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Osteoblast Adhesion Dynamics: A Possible Role for ROS and LMW-PTP

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ABSTRACT

Reactive oxygen species (ROS) modulate a variety of intracellular events, but their role in osteoblast adhesion and spreading remains unclear. ROS is a very-known physiological modulators of Protein Tyrosine Phosphatases activities, mainly to low molecular weight protein tyrosine phosphatase (LMW-PTP) activity. As this biological mechanism is not clear in osteoblast adhesion, we decided to investigate ROS levels and phosphorylations of FAK and Src, identifying these proteins as potential substrates to LMW-PTP activity. Our results showed that during osteoblast adhesion/spreading (30 min and 2 h of seeding) the intracellular ROS content (hydrogen peroxide) is finely regulated by an effective anti-oxidant system [catalase and Superoxide Dismutase (SOD) activities were evaluated]. During the first 30 min of adhesion, there was an increase in ROS production and a concomitant increase in focal adhesion kinase (FAK) activity after its phosphorylation at Tyrosine 397 (Y_{397}). Moreover, after 2 h there was a decrease in ROS content and FAK phosphorylation. There was no significant change in LMW-PTP expression at 30 min or 2 h. In order to validate our hypothesis that LMW-PTP is able to control FAK activity by modulating its phosphorylation status, we decided to overexpress and silence LMW-PTP in this context. Our results showed that FAK phosphorylation at Y_{397} was increased and decreased in osteoblasts with silenced or overexpressed LMW-PTP, respectively. Together, these data show that ROS modulate FAK phosphorylation by an indirect way, suggesting that a LMW-PTP/FAK supra-molecular complex is involved in transient responses during osteoblast adhesion and spreading. J. Cell. Biochem. 115: 1063–1069, 2014.

KEY WORDS: ADHESION; FAK; LMW-PTP; OSTEOBLAST; REDOX; ROS

The development and repair of the vertebrate skeleton require precise coordination among specialized bone cells such as osteoblasts and osteoclasts. A better understanding of the signaling transducers involved in osteoblast adhesion (the first step in the deposition of new bone) is fundamental for areas such as cell biology, bone tissue engineering, and materials sciences. Indeed, proper cell contact with material surfaces and subsequent adhesion/spreading are the first stages in cell-material interactions [Anselme, 2000].

Conflict of Interest: The authors declare that there is no conflict of interest.

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These 2000]. These initial events profoundly influence the integration of dental and orthopedic implants into host tissue and determine the success or failure of a broad range of implanted biomaterials. The importance of these interactions has led to greater interest in understanding bone formation, particularly osteoblast adhesion and differentiation, as a means of predicting the quality of biological responses. In this regard, various studies have examined the molecular signaling involved in guiding osteoblast adhesion in vitro [Zambuzzi et al., 2008; Zambuzzi et al., 2009a,b; Bertazzo et al., 2009, 2010a,b; Milani et al., 2010].

It is very known that Focal Adhesion Kinase (FAK) regulates cytoskeletal re-organization upon integrin activation triggering intracellular events involved with eukaryote cells adhesion. In this way, FAK functions as an active scaffolding platform for the binding others signaling and adaptor proteins, such as Src-kinase and paxillin: upon integrin-mediated adhesion FAK is auto-phosphorylated at Y_{397}, which serves as a binding site for the SH_2 domain of Src family kinases. Once recruited to FAK, Src kinases phosphorylate other tyrosine residues on FAK, some of which serve as binding sites for signaling or adaptor molecules (such as talin, paxilin, Src, and FAK) that activate downstream signaling pathways [reviewed by Milani et al., 2010].

