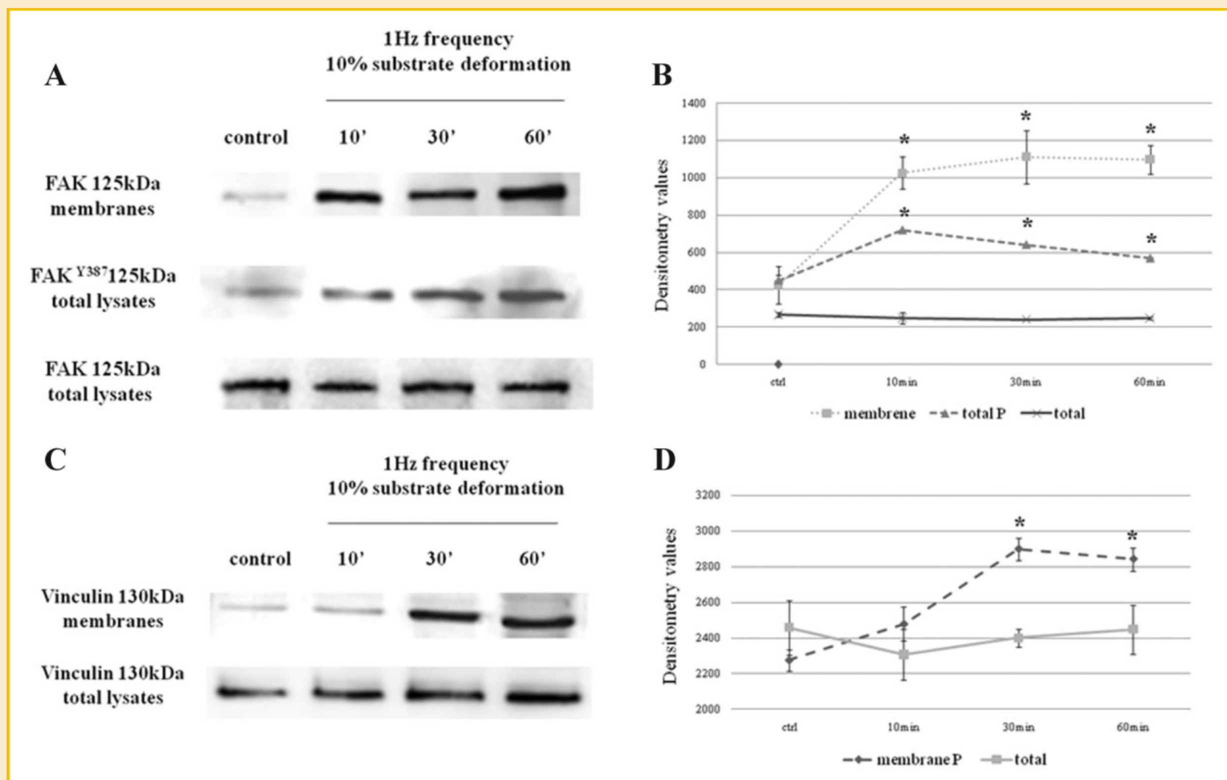

Fig. 2. A–C: Western blot analyses with anti-FAK, anti-FAK^{Y387} and vinculin antibodies on lysates from static controls and cells subjected to dynamic conditions (10% substrate deformation, 1 Hz frequency for 10, 30, and 60 min). Proteins were revealed on membrane fractions and total lysates. B–D: Densitometry graphics obtained from three different experiments are expressed as mean ± SD. *Significant results with respect to control; $P \leq 0.05$.