On the other hand, it is known that reactive oxygen species (ROS) are produced during normal cell metabolism in response to various stimuli. Despite their well-known cytotoxicity, ROS, especially Hydrogen Peroxide (H_2O_2), play a major role in intracellular signaling pathways and in the regulation of gene expression in many organisms [Zhang and Gutterman, 2007; Matsuzawa and Ichijo, 2008]. In addition, in recent years, ROS have been found to modulate protein tyrosine phosphatase (PTP) activity [van der Wijk et al., 2003; Groen et al., 2005; Karisch and Neel, 2013]. Protein-tyrosine phosphatases (PTPs) are now believed to be important targets of ROS once PTPs contain a conserved catalytic cysteine with an unusually low pKa. This property allows PTPs to execute nucleophilic attack on substrate phosphotyrosyl residues, but also renders them highly susceptible to oxidation. Reversible oxidation, which inactivates PTPs, is emerging as an important cellular regulatory mechanism [reviewed by Karisch and Neel, 2013].

Members of the PTP′s family dephosphorylate target proteins and counter the activities of protein tyrosine kinases that are involved in cellular phosphorylation and signaling. Although members of the PTP′s family have crucial roles in various cellular processes, the physiological functions of the individual PTPs are poorly understood owing to the lack of information about the genuine substrates of the respective PTPs. Our group has studied PTP in different biological models over the last years. In 2008, we have shown that a special PTP, known as low molecular weight–protein tyrosine phosphatase (LMW-PTP) plays a crucial role in osteoblast metabolism by modulating Src-kinase activity during biological events of differentiation. These results were similar to those found by Marzia et al. [2000], who showed that Src deficient mice presented osteopetrosis.

Based on this regard, we decided to investigate the involvement of ROS in early osteoblast adhesion and spreading (30 min and 2 h, respectively) and its possible effect on LMW-PTP behavior by monitoring the phosphorylation profile of potentials physiological substrates of LMW-PTP, such as phopsho-Y_{397}-FAK.

MATERIALS & METHODS

ANTIBODIES AND REAGENTS

Antibodies to phospho-FAK (Tyr_{397}; #3283), phospho-Src (Tyr_{416}; #2101), pan-actin (#4968) and anti-rabbit IgG were purchased from Cell Signaling Technology (Boston, MA). Antibody against LMW-PTP was from Abcam (Cambridge, MA). The antibodies were used at the dilutions proposed by the manufacturers. Phalloidin was purchased from Invitrogen (Invitrogen/Molecular Probes, Carlsbad, CA). DAPI (Sigma, St. Louis, MO). All chemicals were of analytical grade. The pan-actin was used as loading control for western blottings analysis.

ESTIMATION OF HYDROGEN PEROXIDE (H_2O_2) PRODUCTION

The intracellular production of ROS was assayed by flow cytometry using the substrate dihydrorhodamine (DHR) 123 (Molecular Probes, Leiden, Netherlands) that diffuses into the cells and is oxidized by ROS to the fluorescent Rhodamine 123. With this method, it is possible to evaluate the response of individual cells. Osteoblasts were incubated with 1 μM DHR123 for 10 min to capture H_2O_2 [Ischiropoulos et al., 1996]. DHR123 was omitted from the blank control. The reaction was stopped on ice, and the fluorescence intensity of the cells was analyzed immediately by flow cytometry using a FACS Calibur flow cytometer. Propidium Iodide (PI, 50 μg/ml, Invitrogen, Grand Island, NY) was used to identify dead cells, once PI intercalates into double-stranded nucleic acids and become fluorescent.

CELL CULTURE AND CONDITIONS

In order to investigate some molecular mechanisms involved with osteoblast adhesion, MC3T3-E1, a mouse calvaria pre-osteoblast cell line, subclone 4, was obtained from ATCC (Manassas, VA) and grown at 37°C in α-MEM (Nutricell, Campinas, Sao Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS, Nutricell, Campinas), 100 U of penicillin/ml, and 100 μg of streptomycin/ml and 4 mM L-Glutamine (Sigma), in a humidified 5% CO_2 atmosphere. Routinely, each 2 days the cells were properly trypsinized (0.25% in PBS) for 5 min at 37°C, re-suspended within supplemented medium and thereafter replaced to new flasks (polystyrene, TPP, St. Louis, MO) and maintained with fresh medium at a ratio 1:2 (cell suspension: supplemented medium). In order to check the cell number per experiment, see the details in each methodology.

CONFOCAL MICROSCOPY

In order to know the rearrangement of actin filaments during unmodified osteoblast adhesion/spreading, we decided to stain the osteoblasts with phalloidin and counterstain with DAPI, in order to identify their nuclei. It was important to estimate the morphological changes during the early osteoblast adhesion. Thus, the cells (3×10^4) cells were seeded at different times on coverslips (13 mm-24 multi-well dish, thermanox, Thermo Scientific, Hudson, NH) as previously described [Zambuzzi et al., 2009a]. Subsequently, the cells were fixed with 2% formaldehyde for 30 min, washed with 0.1 M glycine, permeabilized with 0.01% saponin for 15 min, and washed three times with PBS. The cells were then incubated for 40 min with alexa fluor 488-labelled phalloidin (4 mg/ml; Invitrogen/Molecular Probes). Nuclei were stained with DAPI (20 mg/ml; Sigma) for 20 min.

Coverslips were mounted on glass microscope slides using mounting medium (fluoromount-G). The cells were viewed with a confocal laser scanning microscope (Zeiss, LSM 510 META) using a 40× oil-immersion lens.

IMMUNOBLOTTING

Firstly, in order to estimate the phosphorylation levels of LMW-PTP, FAK and Src during osteoblast adhesion, the cells were cultured and immediately after 30 min or 2 h of seeding the protein extracts were obtained by using a lysis cocktail (50 mM Tris–HCl, pH 7.4, 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM *O*-vanadate, 1 mM NaF and protease inhibitors—1 μg aprotinin/ml, 10 μg leupeptin/ml, and 1 mM PMSF [phenylmethanesulphonyl fluoride]) for 2 h on ice. An equal volume of 2× SDS gel loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to the samples and boiled for 5 min. Protein extracts were resolved into SDS-PAGE (12% polyacrylamide gels) during approximately 3 h and transferred during 40 min to polyvinylidene difluoride (PVDF) membranes. The electrophoresis and transference of proteins were used equipment from Bio-Rad (Hercules, CA). Immunoreactive bands were detected with enhanced chemiluminescence (ECL) kits (Thermo Fisher Scientific, Rockford, IL). β-Actin was used as the internal housekeeping gene and loading control for immunoblotting.

TRANSFECTION OF MC3T3-E1 CELLS WITH LMW-PTP OVEREXPRESSING OR SILENCING PLASMIDS

We decided to modulate LMW-PTP presence by evaluating its importance to control phospho-proteins downstream knowingly upon integrin activation.

Overexpression. MC3T3-E1 cells (50×10^3 cells/ml) were grown to around 60% confluence (six-well plates) and thereafter transiently transfected with LMW-PTP plasmids in order to overexpress it. The transfections were done using an Effectene transfection kit (QIAGEN Benelux, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, the cells were transfected with 2.4 μg of pcDNA3.1/V5-His-TOPO vector with or without an insert containing the sequence of LMW-PTP after a cytomegalovirus promoter. Overexpression efficiency was always verified by immunoblotting (see insert on Fig. 3B).

Silencing. MC3T3-E1 cells (50×10^3 cells/ml) were grown to 60% confluence (six-well plates) and transiently transfected with siLMW-PTP. The transfections were done using a Hiperfect transfection kit (QIAGEN Benelux) according to the manufacturer's instructions. Briefly, the cells were transfected with LMW-PTP siRNA (final concentration: 5 nM) or Mitogen-Activated Protein Kinases (MAPK1) siRNA (also called MAPK2 and Erk2, used as positive control) for 72 h, and then scraped into 100 μl of cell lysis buffer, sonicated, and spun down. The lysates were subsequently mixed with loading buffer for electrophoresis. The efficiency of transfection was assessed based on the expression of LMW-PTP by western blot analysis. To note, the RNAi human/mouse control kit provides positive control siRNA targeted against the protein kinase MAPK1. MAPK1 is ubiquitously expressed in both human and mouse cell line, making it suitable target gene for control experiments.