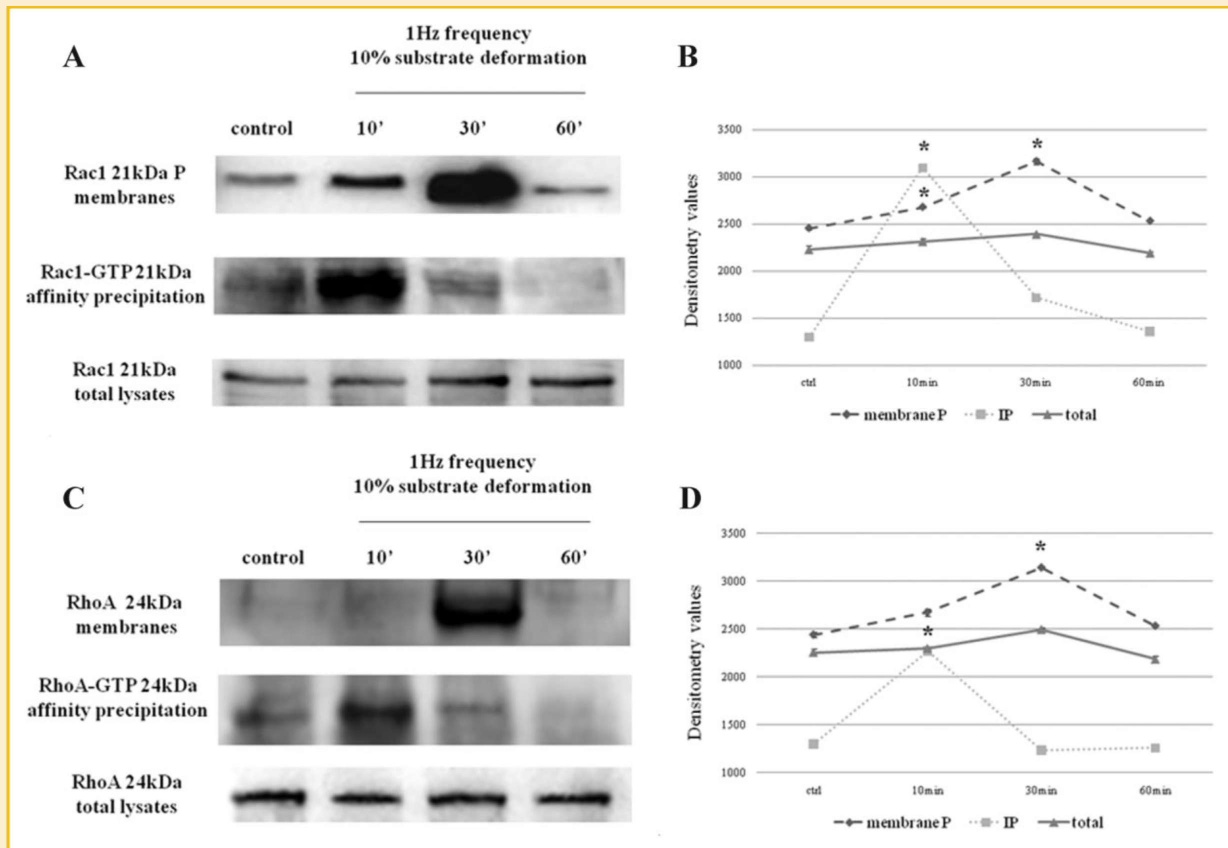

Fig. 3. A–C: Western blot analyses with anti-Rac1and anti-RhoA antibodies on lysates from static controls and cells subjected to dynamic conditions (10% substrate deformation, 1 Hz frequency for 10, 30, and 60 min). Proteins were revealed on membrane fractions and total lysates. Affinity precipitation for Rac1-GTP and RhoA-GTP were performed in order to evaluate Rac1 and RhoA activation in the different experimental conditions. B–D: Densitometry graphics obtained from three different experiments are expressed as mean $\pm$ SD. *Statistically significant results with respect to control; $P \leq 0.05$.

Erk1/2 behaved differently when compared to Akt. In fact, Erk1/2 resulted not detectable in the membrane fraction, its activation was transient between 10 and 30 min of stimulus which did not altered the total amount of the protein over time, as indicated even by the densitometrie's graphic (Fig. 4C,D).

DISCUSSION

Mechanical stress has an important role in morphological changes evidenced in dynamic environments. At first, membrane ruffling characterize these changes, followed by the presence of cytoskeletal protrusions, up to the formation of mature filopodia. It is known that the cytoskeletal system plays an important role in cell migration and proliferation, and lamellipodia, filopodia, membrane ruffling, and focal contacts dynamics are intimately involved in the function of the cytoskeletal system. Thus, mechanical stresses act a dual role in cytoskeletal modifications and cellular behavior in terms of surviving/proliferation and migration. It is interesting notice how Rho family small GTPases are the fundamental point where both these ways converge. It is known from a consistent literature that membrane mechanosensors act as transducers from a mechanical to a biochemical stimulus, and integrins and related focal adhesion proteins are the components of choice indicated as mechanoreceptors and transducers.