MODIFIED OSTEOBLAST ADHESION APPROACH

Osteoblasts were properly transfected with LMW-PTP siRNA or overexpressing plasmids as described in details previously. Those modified osteoblasts were seed (50,000 cells/well) in 24-well culture plates in order to know the influence of LMW-PTP on their adhesion. Indeed, unmodified osteoblasts were used as control of this approach. Briefly, after 2 h of plating, adherent osteoblasts were rinsed in warm 1 phosphate-buffered saline (PBS) and fixed in absolute ethanol-glacial acetic acid (3:1, v/v) for 10 min at room temperature and left to air dry (eventually stored at 4°C, wrapped in aluminum foil). Then, adherent osteoblasts were stained with 0.1% (w/v) crystal violet for 10 min at room temperature. Excess dye was removed by decantation and washed twice with distilled water. The dye was extracted in 10% (v/v) acetic acid, and the absorbance was measured at 550 nm using a microplate reader (Biotek, Winooski, VT). Crystal violet is a basic dye that has been extensively used as a cytochemical stain that binds to chromatin. For each group was considered $n = 6$.

CATALASE (CAT) AND SUPEROXIDE DISMUTASE (SOD) ACTIVITIES

After 30 min and 2 h in culture, unmodified osteoblasts were rinsed three times with cold, sterile PBS, harvested, and centrifuged (1,200*g*, 5 min, 4°C). The supernatant was discarded and the cell pellet was resuspended in 400 μl of ice-cold 10 mmol/L phosphate buffer (pH 7.0) and sonicated in an ice bath for 15 s. After centrifugation (12,000*g*, 10 min, 4°C), the supernatant was collected and used to assay antioxidant enzyme activities and quantify the total amount of protein. CAT activity was measured by monitoring H_2O_2 decomposition based on the change in 230 nm during 30 min incubation in reaction medium containing 50 mmol/L sodium phosphate buffer (pH 7.0), 10 mmol/L H_2O_2 and enzyme sample [Maehly and Chance, 1954]. Total SOD activity was measured by the coupled reaction of *N*, *N*-dimethylaniline (DMA) with 4-aminoantipyrine (4-AAP) and the reaction was monitored at 554 nm [Tang et al., 2002]. The absorbance was measured at 570 nm and the results were expressed in nanomoles/mg of protein. Enzymatic activity was normalized for cellular protein content determined by the method of Lowry et al. [1951] with bovine serum albumin as the standard.

STATISTICAL ANALYSIS

Results were represented as mean ± standard deviation (SD), and also normalized by student's *t*-test (two-tailed) with $P < 0.05$ considered statistically significant and $P < 0.001$ considered highly significant. In experiment where there were >two groups, we used one way ANOVA (non parametric) with post-test of Bonferroni, in order to compare all pairs of groups. In this case, the significance level was considered when alpha = 0.05 (95% confidence interval). The software used was GraphPad Prism 5.

RESULTS

OSTEOBLAST ADHESION PROVOKES DRASTIC CHANGES IN CELL SHAPE

Unmodified osteoblast adhesion involves several morphological alterations in which the cell shape changes from round to spread-out. Figure 1 shows that these shape changes were mediated by actin

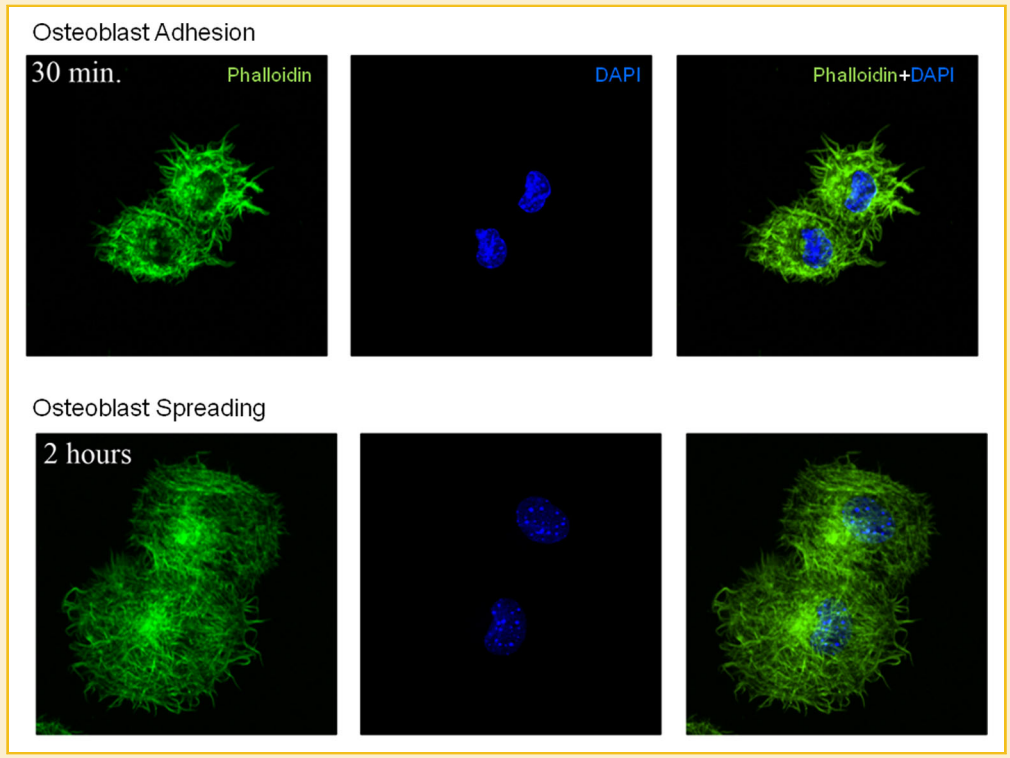

Fig. 1. Morphological changes at early interval of osteoblast adhesion. Osteoblastic cells (MC3T3-E1, subclone 4) were seeded on polystyrene surface and stained with phalloidin and DAPI, respectively for evaluating actin-filaments network and nuclei. *Magnification*: 40× oil-immersion lens.