In this work, we demonstrate that a mechanical stretching participate to intracellular and cytoskeletal dynamics though the involvement of adhesion proteins with an active cross-talk between cytosolic proteins activated and recruited into plasma membrane in presence of mechanical stress. The evident changes in the cytoskeleton are shown when the actin stress fibers, present in static conditions, disappeared to form first actin arcs and lately filopodia and trailing tails. These morphological structures are typical of migrating cells. Vinculin recruitment in membrane shows that a dynamic environment strongly affects focal adhesion assembly. As will be lately recall in the text, RhoA catalyzes the synthesis of phosphatidylinosytol-4,5-biphosphate, which is essential for vinculin activation and for a proper binding of several adhesion molecules involved in focal contacts formation and dynamic (i.e., paxillin, talin). If tyrosine phosphorylation proceeds, as in this case, F-actin and associated cytoskeletal proteins accumulate [Miyamoto et al., 1995]. Moreover, mechanical stress induces integrin accumulation and specific tyrosine phosphorylation resulting in focal accumulation of the same signal transduction molecules, including small Rho GTPases, MAPK, and FAK [Shikata et al., 2005]. FAK plays a key role in bridging the mechanical

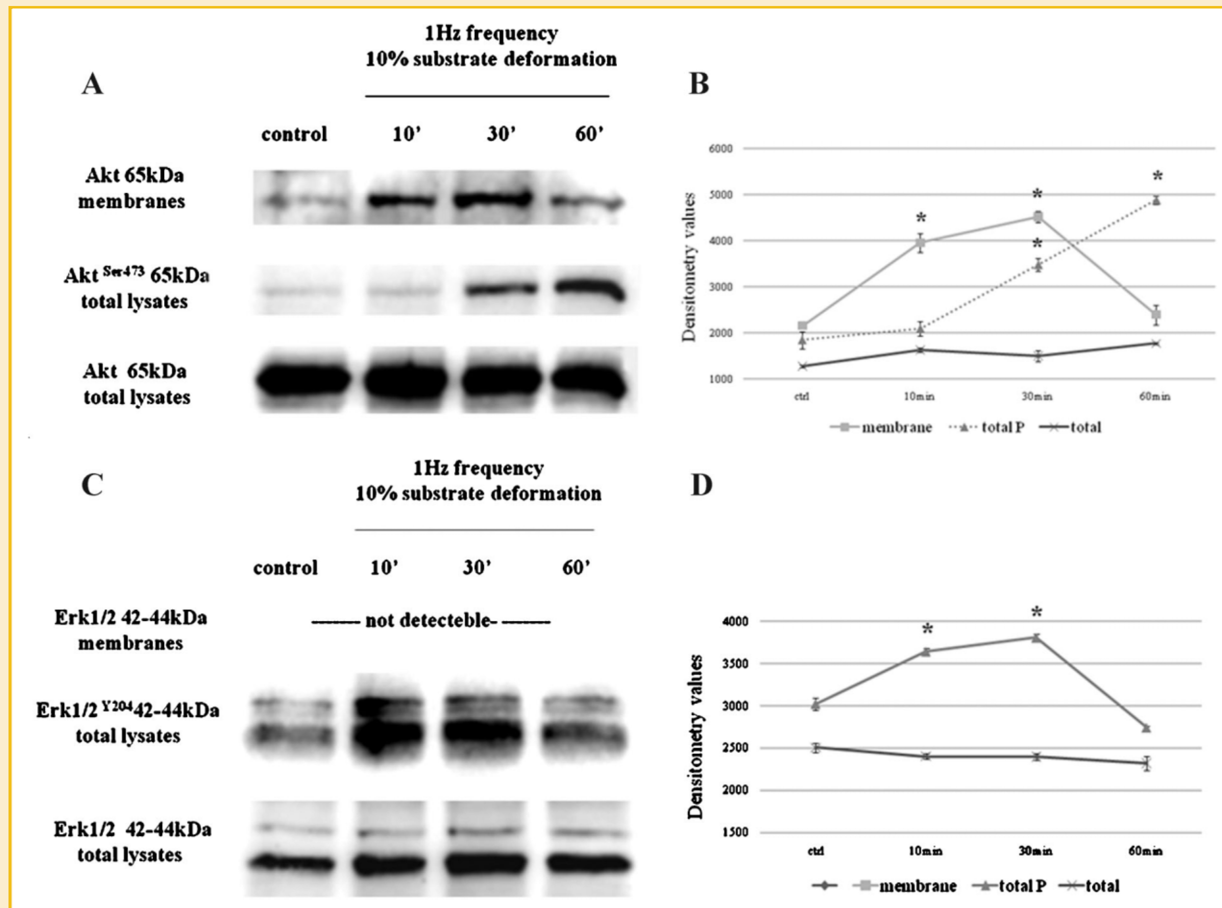

Fig. 4. A–C: Western blot analyses with anti-Akt, anti-Akt^{Ser473}, anti Erk1/2, and anti-Erk^{Y204}antibodies on lysates from static controls and cells subjected to dynamic conditions (10% substrate deformation, 1 Hz frequency for 10, 30, and 60 min). Proteins were revealed on membrane fractions and total lysates. B–D: Densitometry graphics obtained from three different experiments are expressed as mean ± SD. *Statistically significant results with respect to control; $P \leq 0.05$.