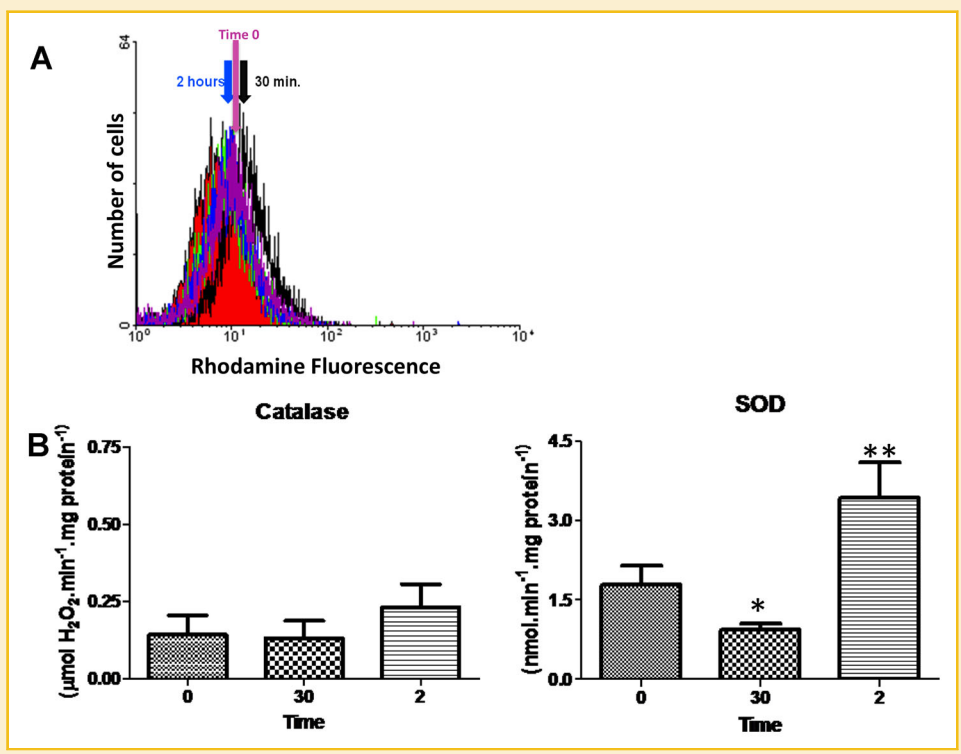

Fig. 2. ROS content during osteoblast adhesion and its modulation. A: Flow cytometry indicated ROS production for up to 2 h after plating, with early (30 min) production being greater than at 2 h. B: As showed previously, the amount of ROS was decreased at 2 h, suggesting a potent efficiency of antioxidant machinery. In order to check this hypothesis, we decided to evaluate catalase (CAT) and superoxide dismutase (SOD) activities. The results suggest that the decrease in ROS production at 2 h was probably related to the increased CAT and SOD activities at this interval compared to 30 min. The results were considered significant when $P < 0.05$ (*, **)

rearrangement. This observation suggested that cell spreading involved rigorous control of the assembly of focal adhesion components. Since most focal adhesion components are PTKs their activity is very likely to be modulated by PTPs. As mentioned previously in this work, most of PTP's activities are modulated by physiological content of ROS.

OSTEOBLAST ADHESION GENERATES AN EARLY, TRANSIENT INCREASE IN ROS

ROS production during osteoblast adhesion was assessed based on H_2O_2 production. Flow cytometry indicated ROS production for up to 2 h after plating, with early (30 min) production being greater than at 2 h (Fig. 2A). The decrease in ROS production at 2 h was probably related to the increased CAT and SOD activities at this interval compared to 30 min (Fig. 2B).

MODULATION OF FAK AND SRC PHOSPHORYLATIONS BY LMW-PTP: A POSSIBLE ROLE FOR LMW-PTP IN OSTEOBLAST ADHESION

In view of the altered redox status and possible involvement of PTPs in osteoblast adhesion we examined the phosphorylation profile of FAK (Y_{397}) and Src (Y_{416}), both of which are potential physiological substrates for LMW-PTP. In this way, immunoblotting bands showed that endogenous LMW-PTP expression was unchanged even though FAK ($P < 0.05$) and Src were phosphorylated at 30 min and 2 h, with greater phosphorylation at the earlier interval (Fig. 3A). For this reason (endogenous LMW-PTP remains unchanged), we decided to examine the possibility of LMW-PTP in negatively modulating FAK and Src phosphorylations by exogenously affecting LMW-PTP: we silenced and overexpressed LMW-PTP in osteoblasts and then assessed FAK and Src phosphorylation. The phosphorylation of both of these PTKs was enhanced ($P < 0.05$) in LMW-PTP-silenced osteoblasts but decreased ($P < 0.05$) in LMW-PTP-overexpressed osteoblasts (Fig. 3B). The phosphorylation levels of the proteins were confronted with the total-FAK and total-Src. Our results showed that there is no significant difference on the both Src and FAK expressions between the groups (data not shown).

Thereafter, in order to check an influence of LMW-PTP during osteoblast adhesion, we decided to silence or over express LMW-PTP and thereafter re-seeded the cells on polystyrene. By using crystal violet assay, our results showed that there was a discrete increase of osteoblast adhesion when LMW-PTP was silenced, but it was very similar with the control group, when no significance was found

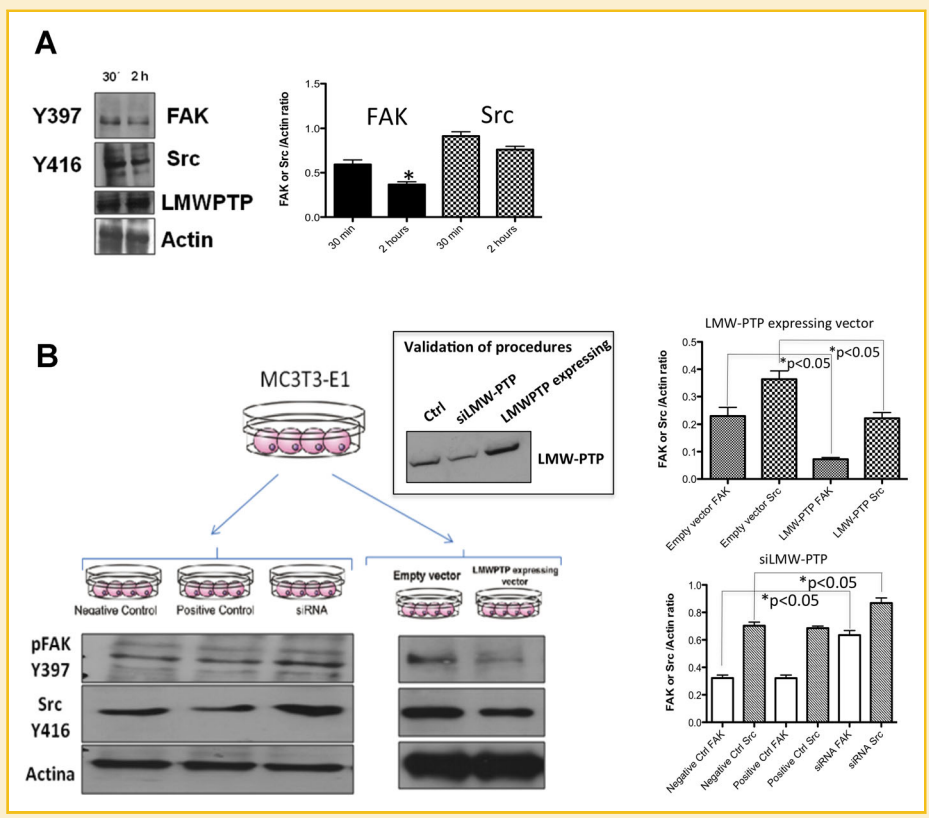

Fig. 3. Effect of LMW-PTP knock down with siRNA on pY397FAK and Y416Src levels. A: Immunoblottings show the phosphorylation levels of FAK (Y397) and Src (Y416) during osteoblast adhesion. Both phosphorylations were greater at 30 min than 2 h; B: MC3T3-E1 cells were transiently transfected with siLMW-PTP for 72 h. The negative control was an irrelevant RNA sequence and the positive control was a sequence directed against MAPK1 (knock down not shown). The cells were subsequently scraped into cell lysis buffer and transfection was confirmed by immunoblotting. In parallel, the cells were transfected with empty vector or an LMW-PTP expressing construct. In addition, total-Src and total-FAK were performed as a comparative control and actin expression was used as an internal loading control of the protein samples and it was unchanged (data not shown). β-Actin was used as the internal housekeeping and loading control for immunoblotting. Briefly, our data showed that LMW-PTP is involved with FAK and Src activations during osteobalst adhesion. Insert: the validation of the transfections procedures. The results were considered significant when $P < 0.05$ (*). The bar graph shows the relative amounts of protein expressions (mean ± SD) normalized to actin expression.

($P > 0.05$). Moreover, the adhesion of osteoblast was impaired ($P < 0.05$) in cells over-expressing LMW-PTP (Fig. 4) by evaluating the adherent cells up to 2 h of seeding.

Altogether, our findings indicate that the early events of osteoblast adhesion require a rigorous map of biochemical events, involving a strict balance of ROS production and FAK and Src phosphorylations. The modulation of phosphorylation of these two PTKs may reflect the inhibition of LMW-PTP activity by ROS.

DISCUSSION

FAK is an important signaling protein involved in signal transduction after integrin activation and has a role in guiding cell adhesion and migration. When stimulated by integrin activation, FAK autophosphorylates at Y_{397}, which creates sites for interaction with Src and subsequent activation of this protein. In recent years, we have studied the signaling proteins involved in osteoblast adhesion [Zambuzzi et al., 2009a; Milani et al., 2010; Zambuzzi et al., 2011a,b]. Based on the morphological changes observed during this phenomenon, we hypothesized that LMW-PTP modulates FAK phosphorylation at Y_{397} while the oxidative state of the cell is rigorously controlled by its potent antioxidant machinery in osteoblastic cells.