stimulus from the membrane adhesion plaque to the cytosolic molecular pathways. In fact, following its own membrane recruitment, the concerted regulation of RhoGTPases proteins occurs, together with Akt recruitment in membrane and related activation and Erk activation. Supporting these data, considerable evidence implicates FAK in the regulation of cell migration, providing one pathway to the recruitment and activation of the previous cited proteins, all required for actin assembly, protrusive activity or modulation of adhesive complex stability, as well as for cell surviving and proliferation. Small GTPases of the Rho family are pivotal regulators of several signaling networks that are activated by a wide variety of receptors types. When activated, Rho GTPases affect many aspects of cell behavior, including actin cytoskeleton dynamics and membrane trafficking. When bound to GDP they are inactive; upstream events lead to the exchange of GDP for GTP and the protein switches into an active conformation. The activation of Rho GTPases in cells requires the interaction with molecules which can affect the GDP/GTP cycle. It is presumed that even such interactions take place at the plasma membrane when their own recruitment occurs.

As demonstrated in literature concerning the role of RhoA and Rac1 following growth factor stimulation, the activation of Rac and the consequent membrane ruffling are tightly associated with PI3K/Akt activation [Hawkins et al., 1995; Nobes and Hall, 1995]. Strengthening this issues, the Rho GTPases family is activated by adhesion receptors, promoting integrin-mediated spreading on the matrix [Clark et al., 1998], even if the mechanisms by which integrins signal to GTPases are still unclear. So consequent to cell adhesion, integrins clustering recruits adhesion, and regulatory proteins to the membranes. Thus, a cross-talk between small GTPases is suggested, which act sequentially on actin fibers and polymerization.

Rac1 seems to be first involved in membrane changes related to actin and focal contacts organization, and, presumably through its ability to promote actin polymerization at the cell periphery (actin arcs), provides the driving force for the protrusive activity required for cell migration.

RhoA involvement is required for focal contacts assembly and actin stress fibers formation. It has been suggested that phosphatidylinositol 4-phosphate 5-kinase is a candidate RhoA target that might control actin polymerization leading to filopodia formation though localized increase in phosphatidylinositol (4,5) biphosphate (PIP_2) levels [Stossel, 1989; Chong et al., 1994]. Moreover, this molecule is also responsible of vinculin activation

when bind to vinculin tail, acting a conformational change able to stabilize the multiprotein complex related to focal contacts. A dynamic response of vinculin is possible be involved in migration events, even related to the presence of mechanical stresses. From this point of view, a hierarchical relationship between these two members of RhoGTPases family has shown and actin structural changes, membrane ruffling, lamellipodia formation, and focal adhesion assembly are distinct downstream effects of the same regulatory molecules, RhoA and Rac1, together with other molecular pathways typically involved in cell surviving (PI3K/Akt) and proliferation (MAPK/Erk).

In summary, mechanical stress induces some cellular morphological changes due to the ability of the adhesion sites to act as mechanosensors. The supposed integrin clustering occurred in presence of a dynamic environment leads to cytoskeletal reorganization via several pathways. The initial activation and membrane recruitment of vinculin and FAK leads to the involvement of regulatory proteins of the small GTPases family (Rac1, RhoA) which play pivotal roles in focal adhesion assembly, as well as in stress fibers reorganization with the consequent formation of actin structures such as actin arcs, membrane ruffles, and filopodia. Moreover, the cross-talk between the Rho GTPases and other molecular intracellular pathways has direct effects on proliferation, through the MAPK/Erk cascade, and on surviving, through PI3K/Akt activation.

Thus, this work supports some issues concerning the key role of RhoGTPases in cell adhesion and migration, though the morphological changes occurring in presence of mechanical stress. Moreover, the involvement of these events in tight correlation with cell proliferation and surviving is also reinforced by the strong influence that a substrate stretching has on PI3K/Akt and MAPK/Erk pathways. These findings have an important role in vascular tissue engineering application, where the use of dynamic environments is more and more attested as a successful way to improve the biomechanical characteristics of the engineered tissues. In fact, future works will focus on the influence of the mechanical stresses on the induction of a contractile phenotype on C_2C_{12} cell line, even in relation with the findings presented in this work.

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