To examine this hypothesis, MC3T3-E1 pre-osteoblastic cells were seeded on polystyrene surfaces and studied after 30 min and 2 h. Hydrogen peroxide production was greater at 30 min than at 2 h, indicating enhanced oxidation in the early stages of adhesion. Besides mitochondrial respiratory chain malfunction, there are several potential intracellular sources of ROS, including NADPH oxidase and 5-lipoxygenase (LOX), which catalyze the synthesis of superoxide anion rapidly converted in H_2O_2 [Finkel, 2001; Pani et al., 2001; Chiarugi et al., 2003].

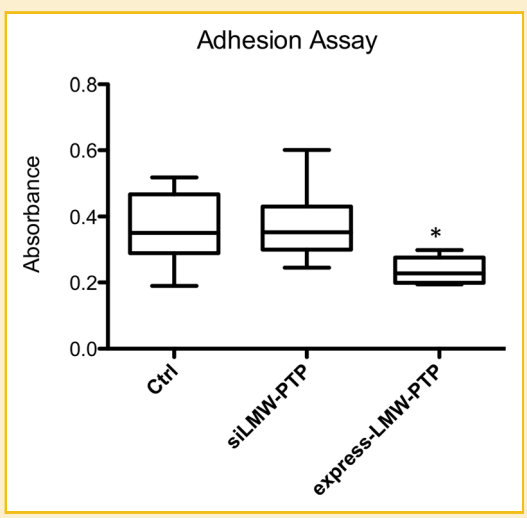

Fig. 4. LMW-PTP affects osteoblast adhesion. In order to check the LMW-PTP effects on osteoblast adhesion we decided to silence or over-express LMW-PTP prior to seed the cells on polystyrene. The adherent cells were stained with violet crystal as described in material and methods section. The results were considered significant when $P < 0.05$ (*).

In 2003, Chiarugi et al. provide evidence that ROS take a role in integrin signaling. Moreover, the production of ROS during integrin receptor engagement is dramatically more pronounced than during growth factor administration. In suggesting a role for oxidant species in integrin signaling, they propose that the production of ROS during cell adhesion leads to an up-regulation of FAK.

Also in osteoblasts, this enhanced level of ROS formation appeared to inhibit LMW-PTP activity (without affecting protein expression) since FAK and Src phosphorylation (which is modulated by LMW-PTP) mirrored the changes in ROS production. The reduction in ROS production seen at 2 h was probably related to the greater activity of CAT and SOD during this period.

To confirm the involvement of LMW-PTP in FAK (Y_{397}) and Src (Y_{416}) phosphorylations during adhesion this PTP was either silenced or overexpressed in osteoblasts. Phosphorylation of both kinases was enhanced in LMW-PTP-silenced cells and attenuated in LMW-PTP-overexpressed cells. In relation to Src, this result was in agreement with our previous data [Zambuzzi et al., 2008]. Moreover, we confirm the influence of LMW-PTP during osteoblast adhesion by either LMW-PTP silencing or overexpressing prior to seed on polystyrene. Our results showed that there was a significance reduction on osteoblasts adhesion when they were over expressing LMW-PTP. These results suggest that there are other PTPs able to modulate these transient FAK and Src phosphorylations than LMW-PTP, enhancing the complexity of the supramolecular complex (FAK, Src, Paxillin, and others) necessary to maintain the activation of signaling proteins downstream of integrin activation, what justify their dynamic activity in cell metabolism.

Together, these results show a transient nature of FAK and Src phosphorylation during osteoblast adhesion, suggesting the formation of a supra-molecular interaction or complex involving at least FAK/Src/LMW-PTP. The formation and activity of this complex in subsequent cell adhesion may be modulated by ROS. The results described here may be useful in producing "smart" biomaterial that will enhance cell adhesion and lead to more predictable biological responses.

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